The Halophyte Seashore Paspalum Uses Adaxial Leaf Papillae for Sodium Sequestration

John J. Spiekerman,a and Katrien M. Devos,a,b,2,3

aDepartment of Plant Biology, University of Georgia, Athens, Georgia 30602
bInstitute of Plant Breeding, Genetics and Genomics, Department of Crop and Soil Sciences, University of Georgia, Athens, Georgia 30602

ORCID IDs: 0000-0003-0776-505X (J.J.S.); 0000-0002-0358-3278 (K.M.D.).

Salinity is a growing issue worldwide, with nearly 30% of arable land predicted to be lost due to soil salinity in the next 30 years. Many grass crops that are vital to sustain the world’s caloric intake are salt sensitive. Studying mechanisms of salt tolerance in halophytic grasses, plants that thrive in salt conditions, may be an effective approach to ultimately improve salt-sensitive grass crops. Seashore paspalum (Paspalum vaginatum) is a halophytic Panicoid grass able to grow in salt concentrations near that of seawater. Despite its widespread cultivation as a sustainable turfgrass, the mechanism underlying its ability to retain high Na⁺ concentrations in photosynthetic tissue while maintaining growth remains unknown. We examined the leaf structure and ion content in P. vaginatum ‘HI10,’ which shows increased growth under saline conditions, and Paspalum distichum ‘Spence,’ which shows reduced growth under salt, to better understand the superior salt tolerance of cv HI10. A striking difference between cv HI10 and cv Spence was the high steady-state level of K⁺ in cv HI10. Imaging further showed that the adaxial surface of both cv HI10 and cv Spence contained dense costal ridges of papillae. However, these unicellular extensions of the epidermis were significantly larger in cv HI10 than in cv Spence. The cv HI10 papillae were shown to act as Na⁺ sinks when plants were grown under saline conditions. We provide evidence that leaf papillae function as specialized structures for Na⁺ sequestration in P. vaginatum, illustrating a possible path for biotechnological improvement of salt-sensitive Panicoid crops with analogous leaf structures.

About 20% of irrigated land is considered saline, with the amount of saline soils increasing worldwide (Mayak et al., 2004). This is due to increased irrigation in agricultural fields necessitated by more frequent droughts due to climate change. This trend is alarming due to the high salt sensitivity of most crop species that we rely on for vital resources. Yield reduction in crops in saline soils amounts to losses on the order of 12 to 27.3 billion U.S. dollars annually (Qadir et al., 2014). Thus, the improvement of salt tolerance in plants will become key in the coming decades. Breeding salt-tolerant crops is a cost-effective approach to improve growth in saline soils. Although much work has focused on breeding salt-tolerant species, progress in this area has been slow due to the complex genetic and physiological nature of the salt response. Furthermore, most research has been conducted on glycophytic model systems that are salt sensitive (Munns and Gilliham, 2015). Unraveling the salt-tolerance mechanisms in halophytes, species that can complete their life cycle in 200 mM salt concentrations, and transferring these pathways into glycophytes is therefore of great interest (Rajalakshmi and Parida, 2012; Roy and Chakraborty, 2014).

Both glycophytes and halophytes have evolved a multitude of salt-tolerance mechanisms, including sodium (Na⁺) exclusion, sequestration, and secretion; osmolyte production; ion homeostasis; and reactive oxygen species (ROS) detoxification (Meng et al., 2018). Often, mechanisms present in glycophytes, such as osmolyte production and Na⁺ exclusion, are utilized in halophytes at higher efficiency (Wyn Jones and Storey, 1981; Grieve and Maas, 1984). However, halophytes also use mechanisms that are absent in glycophytes. Salt sequestration and secretion via salt glands is a halophyte-specific mechanism of coping with salt (Flowers and Colmer, 2008). Salt glands are found in over 50 species in 14 angiosperm families with four subtypes: epidermal bladder cells, complex multicellular glands, bicellular glands, and unicellular glands (Dassanayake and Larkin, 2017). The Poales order contains ~8% of all halophytes (Flowers et al., 2010).
and has therefore been the focus of much salt-gland-focused work (Ceccoli et al., 2015). As salt tolerance has independently evolved >70 times in grass lineages (Bennett et al., 2013), studying these salt sequestering/secretion structures in grasses is an excellent approach to better understand salt tolerance mechanisms in halophytes.

Most structural and physiological work on salt glands in grasses has been conducted in the Chloridoideae and Oryzoideae subfamilies. Grasses carry either unicellular or bicellular glands, often referred to as glandular trichomes or microhairs, on the leaf surface (Dassanayake and Larkin, 2017). Microhairs have been observed on the leaf surface in all grass subfamilies except the Pooidae, and have evolved diverse functions including the sequestration or secretion of substances such as callose and heavy metals (Burke et al., 2000; Ceccoli et al., 2015). Unicellular structures on the adaxial leaf side able to secrete salt are only found in the Oryzoideae wild rice species Porteresia coarctata (Flowers et al., 1990; Sengupta and Majumder, 2009). Salt glands in the Chloridoideae are bicellular, consisting of a cap cell and a lower basal cell, both of which are dense in cytoplasm and mitochondria (Ceccoli et al., 2015). The cuticle is thickened above the cap cell in some species, forming a cuticular chamber used for storing secreted salts (Amarasinghe and Watson, 1988). In the Panicoideae, a few cases of Na+ secretion have been reported (McWhorter et al., 1995; Ramadan and Flowers, 2004), but to date, no sequestration structures have been identified.

The Panicoideae subfamily includes the agronomically important food crops maize (Zea mays) and sorghum (Sorghum bicolor) in addition to the biofuel grasses miscanthus (Miscanthus sinensis), switchgrass (Panicum virgatum), and sugarcane (Saccharum officinarum). One of the most salt-tolerant species in the Panicoideae is the halophyte seashore paspalum (Paspalum vaginatum). It is cultivated as a turfgrass worldwide and derives its popularity from its ability to be irrigated with brackish water. P. vaginatum can survive in salt concentrations near that of seawater (Uddin et al., 2012) and uses osmolyte production, ion homeostasis, and Na+ exclusion to cope with salt stress (Peacock and Dudeck, 1985; Lee et al., 2008; Guo et al., 2016). However, its ability to maintain growth while accumulating high levels of Na+ in leaf tissue remains perplexing.

Here, we studied the leaf structure and Na+ sequestration in ‘HI10’, a P. vaginatum cultivar, and ‘Spence’, a Paspalum distichum cultivar. P. vaginatum and P. distichum are closely related (and possibly the same species; Eudy et al., 2017), and constitute group “Disticha” in the tribe Paspalaeae. P. distichum is less salt tolerant than P. vaginatum and is typically found in freshwater habitats (Leithhead et al., 1971). P. vaginatum and P. distichum therefore represent a useful species pair to study salt tolerance. Furthermore, their salt responses can be compared with those of sorghum, a Panicoid glycophyte. Our main research objective was to identify the phenotypic and physiological factors that contribute to the differential tolerance to salt stress of the two Paspalum spp. cultivars and sorghum ‘BTx623’.

We show that both Paspalum species contain dense rows of translucent papillae on the adaxial surface. The papillae are unicellular protrusions from epidermal cells and are much larger in cv HI10 than in cv Spence. We further demonstrate that the papillae sequester Na+ under salt stress. This study thus provides evidence of Na+ sequestration in specialized leaf-borne organs within the Panicoideae.

RESULTS

cv HI10 and cv Spence Differ in Their Salt Response at 0, 10, and 30 dS m⁻¹ Salt Levels

P. vaginatum ‘HI10’ has enhanced growth in salt water compared to freshwater, while cv Spence has reduced growth. The growth behavior of cv Spence under salt stress is similar to what is typically observed in glycophytes like sorghum (Fig. 1, A–C). cv HI10 and cv Spence also exhibit different leaf morphologies, with cv Spence having larger, coarse-textured leaves compared to cv HI10 (Fig. 1D).

Comparison of Na+ levels in above- and belowground organs of cv HI10, cv Spence, and cv BTx623 revealed significant differences in the amount as well as distribution of Na+ between the two Paspalum species and cv BTx623 (Fig. 2, A–C; Supplemental Fig. S1, A–C). Across organs, average Na+ levels under 30 deciSiemens (dS) m⁻¹ salt stress were 44,968 ppm in cv BTx623, 14,917 ppm in cv HI10, and 12,175 ppm in cv Spence. At 30 dS m⁻¹ salt stress, cv BTx623 accumulated the highest amount of Na+ in stem tissue, while the highest Na+ levels in cv HI10 and cv Spence were seen in roots (Fig. 2, A–C). Within Paspalum spp. leaf tissue, cv HI10 sequestered a significantly higher amount of Na+ in the leaf sheath relative to the leaf blade under both 10 and 30 dS m⁻¹ salt (Fig. 2C). This same trend was seen in cv Spence, though the difference was not statistically significant.

Potassium (K+) content (Fig. 2, D–F; Supplemental Fig. S1, D–F), which averaged 22,780 ppm across organs in cv BTx623, 34,767 ppm in cv HI10, and 19,428 ppm in cv Spence under freshwater, increased significantly in aboveground organs in cv BTx623 under 30 dS m⁻¹ salt (Fig. 2D), but remained largely constant in cv HI10 in all organs except stolons (Fig. 2F), where a significant decrease in K+ content was observed under salt. In cv Spence, K+ concentration was significantly lower in all aboveground tissues in plants grown under 30 dS m⁻¹ salt compared to freshwater (Fig. 2E).

Across organs, the K⁺/Na⁺ ratios in freshwater were comparable in cv BTx623 and cv Spence, with average ratios of 4,625.1 and 2,419.7, respectively, but were two orders of magnitude lower in cv HI10 (average of 61.8; Fig. 2, G–I). Ratios were consistently higher in aboveground tissues compared to roots (Supplemental Fig. S1, G–I). Salt stress led to a significant reduction in K⁺/Na⁺.
ratios in all accessions, but the fold change was much higher in cv BTx623 and cv Spence than in cv HI10. In fact, although significantly lower under freshwater, $K^+ / Na^+$ ratios in aboveground tissues of plants grown under 30 dS m$^{-1}$ salt were higher in cv HI10 (average of 3.1) than in either cv BTx623 (average of 1.0) or cv Spence (average of 1.8; Supplemental Fig. S1, G–I).

cv HI10 and cv Spence Exhibit Distinct Leaf Surface Morphologies

To further investigate the different salt accumulation patterns in cv HI10, cv Spence, and cv BTx623 under salt stress, we compared their leaf anatomy. Light microscopy indicated that the leaf sheath structure was similar in cv HI10, cv Spence, and cv BTx623 (Fig. 3, A, C, and E). In the leaf blade, however, unicellular extended epidermal cells, termed papillae, were observed above each major longitudinal vein in the $Paspalum$ spp. (Fig. 3, B and D), but not in cv BTx623 (Fig. 3F).

Abaxial and adaxial epidermal cell diameter did not significantly differ between salt treatments in $Paspalum$ spp. genotypes nor in cv BTx623 (Fig. 4, A and B). Average abaxial cell diameters were significantly higher in cv HI10 compared to cv Spence and cv BTx623, averaging 23.0 $\mu$m across treatments compared to 19.2 and 20 $\mu$m for cv Spence and cv BTx623, respectively (Fig. 4A). Significant differences in adaxial cell diameter were also observed between cv HI10, cv Spence, and cv BTx623, with cv HI10 averaging 32.3 $\mu$m across treatments compared to 24.8 $\mu$m and 21.5 $\mu$m in cv Spence and cv BTx623, respectively (Fig. 4B). The differences in epidermal cell size on the adaxial leaf side were even more striking when only papilla cells (topmost six cells above each major vein) were measured in cv HI10 and cv Spence, with cell diameters averaging 37.1 $\mu$m in cv HI10 papilla compared to 20.1 $\mu$m in cv Spence across treatments (Fig. 4C). The number of epidermal cells above each major vein was similar between cv HI10 and cv Spence, averaging 8.3 cells and 7.6 cells per major vein across treatments for cv HI10 and cv Spence, respectively (Fig. 4D).

Images obtained from a high-powered dissecting microscope indicated that the papillae are translucent and present in rows across the entire adaxial leaf surface (Fig. 5, A and B). Scanning electron microscopy (SEM) confirmed that the papillae were considerably larger in cv HI10 than in cv Spence (Fig. 5, C and D). SEM imaging of two additional $P. vaginatum$ accessions, cv 509018-3 and cv KC9, showed that both had large papillae that were similar in size to those of cv HI10 (Supplemental Fig. S2, A–D). The SEM images further revealed that the costal ridges with papillae overarched grooves containing stomata (Supplemental Fig. S2E).

The abaxial surfaces of cv HI10, cv 509018-3, cv KC9, and cv Spence carried microhair structures (Supplemental Fig. S3, A–D) that are morphologically similar to the bicellular trichomes found in the Panicoide grass Johnson-grass ($Sorghum halepense$; McWhorter et al., 1995). Their density was significantly higher on the abaxial surface in cv Spence than in the three $P. vaginatum$ accessions (Supplemental Fig. S3F). The microhairs were also observed on the adaxial surface in cv Spence (Supplemental Fig. S3E). They were not seen on the adaxial surface in the three $P. vaginatum$ accessions imaged, although it is possible that they were present but hidden by the large papillae. Adaxial microhair density in cv Spence was similar to the abaxial microhair density in the three $P. vaginatum$
accessions. No significant differences in density were observed between freshwater and salt treatments (Supplemental Fig. S3F).

In cv Spence, stomatal density was significantly higher on the adaxial side than on the abaxial side (Supplemental Fig. S3G). Although adaxial stomata were also present in *P. vaginatum* accessions, as can be seen in transverse leaf sections (Fig. 3B), they were mostly hidden by the large papillae on the SEM images, and hence not counted. Excluding cv 509018-3, which exhibited an increase in abaxial stomatal density under salt stress, no significant differences in stomatal density were observed between freshwater and salt treatments (Supplemental Fig. S3G).

### Contact Angle Measurements: A Proxy for Papilla Size

To determine whether papilla size affects leaf surface hydrophobicity, we measured the contact angle of water droplets placed on the adaxial and abaxial leaf surfaces of cv HI10, cv Spence, and cv BTx623. Adaxial leaf surfaces containing papillae were significantly more hydrophobic than the abaxial surfaces in cv HI10.
and cv Spence (Fig. 5E; Table 1). In cv BTx623, which lacks papillae, both surfaces showed similar levels of hydrophobicity (Fig. 5E; Table 1). The adaxial surface hydrophobicity was significantly higher in cv HI10 than in cv Spence, and both had significantly higher adaxial hydrophobicity than cv BTx623. In contrast, there were no differences in abaxial surface hydrophobicity between cv HI10 and cv Spence, and only marginal differences between cv BTx623 and the two Paspalum accessions. Adaxial and abaxial hydrophobicity measurements did not significantly differ between leaves from Paspalum spp. plants grown under freshwater (0 dS m\(^{-1}\)) and 30 dS m\(^{-1}\) salt (Supplemental Table S1).

To determine whether hydrophobicity was correlated with papilla size, contact angle measurements were made on two additional accessions, P. vaginatum ‘PI 299042’ and P. distichum ‘Tropic Shore’ (Table 1). In addition, we measured the width of a minimum of 100 papillae from these accessions, as well as from cv HI10 and cv Spence, from dissecting microscope images (Supplemental Fig. S4). cv PI 299042 and cv Tropic Shore have a leaf size and texture similar to those of cv Spence, and cv PI 299042 performs similarly to cv Spence under salt stress (Supplemental Fig. S5). Although cv PI 299042 is considered to belong to P. vaginatum, and cv Spence and cv Tropic Shore have been classified as P. distichum, genotyping analysis with simple sequence repeat (SSR) markers showed that the three accessions belonged to the same genetic subgroup within group Disticha. cv HI10 belonged to a different genetic population group (Eudy et al., 2017). Papillae in cv PI 299042 and cv Tropic Shore were comparable in size but were larger than those of cv Spence and smaller than those of cv HI10 (Supplemental Fig. S4A). Adaxial surface hydrophobicity in the four Paspalum spp. accessions was significantly positively correlated with mean papilla cell width (\(R = 0.98, P = 0.0162\)).

Leaf Papillae Act as a Na\(^+\) Sink under Salt Stress

Strings of papillae-enriched cells can easily be peeled from the Paspalum spp. leaf surface (Supplemental Fig. S6), allowing for further quantification of salt levels in papillae versus the rest of the leaf blade (referred to as “peeled leaves”). Ion levels were measured in P. distichum ‘Spence’ and P. vaginatum ‘HI10’, ‘509018-3’, and ‘PI 299042’ (Fig. 6). cv 509018-3 has large papillae (Supplemental Fig. S2D) and performs similarly to cv HI10 under salt stress (Supplemental Fig. S5). In freshwater, Na\(^+\) content was similar across peeled leaves and papillae-enriched peels in all four Paspalum accessions tested (cv Spence, cv HI10, cv 509018-3, and cv PI 299042; Fig. 6A). Under 30 dS m\(^{-1}\) salt stress, Na\(^+\) content was significantly higher in papillae-enriched peels than in underlying leaf tissue in both cv HI10 and cv 509018-3, while no significant differences were seen in cv Spence and cv PI 299042 (Fig. 6B). No significant differences were observed between accessions for Na\(^+\) content in underlying leaf tissue without papillae.

Under freshwater, K\(^+\) content in papillae-enriched peels was significantly higher than in peeled leaves in cv 509018-3 and cv HI10, while no significant differences were found for cv PI 299042 and cv Spence (Fig. 6C). All species exhibited significantly higher K\(^+\) levels in papillae-enriched peels compared with underlying tissue under 30 dS m\(^{-1}\) salt stress (Fig. 6D). K\(^+\) content in cv HI10 and cv 509018-3, however, was significantly higher in both tissue types compared with cv PI 299042 and cv Spence (Fig. 6D). No significant differences were found for the K\(^+/\)Na\(^+\) ratio across tissue fractions, nor across accessions, under freshwater or salt conditions (Fig. 6, E and F).

To confirm Na\(^+\) sequestration, the CoroNa Green sodium stain was used to stain peeled papillae in cv HI10 and cv Spence (Fig. 7). The significantly higher fluorescence of papillae from cv HI10 plants grown under 30 dS m\(^{-1}\) salt compared to those from plants grown under freshwater indicates that Na\(^+\) is present in cv HI10 papillae under salt stress (Fig. 7, C–E). In cv Spence papillae, however, similar levels of fluorescence were observed under both freshwater and salt conditions (Figs. 7, A, B, and E). Interestingly, high fluorescence was observed in cv Spence in the cell layer running underneath the costal ridges, irrespective of the presence of salt (Fig. 7, A and B). Energy dispersive spectroscopy (EDS) conducted on SEM samples illustrated that this cell layer contains elemental silicon, likely in the form of silica (Supplemental Fig. S7, A and B). Though more difficult to observe due to the large

Figure 3. Transverse images of the leaf sheath and leaf blade. Transverse sections of the leaf sheath (A, C, and E) and leaf blade (B, D, and F) in P. vaginatum ‘HI10’ (A and B), P. distichum ‘Spence’ (C and D), and sorghum cv BTx623 (E and F) grown in freshwater were imaged under a light microscope. Scale bars = 50 μm.
papilla size, siliconized cells were also observed in cv HI10 in the same location (Supplemental Fig. S7, C and D).

DISCUSSION

Ion Distribution Differs in cv BTx623, cv Spence, and cv HI10

We observed differences in Na\(^+\) and K\(^+\) levels at the whole-plant level as well as across organs in cv BTx623, cv Spence, and cv HI10 under both freshwater and salt stress conditions (Fig. 2), indicating that the mechanisms for dealing with Na\(^+\) may differ between these cultivars. A higher concentration of Na\(^+\) in roots than in leaves indicates that Na\(^+\) is being excluded from the aboveground tissues (Munns, 2005). Based on this criterion, sorghum ‘BTx623’ is a more efficient Na\(^+\) excluder than P. vaginatum ‘HI10’ under 10 dS m\(^{-1}\) salt, while the reverse is true at 30 dS m\(^{-1}\) salt (Fig. 2, A–C; Supplemental Fig. S1, A–C). The fact that Na\(^+\) levels were lower in roots than in aboveground tissues in cv BTx623 at 30 dS m\(^{-1}\) salt suggests that Na\(^+\) was transported from the roots to the stem and leaves. The high tissue Na\(^+\) levels likely caused, or at least contributed to, the eventual death of cv BTx623 under prolonged growth at 30 dS m\(^{-1}\) salt.

In addition to a reduced rate of Na\(^+\) transport from the roots to the shoots under high salt in cv HI10, we also noted that in leaf tissue, Na\(^+\) was significantly more concentrated in the leaf sheath than in the leaf blade (Fig. 2C). The same trend was seen in cv Spence and cv BTx623, but the difference was not statistically significant (Fig. 2, A and B). A higher capacity of the leaf sheath to extract and sequester Na\(^+\) has previously been associated with higher levels of salt tolerance in durum wheat (Triticum turgidum; Davenport et al., 2005).

In plants, ion homeostasis via K\(^+\) uptake is essential to maintain low concentrations of toxic Na\(^+\) ions under salt stress (Zhu, 2003). An ability to retain K\(^+\) has been linked to increased salt tolerance in a number of crop species (Chen et al., 2005; Wu et al., 2014; Gao et al., 2016). Surprisingly, in the glycophyte sorghum, we saw a significant increase in K\(^+\) concentrations in salt versus freshwater treatment (Fig. 2D). Some studies have made similar observations in sorghum (Ahmed et al., 2013; Almodares et al., 2014), while others noted a reduction in K\(^+\) under salt stress compared to freshwater conditions (Wang et al., 2014). One possible explanation for this discrepancy is that tissue K\(^+\) levels are linked to the amount of K\(^+\) available for uptake (Botella et al., 1997; Liu et al., 2013; Li et al., 2014). In our study, which used a proprietary sea salt mix (Oceanic 81050), the K\(^+\) levels were higher in the salt compared to the freshwater treatment. We do not know the precise concentration of K\(^+\) present in the Oceanic sea salt mix used in our salt treatments. However, this salt mix mimics the

Figure 4. Diameter of abaxial and adaxial epidermal cells. Graphs show the diameters of abaxial epidermal cells (A), adaxial epidermal cells (B), papilla cells (C), and the number of adaxial cells above each major vein (excluding stomatal and bulliform cells; D) in leaves of sorghum cv BTx623, P. distichum ‘Spence’, and P. vaginatum ‘HI10’ grown for 6 weeks under 0 (freshwater), 10, and 30 dS m\(^{-1}\) salt. Papilla cell diameter was determined by measuring the six topmost cells above each major vein. Values are represented as the mean ± SE per treatment for n = 162–227 cells (abaxial cell diameter), 199–260 cells (adaxial cell diameter), 72–100 cells (papilla cell diameter), and 18–24 major veins used for cell number counts. Lowercase letters above each bar indicate statistical significance at the level of P < 0.05 as indicated by one-way ANOVA analyses with Tukey’s honestly significant difference (HSD) mean-separation test conducted by cell type across accessions and salt treatments.
composition of seawater, which has an average of 10.2 mM K$^+$ (Atkinson and Bingman, 1997). We therefore estimate K$^+$ to be present at a concentration of 2.0 and 6.1 mM in 10- and 30-dS m$^{-1}$ salt solutions, respectively (seawater has a conductivity of 50 dS m$^{-1}$ [FAO, 1992]). The amount of K$^+$ in the fertilizer added to both the freshwater and salt treatments was 0.73 mM. Providing supplementary K$^+$ under salt treatments has been shown to increase K$^+$ levels in tissues, decrease Na$^+$ levels, and increase the K$^+$/Na$^+$ ratio (Botella et al., 1997). However, despite the increased K$^+$ uptake under salt, K$^+$/Na$^+$ ratios dropped significantly under salt stress in cv BTx623. A similarly large drop in the K$^+$/Na$^+$ ratio was seen in cv Spence. In cv HI10, the K$^+$/Na$^+$ ratio also decreased under salt, but the difference between the K$^+$/Na$^+$ ratio in freshwater compared to 30 dS m$^{-1}$ saltwater was several orders of magnitude smaller than in cv Spence and cv BTx623. This was mainly caused by the high K$^+$ level already present in cv HI10 under freshwater conditions (Fig. 2F; Supplemental Fig. S1D). Active K$^+$ uptake has a considerable energy cost, requiring energy produced through respiration in root tissue (Kant et al., 2005). We hypothesize that the superior salt tolerance of cv HI10 may be due in part to this accession having a largely constitutive response rather than an induced response to saline conditions. A constitutive response could explain, or contribute to, the slow growth of cv HI10 relative to cv Spence under freshwater conditions. In contrast to sorghum, no increases in tissue K$^+$ levels

### Table 1. Mean contact angle measurements of static water droplets on the adaxial and abaxial leaf surfaces

<table>
<thead>
<tr>
<th>Cultivar (Species)</th>
<th>Adaxial Contact Angle</th>
<th>Abaxial Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>'HI10' (P. vaginatum)</td>
<td>124.65 ± 1.77 a</td>
<td>63.35 ± 1.87 d</td>
</tr>
<tr>
<td>'PI 299042' (P. vaginatum)</td>
<td>110.08 ± 2.60 b</td>
<td>63.55 ± 1.32 d</td>
</tr>
<tr>
<td>'Tropic Shore' (P. distichum)</td>
<td>114.38 ± 0.86 b</td>
<td>64.12 ± 1.23 d</td>
</tr>
<tr>
<td>'Spence' (P. distichum)</td>
<td>101.76 ± 1.20 c</td>
<td>65.20 ± 1.39 d</td>
</tr>
<tr>
<td>'BTx623' (S. bicolor)</td>
<td>61.80 ± 1.18 d</td>
<td>60.81 ± 1.30 d</td>
</tr>
</tbody>
</table>

Values shown represent means ± st. Lowercase letters indicate statistical significance at the level of $P < 0.05$ as indicated by one-way ANOVA analyses using Tukey's honestly significant difference (HSD) mean separation test. $n = 9–15$ measurements per accession per treatment.
were observed in either cv Spence or cv HI10 under salt, despite the higher K$^+$ levels present in the saltwater versus the freshwater treatment. K$^+$ levels significantly decreased under 30 dS m$^{-1}$ salt versus freshwater conditions in aboveground tissue in cv Spence and in stolons in cv HI10, but remained largely constant in the leaf blade and sheath of cv HI10 (Fig. 2, E and F), the two aboveground tissues with the highest concentrations of Na$^+$ (Fig. 2C). This suggests that in contrast to sorghum, uptake efficiency of K$^+$ in *P. vaginatum* ‘HI10’ is largely independent of both K$^+$ level and Na$^+$ level in the irrigation solution.

**Adaxial Leaf Papillae Sequester Salt in Turf-Type *P. vaginatum***

Imaging of the adaxial surface of leaves from several *P. vaginatum* and *P. distichum* cultivars showed the presence of dense rows of papillae atop costal ridges. Papilla size varied significantly across accessions. ‘HI10’, ‘KC9’ and ‘509018-3’, three turf-type *P. vaginatum* cultivars with a fine leaf texture that belonged to the same genetic subpopulation based on 43 SSR markers (Eudy et al., 2017), had similar-sized large papillae, while ‘Spence’, a *P. distichum* cultivar, which belonged to a different genetic subpopulation, had broad leaves and significantly smaller papillae (Supplemental Fig. S2, A–D). cv Tropic Shore and cv PI 299042, two other broad-leaved accessions that had patterns of genotypic variation similar to those of cv Spence and had been identified as *P. distichum* and *P. vaginatum*, respectively (Eudy et al., 2017), had intermediate-sized papillae (Supplemental Fig. S4). In our salt screens, cv HI10 and cv 509018-3 increased growth under salt compared to freshwater, a characteristic typical of halophytes, while cv Spence and cv PI 299042 behaved more like glycophytes.

Figure 6. Ion levels in peeled papillae and leaf peels. Na$^+$ content (A and B), K$^+$ content (C and D), and K$^+$/Na$^+$ ratio (E and F) in peeled leaf and papillae-enriched fractions for *P. distichum ‘Spence’* and *P. vaginatum ‘HI10’, ‘509018-3’, and ‘PI 299042’ grown under freshwater (0 dS m$^{-1}$; A, C, and E) and 30 dS m$^{-1}$ salt (B, D, and F). Lowercase letters above each bar indicate statistical significance at the level of $P < 0.05$ as indicated by one-way ANOVA analyses with Tukey’s honestly significant difference (HSD) mean-separation tests conducted by ion type within treatments. All values shown represent means ± se.
with growth decreasing under salt (Supplemental Fig. S5).

There is debate as to whether *P. vaginatum* and *P. distichum* are indeed separate species. They have historically been distinguished by the presence of glabrous (*P. vaginatum*) or pubescent glumes (*P. distichum*; Echarte and Clausen, 1993). cv PI 299042 was shown by Eudy et al. (2017) to have glabrous glumes, as did cv HI10 and cv 509018-3, which led to classification of these accessions as *P. vaginatum*. cv Spence and cv Tropic Shore, on the other hand, were shown to have pubescent glumes, and were classified as *P. distichum* (Eudy et al., 2017). However, in a population structure analysis with SSR markers, cv PI 299042 clustered with cv Spence and cv Tropic Shore (Eudy et al., 2017). This subpopulation consisted of both *P. distichum* and *P. vaginatum* accessions with mixed leaf texture and glume pubescence phenotypes. Our results indicate that this subpopulation may have small or intermediate-sized papillae compared to turf-type accessions like cv HI10 (large papillae) and cv 509018-3, which belong to a distinct subpopulation grouping is based on a small number of accessions distributed across the different Distichia genetic subpopulations.

To determine whether the papillae played a functional role in salt sequestration, papillae peels from cv HI10 (large papillae) and cv Spence (small papillae) were incubated with the Na\(^+\) stain CoroNa Green. A significant increase in fluorescence of the papillae was seen in cv HI10, but not in cv Spence, when plants were grown in 30 dS m\(^{-1}\) salt compared to freshwater (Fig. 7). This supports our hypothesis that papillae can sequester salt, and it is congruent with a recent study that detected high Na\(^+\) concentrations in “bladder-like” structures on the adaxial leaf surface of *P. vaginatum* ‘UGP3’ using SEM-EDS (Chavarria et al., 2020). However, our study also indicated that the ability to sequester Na\(^+\) might be determined at least partly by papilla size. When grown under 300 mM salt stress in a separate experiment, cv 509018-3 and cv PI 299042 showed patterns of staining and fluorescence intensity similar to those of cv HI10 and cv Spence, respectively (Supplemental Fig. S8). Although CoroNa Green staining was repeated multiple times with similar results, we could not exclude that the lack of fluorescence in the cv Spence and cv PI 299042 papillae was caused by inefficient uptake of CoroNa Green. This may have been exacerbated by the incorporation of dye in the silicon-containing cells in cv Spence and cv PI 299042, a phenomenon likely unrelated to Na\(^+\) content, as it was observed under both freshwater and salt conditions (Fig. 7, A and B; Supplemental Fig. S8, E–H). We therefore measured Na\(^+\) levels directly in peeled papillae and leaf peels of cv HI10, cv 509018-3, cv Spence, and cv PI 299042 (Fig. 6). Na\(^+\) concentrations were significantly higher in the papillae peels than in the peeled leaf in accessions with large papillae (cv HI10 and cv 509018-3) when plants were grown under 30 dS m\(^{-1}\) salt (Fig. 6B). The same trend was seen in cv Spence and cv PI 299042, although the difference was not statistically significant, supporting the results from the CoroNa Green staining that Na\(^+\) is sequestered in the large papillae of cv HI10 and cv 509018-3 but not, or to a lesser extent, in the smaller papillae of cv Spence and cv PI 299042. A differential capacity of structurally similar glands to sequester/secrete salt has also been reported in the Chloridoideae (Maricle et al., 2009; Dassanayake and Larkin, 2017).

**Leaf Papillae and Their Roles across Grasses**

Papillae are not unique to *Paspalum* spp. (Ensikat et al., 2011; Duarte-Silvá et al., 2013). Indeed, they are
found on the leaf surface atop costal ridges and within intercostal grooves in many Poaceae subfamilies, including the Chloridoideae (Milby, 1971; Barhoumi et al., 2008), Oryzioideae (Flowers et al., 1990), Bambusoideae (Leandro et al., 2017; Zhang et al., 2017), Pooidae (Macfarlane and Watson, 1982; Ospina et al., 2015), and Panicoideae (Sanyal et al., 2006; Aliscioni et al., 2016). A striking difference, however, between the papillae in our study species and those in other grasses is their large size and high density. The structures that the *P. vaginatum* papillae most strongly resemble are the adaxial salt-secreting unicellular hairs found in *P. coarctata*, a wild rice species (Flowers et al., 1990; Sengupta and Majumder, 2010). Similar to *Paspalum* spp., *P. coarctata* contains costal ridges and grooves, but in stark contrast to *Paspalum* spp., the papillae-like hairs in *P. coarctata* are found in the grooves instead of atop costal ridges (Flowers et al., 1990).

In grasses, virtually all salt glands and microhairs shown to play a role in salt tolerance are bicellular (Ceccoli et al., 2015). In the Panicoideae, only a few examples of these structures functionally sequestering or secreting salt exist. Leaf washes indicated that bicellular microhairs may secrete Na⁺ in maize (Ramadan and Flowers, 2004). Johnsongrass was able to secrete Na⁺ from bicellular trichomes, but only when grown on high lime soil (McWhorter et al., 1995). To date, no grass species except *Paspalum* spp. and *P. coarctata* have been shown to sequester or secrete salt using densely populated unicellular structures. The mechanisms behind Na⁺ loading into both the adaxial unicellular salt hairs of *P. coarctata* and the papillae of *P. vaginatum* are unknown. The presence of papillae with a similar unicellular structure does not necessarily equate to both species having similar Na⁺ sequestration/secretion mechanisms. Nevertheless, the prevalence of costal/intercostal leaf papillae and the evolution of parallel Na⁺ sequestration structures in the Oryzioideae and Panicoideae grass subfamilies make it likely that other salt-tolerant grass species have co-opted them for similar roles.

In addition to salt sequestration, the leaf papillae in *Paspalum* spp. may act with the ridge-and-furrow structure of the leaf to control the transpiration rate. Grasses contain large thin-walled bulliform or motor cells that contract under water deficit, which results in leaf folding (Redmann, 1985). In the genus *Spartina* (Chloridoideae), saltwater species differ from freshwater species in that their stomata are predominantly located on the adaxial side of the leaf and are overarched by large ridges, which fit together during leaf rolling. These adaptations to saline environments allow plants to modulate water loss under stress conditions (Maricle et al., 2009). Similarly, ridges on *Paspalum* spp. leaves fit together when leaf rolling occurs (Ellis, 1974), and stomata on the adaxial leaf side are covered by papillae (Supplemental Fig. S2E). Stomatal density on the adaxial surface was not measured in *P. vaginatum* ‘H110’ due to obstruction by the large papillae. However, similar to saltwater *Spartina* species, cv Spence had a significantly higher adaxial than abaxial stomatal density (Supplemental Fig. S3G). *Paspalum* species may therefore modulate water loss through adaxial stomata using their ridge-and-furrow leaf structure. The larger papillae and ridges present in cv H110 are likely more efficient in preventing water loss than the smaller structures in cv Spence.

**Bicellular Microhairs in *P. vaginatum* and *P. distichum* Are Reminiscent of Inactive Salt Glands**

It has previously been reported that *P. distichum* has bicellular microhairs that have the same basic structure as the salt glands in the Chloridoideae (Liphschitz and Waisel, 1982). Our SEM images show that *P. distichum* ‘Spence’ carries bicellular microhairs on both the adaxial and abaxial surfaces (Supplemental Fig. S3). In *P. vaginatum*, these microhairs were observed only on the abaxial surface, but they may have been obscured by the large papillae on the adaxial side. The existence of the gland-like structures on the leaf surface in *P. vaginatum* and *P. distichum* is intriguing, because neither *P. vaginatum* nor *P. distichum* exhibits secretion capacity under salt stress (Liphschitz and Waisel, 1982; Chen et al., 2009). It is possible that they are inactive salt glands, similar to the inactive salt glands present on the abaxial leaf side in the Chloridoideae *Zoysia* (Marcum and Murdoch, 1990). The length of the microhairs in *Paspalum* (30–40 µm; Supplemental Fig. S3) is comparable to the size of the salt glands in *Zoysia matrella* and somewhat bigger than those in *Zoysia japonica* (35.2 ± 0.77 and 20.5 ± 0.47 µm, respectively; Marcum and Murdoch, 1990). Salt gland density varies significantly both between and within *Zoysia* species (Yamamoto et al., 2016), and while the abaxial microhair density in the broad-leaved *P. distichum* ‘Spence’ was about 2-fold higher than in the three fine-leaved *P. vaginatum* accessions analyzed (cv H110, cv 509018-3, and cv KC9; Supplemental Fig. S3F), the density in both *Paspalum* species fell within the range observed in *Zoysia* spp. Further work is needed to elucidate whether the glandular structures in *Paspalum* spp. play a role in salt stress tolerance.

**CONCLUSION**

Our study shows that *P. vaginatum* ‘H110’, which shows increased growth under saline conditions, differs from *P. distichum* ‘Spence’, which shows a decrease in growth under salt treatment, in a number of characteristics that may contribute to their differential salt response. cv H110 has high levels of K⁺ under freshwater conditions, which likely helps to mitigate the effects of increased Na⁺ uptake under salt. Although cv Spence appears to be better than cv H110 at excluding Na⁺ from the shoot, the high steady-state level of K⁺ in cv H110 results in K⁺/Na⁺ ratios that are significantly
higher in aboveground tissues in cv HI10 than in cv Spence. In addition to being able to maintain a better ion homeostasis, cv HI10 has large papillae across the adaxial leaf surface that sequester Na\(^+\) under salt stress. No significant sequestration was observed in the smaller papillae that adorned the adaxial surface in cv Spence.

Our study raises the question of whether there is a link between papilla size and an ability to act as a Na\(^+\) sink. While it is very time consuming to measure papilla size from bright-field images, and very expensive to conduct SEM on large numbers of samples, the high correlation observed between papilla size measured in four accessions and the level of hydrophobicity as determined by drop contact angle measurements suggests that the latter can be used as a proxy for papilla size. Availability of a fast and efficient approach to measuring papilla size would also pave the way to study the genetics of papilla size and Na\(^+\) sequestration in these structural adaptations in *Paspalum* spp.

In summary, our study provides evidence of unicellular Na\(^+\)-sequestration structures in the Panicoideae, an economically crucial family of plants. Future studies will focus on the underlying mechanisms behind Na\(^+\) loading into papilla cells and will provide the groundwork to determine whether these mechanisms can be leveraged in salt-sensitive crops currently unable to sequester Na\(^+\) in leaf surface structures.

**MATERIALS AND METHODS**

**Plant Material and Growth**

*Paspalum vaginatum* ‘HI10’, ‘509018-3’, ‘K9’, and ‘PL299042’, and *Paspalum distichum* ‘Spence’ and ‘Tropic Shore’ were obtained from Paul Raymer at the University of Georgia-Griffin. Source material was maintained in 12-inch pots. Individual ramets of *Paspalum* spp. were grown via nodal propagation. Single nodes with 1.5 to 2 cm of surrounding stolon tissue were cut from growing stolons. Stolon segments were placed in a tray of soil, consisting of 3 parts sand, 1 part pine bark mix (mix of 500 L fine-grade pine bark, 2 L coarse vermiculite, 500 mL superphosphate, 250 mL potassium nitrate, 250 mL calcium nitrate, 250 mL gypsum, and 250 mL Micromax micronutrient blend), with the node below the soil line. After 1 week, nodes with emerging shoot tissue were transferred to UV-stabilized SC-10 (164 mL) Ray Leach Cone-tainers (Stuewe and Sons). The cone-tainers for each genotype were randomly placed in 98-cell trays under freshwater irrigation, we initiated salt treatments by irrigating with a 1-mM NaCl solution consisting of a sea salt mix (Oceanic 81050) with added magnesium sulfate. Leaf blades (second fully developed leaf on a growing stolon segment) from *P. vaginatum* ‘HI10’, ‘509018-3’, ‘K9’, and ‘PL299042’, and *P. distichum* ‘Spence’ grown under freshwater (0 dS m\(^{-1}\)) and salt stress (30 dS m\(^{-1}\)) for 6 weeks were excised using a razor blade, placed in vials of fixative containing 2.5% (v/v) glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2), and stored overnight at 4°C. Two leaves per accession per treatment were analyzed. Samples were washed with buffer and postfixied for 2 h at 4°C in similarly buffered 1% (v/v) osmium tetroxide. Samples were then rinsed in distilled water, dehydrated in a graded ethanol series (25%, 50%, 75%, 95%, and 100% [v/v]) and critical point dried using a Samdri model 780-A Critical Point Dryer (Tousimis). Samples were mounted on sticky carbon tabs placed on top of aluminum stubs, sputter-coated with gold-palladium (EM Ace 600 Sputter Coater, Leica), and viewed using a FEI FE-SEM Teneo Scanning Electron Microscope (Thermo Fisher Scientific) operating at 10 kV. For EDS elemental analysis, full EDS spectra comprising all detectable elements were acquired. Subsequently, EDS maps were examined for elements of interest. Abaxial and adaxial microwhiskers and stomata were counted within a 500-μm\(^2\) area on three to six SEM images for each accession grown under 0 and 30 dS m\(^{-1}\) salt conditions.

**Light Microscopy**

Leaf blades (second fully developed leaf on a growing stolon segment) from *P. vaginatum* ‘HI10’, ‘509018-3’, ‘K9’, and ‘PL299042’, and *P. distichum* ‘Spence’ grown under freshwater ebb-and-flow bins for 6 weeks were analyzed by light microscopy. The second fully developed leaf from a growing stolon segment of *Paspalum* species or a sorghum plant was excised from three independent plants and fixed in 2% (v/v) formaldehyde for 48 h. Peeled leaves and papilla-enriched fractions were generated from a second set of leaves of the same accessions, except that papillae were scraped from the surface prior to fixation. Leaf sheaths were also sampled from the same numbered leaf for both *Paspalum* species and sorghum. Samples were dehydrated in a graded ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, and 100% [v/v]) and critical point dried using a Samdri model 780-A Critical Point Dryer (Tousimis). Samples were mounted on sticky carbon tabs placed on top of aluminum stubs, sputter-coated with gold-palladium (EM Ace 600 Sputter Coater, Leica), and viewed using a Reichert UltraCut ES microtome and stained with 0.1% (v/v) toluidine blue. Samples were then examined using a bright-field light microscope. For dissecting microscope imaging, the same numbered leaf was excised from a growing stolon fragment from three independent plants per accession and immediately visualized using Z-stack imaging on a Leica DVM 6 digital microscope.

Epidermal cell diameter, papilla cell diameter, and cell number above each major vein were counted on images of sections taken at 20× magnification from three leaves per accession grown at 0, 10, and 30 dS m\(^{-1}\) for 6 weeks, each from an independent plant. Adaxial and abaxial epidermal cell size was determined by measuring the cell diameter at the widest point for each cell. Papilla cell diameter was measured using the same specifications, but only measuring the six topmost cells above each major vein. All adaxial cells above each major vein excluding bulliform cells and stomatal guard cells were counted to determine...
the cell number above each major vein. Papilla cell width was measured from images obtained from the Leica DVM 6 digital microscope.

Sodium Staining

CoroNa Green (Invitrogen) was used to stain papillae for Na⁺ content. The stain was first reconstituted in anhydrous dimethyl sulfoxide at 150 μM and then diluted in water to 15 μM for staining. CoroNa Green has absorbance and emission spectra of 492 and 516 nm, respectively. Papillae were peeled from the adaxial surface of leaves (second fully developed leaf on the growing stolon segment) from plants grown under freshwater (0 dS m⁻¹) and salt stress (30 dS m⁻¹) for 6 weeks. Strings of papillae were incubated with the dye for 2 h in the dark, rinsed with water, and then mounted in 80% (v/v) glycerol for imaging. Samples were taken from three independent plants per genotype per treatment. Samples from the same leaves were also incubated in deionized water as a negative control. Imaging was conducted using a Zeiss LSM 880 upright confocal microscope using a 20× dry objective with the excitation wavelength set at 488 nm and emission detected at 516 nm using an argon laser. Z-stack imaging was conducted to obtain a three-dimensional view of the papillae. Using ImageJ, fluorescence intensity of papilla cells in cv HI10 and cv Spence normalized to background levels was calculated for three images (15–20 papilla on each image) per accession per treatment, each from a separate ramet.

Contact Angle Measurements

The second fully developed leaf from a growing stolon segment of *Paspalum* species or a sorghum plant growing in freshwater (0 dS m⁻¹) and salt (30 dS m⁻¹) for ~2.5 months was used to determine leaf surface hydrophobicity. Three leaves, each from an independent plant, were excised and used for analysis. Each leaf was cut lengthwise, and one half was used for adaxial measurements while the other half was used for abaxial measurements. To assess hydrophobicity, three to five drops of 1 μL water were pipetted on each adaxial and abaxial leaf surface for a total of 9 to 15 measurements per accession per treatment. Contact angles were measured using a Kruss DSA 100 Drop Shape Analyzer.

Statistical Analysis

One-way ANOVA analyses with Tukey’s honestly significant difference (HSD) mean-separation tests were conducted to determine statistical significance at the level of $P < 0.05$.

Supplemental Material

The following supplemental materials are available.

**Supplemental Figure S1.** Ion levels in different organs of *Paspalum* species and *S. bicolor*.

**Supplemental Figure S2.** Papillae are present on the adaxial leaf surface of *Paspalum* species.

**Supplemental Figure S3.** Microhair structures are present on both the abaxial and adaxial leaf surfaces of *Paspalum* species.

**Supplemental Figure S4.** Variation in papilla size across *Paspalum* spp. accessions.

**Supplemental Figure S5.** Growth response of plants under freshwater conditions and salt stress.

**Supplemental Figure S6.** Imaging of papillae peeled from the adaxial leaf surface.

**Supplemental Figure S7.** Silicon EDS maps of the adaxial leaf surface.

**Supplemental Figure S8.** Na⁺ localization by CoroNa Green staining.

**Supplemental Table S1.** Mean contact angle measurements of static water droplets on the adaxial and abaxial leaf surfaces for *P. vaginatum* ‘HI10’ and *P. distichum* ‘Spence’ grown in freshwater (0 dS m⁻¹) and 30 dS m⁻¹ salt.

ACKNOWLEDGMENTS

The authors thank Beth Richardson and John Shields (Georgia Electron Microscopy Lab at the University of Georgia [UGA]) for their assistance with SEM/EDS and Jason Locklin (UGA New Materials Institute) for assistance with drop contact angle measurements. The authors also thank Paul Raymer (UGA-Griffin Campus) for providing the Paspalum genotypes used in this study and David Jespersen (UGA-Griffin Campus) for assistance with ion measurements.

Received June 19, 2020; accepted October 7, 2020; published October 20, 2020.

LITERATURE CITED


