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Diversity in leaf anatomy, and stomatal distribution and conductance, between salt marsh and freshwater species in the C_4 genus *Spartina* (Poaceae)

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Summary

- Leaf anatomy, stomatal density, and leaf conductance were studied in 10 species of *Spartina* (Poaceae) from low versus high salt marsh, and freshwater habitats.
- Internal structure, external morphology, cuticle structure, and stomatal densities were studied with light and electron microscopy. Functional significance of leaf structure was examined by measures of CO_2 uptake and stomatal distributions.
- All species have Kranz anatomy and C_4 $\delta^{13}C$ values. Freshwater species have thin leaves with small ridges on adaxial sides and stomata on both adaxial and abaxial sides. By contrast, salt marsh species have thick leaves with very pronounced ridges on the adaxial side and stomata located almost exclusively on adaxial leaf surfaces. Salt marsh species also have a thicker cuticle on the abaxial than on the adaxial side of leaves, and CO_2 uptake during photosynthesis is restricted to the adaxial leaf surface.
- Salt marsh species are adapted to controlling water loss by having stomata in leaf furrows on the adaxial side, which increases the boundary layer, and by having large leaf ridges that fit together as the leaf rolls during water stress. Differences in structural–functional features of photosynthesis in *Spartina* species are suggested to be related to adaptations to saline environments.

Introduction

Coastal salt marshes have many abiotic stresses that change with elevation in the intertidal zone. At low elevations, tidal inundations are frequent and long in duration (Pennings *et al.*, 2005). At higher elevations, tidal inundations are less frequent. This often leads to soil drying between tidal cycles and a consequent increase in soil salinity (Pennings & Callaway, 1992). Marsh salinity gradients have been shown to influence species distributions (Crain *et al.*, 2004) and to affect leaf size in individual plants (Maricle *et al.*, 2007a).

High salinity can be damaging to plants, both by salt toxicity and by dehydration caused by low water potential. Plants that live in saline, high-light environments may thus be adapted to minimize water loss to prevent dehydration. Although there have been some attempts to investigate changes in the ultrastructure of grass leaves in response to salinity (Barhoumi *et al.*, 2007), there have been few studies linking grass leaf micromorphology and anatomy to environment (Abernethy *et al.*, 1998; Gielwanowska *et al.*, 2005).

In Poaceae, there are clearly systematic differences with respect to leaf morphology and anatomy (especially epidermal organization; Thomasson, 1978; Ellis, 1979, 1986) which have long been ascribed to systematic groups (Metcalfe, 1960; Grass Phylogeny Working Group, 2001). Many adaptations of leaves can also be attributed to environmental conditions, particularly with water stress. In order to study these relationships with respect to salinity, we investigated leaf structure and gas exchange in salt marsh and freshwater species of *Spartina* (Poaceae), which are dominant macrophytes in many salt marshes in North America (Teal & Teal, 1969). There are at least 13 *Spartina* species (Mobberley, 1956), including low to high intertidal species and freshwater species, suggesting a large degree of variability in environmental tolerance and making it an ideal system to study adaptations to salinity within closely related species.

Table 1 A list of *Spartina* species used in this study, including the collection location, as well as chromosome counts (2*n*), the phylogenetic clade in which they occur (Baumel *et al*., 2002; Fortune *et al*., 2008) and an environmental grouping according to the habitat where they grow

Spartina species	2*n*	Clade (Baumel *et al*., 2002)	Collected from	Natural habitat
S. alterniflora Loisel.	62[b]	I	Willapa Bay, WA, USA	Low marsh[AB]
S. anglica C.E. Hubbard	122, 124[a]		Livingston Bay, WA, USA	Low marsh[C]
S. argentinensis (Trin.) Parodi	40[b]	I	Argentina	High marsh[A]
S. bakeri Merr.	40[b]	II	Sapelo Island, GA, USA	High marsh[A]
S. densiflora Brongn.	70[c]		Odiel Salt Marshes, Spain	High marsh[ADEF], Low to high marsh[G]
S. patens (Aiton) Muhl.	40[b]	II	Panacea, FL, USA	High marsh[AB]
S. spartinae (Trin.) Merr. ex A.S. Hitchc.	40[d]		Galveston Bay, TX, USA	High marsh[A]
S. cynosuroides (L.) Roth	40[b]	II	Sapelo Island, GA, USA	Brackish marsh, Freshwater[AHI]
S. pectinata Bosc ex Link	40[b]	II	Stafford County, KS, USA	Freshwater[A]
S. gracilis Trin.	40[b]		Grant County, WA, USA	Freshwater[A]

Natural habitat designations follow Mobberley (1956) and other sources as indicated.
2*n*: [a]Marchant (1968a); [b]Marchant (1968b); [c]Fortune *et al*. (2008); [d]Reeder (1968).
Natural habitat: [A]Mobberley (1956); [B]Bertness (1991); [C]Frenkel (1987); [D]Vicari *et al*. (2002); [E]Castillo *et al*. (2000); [F]Bortolus (2006); [G]Costa *et al*. (2003); [H]Higinbotham *et al*. (2004); [I]Stribling (1997).

Studies on leaf structure within *Spartina* are limited, and mostly restricted to a few species in relation to Kranz anatomy and C_4 photosynthesis (Long *et al*., 1975; Koyro & Huchzermeyer, 2004). Thus, there have been no studies that compare structure and function, relative to habitat differences and phylogeny. Since leaf characteristics are generally considered a product of environment (Dickison, 2000), specific adaptations are expected to enable plants to survive in saline sediments. To evaluate this in *Spartina*, leaf forms and stomatal features were examined by light microscopy and scanning and transmission electron microscopy in 10 species of *Spartina* adapted to different habitats. The functional significance of leaf anatomy was tested in some species by measuring photosynthesis rates on adaxial and abaxial leaf surfaces in relation to stomatal distribution and leaf conductance to water vapor.

Materials and Methods

Plant material

Spartina grasses were collected from field sites listed in Table 1. Species identities were verified against herbarium specimens and Flora of North America keys. All species were grown in potting soil in 11 × 11 cm pots, and were maintained under glasshouse conditions on the campuses of both Fort Hays State University (Hays, KS, USA) and Washington State University (Pullman, WA, USA). Plants were watered as needed with tap water.

Light microscopy and transmission electron microscopy (TEM)

All microscopy work on leaves was performed on the middle of the first fully-expanded mature leaf, usually the third leaf from the apex. Samples were fixed at 4°C in 2% (v : v) paraformaldehyde and 2% (v : v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), postfixed in 2% (v : v) OsO_4, and then, after a standard acetone dehydration procedure, embedded in Spurr's epoxy resin. Cross-sections were made on a Reichert Ultracut R ultramicrotome (Reichert-Jung GmbH, Heidelberg, Germany). For light microscopy, semithin sections (0.8–1.0 μm thick) were stained with 1% (w : v) Toluidine blue O in 1% (w : v) $Na_2B_4O_7$. Ultra-thin sections (70–100 nm thick) were stained for TEM with 2% (w : v) uranyl acetate followed by 2% (w : v) lead citrate. Hitachi H-600 (Hitachi Scientific Instruments, Mountain View, CA, USA) and JEOL JEM-1200 EX (JEOL USA, Inc., Peabody, MA, USA) TEM were used for observation and photography.

Thickness of cell walls and cuticle were measured on TEM micrographs of leaf cross-sections with UTHSCSA, Image Tool for Windows (version 3.00; University of Texas Health Science Center, San Antonio, TX, USA). Leaf thickness and distance between veins were measured on light micrographs of leaf cross sections using Image Tool or Adobe Photoshop (version 7.0; Adobe Systems, San Jose, CA, USA), calibrated against a stage micrometer. Maximal leaf thickness was the distance from the abaxial epidermis to the top of the tallest adaxial leaf ridge. Minimal leaf thickness was the distance from the abaxial epidermis to the bottom of the adaxial leaf furrows. Leaf measurements were performed on four individuals per species.

To distinguish between silica cells and cork cells in *Spartina* (both are 'short cells' and appear similar under the microscope), paradermal sections were made of the abaxial and adaxial leaf epidermis from all species. Sections were stained with 0.1% (w : v) Sudan IV, which stains cork cells red.

Scanning electron microscopy

The first fully-expanded, mature leaves from each species were fixed in 5% (v : v) glutaraldehyde in 0.1 M phosphate

buffer (pH 7.0) and then dehydrated in an ethanol series. Following this, leaves were cryofractured under liquid nitrogen with forceps. Specimens were then immersed in steps of increasing hexamethyldisilazane (HMDS) concentration (Oshel, 1997). Samples were mounted on a brass plate and were sputter-coated with gold and platinum for 3 min in a SPI Sputter (SPI Supplies, West Chester, PA, USA). Specimens were examined in an ISI-SX30 scanning electron microscope (International Scientific Instruments, Prahran, Victoria, Australia) at 15 kV.

Photosynthesis measures

Leaf photosynthesis rates were measured in a FastEst gas exchange system (Tartu, Estonia) as described by Laisk & Edwards (1998). Intercellular CO_2 concentration in the leaf (C_i) was calculated from the rate of photosynthesis, the CO_2 concentration in the air, and the diffusive resistance of CO_2 from the atmosphere to the intercellular space (for description see Ku *et al.*, 1977; von Caemmerer & Farquhar, 1981). The CO_2 uptake rates and transpiration rates, measured at air flow rates through the cuvette at 0.5 mmol s^{-1}, allowed calculation of leaf conductance to water vapor (g_v, mmol m^{-2} s^{-1}). This value represents the gas-phase conductance from air outside the leaf to the intercellular cell walls. Values for g_v are presented instead of stomatal conductance (g_s) because of the long path length to reach stomata through the boundary layer. At the flow rate in the cuvette, the calculated boundary layer conductance was 830 mmol m^{-2} s^{-1}. The CO_2 compensation point was determined by extrapolating the CO_2 response curve through the *x*-axis and taking the zero intercept for CO_2 assimilation.

Rates of photosynthesis were measured from intake of CO_2 from the abaxial versus adaxial side of leaves. Abaxial and adaxial surfaces were isolated by taping one side with Scotch tape. Leaves were illuminated from the taped side, and the measured (nontaped) side faced the inside of the chamber where gas circulated around it. Using an intact plant, the middle part of the first fully expanded mature leaf (the third visible leaf) was enclosed in the chamber and illuminated with a photosynthetic photon flux density (PPFD) of 780 μmol quanta m^{-2} s^{-1}, 25°C, and 21% O_2. $A : C_i$ response was measured at CO_2 concentrations from 0 to 1500 μbar. Measurements were made on two separate plants for each species.

Leaf conductance

The g_v was measured on adaxial and abaxial leaf surfaces. Measurements were made with a SC-1 Leaf Porometer (Decagon Devices, Inc., Pullman, WA, USA) during mid-day hours on clear days. The first fully-expanded, mature leaf was measured at locations 3–5 cm from the base of the lamina. Leaves from at least six individuals were measured for each species. Owing to sample-to-sample variation in leaf conductance, measurements are expressed as the ratio of adaxial g_v (g_{vad}) to abaxial g_v (g_{vab}).

Leaf carbon isotope composition

Measures of leaf $\delta^{13}C$ values relative to Pee Dee Belemnite limestone were determined (Bender *et al.*, 1973). Entire leaves were sampled from three separate individuals from each species, dried at 60°C for 24 h, and milled to a fine powder. Samples (2 mg) were placed in tin capsules and combusted in a Eurovector elemental analyser. Nitrogen (N_2) and CO_2 gases were separated by gas chromatography and admitted into the inlet of a Micromass Isoprime isotope ratio mass spectrometer for determination of $^{13}C : {}^{12}C$ ratios (R). The $\delta^{13}C$ values were determined as δ (‰) = $1000 \times [(R_{sample}/R_{standard}) - 1]$.

Leaf stomatal counts

Numbers of stomata were measured using different techniques for abaxial versus adaxial leaf sides. For relatively flat abaxial surfaces of leaves, nail polish replicas (Horanic & Gardner, 1967), taken from two leaves, were used; stomata were counted on micrographs of five to seven 0.66 mm^2 areas from each replicate leaf.

For rather flat adaxial surfaces of *Spartina cynosuroides*, stomata were counted on five to seven micrographs of handmade paradermal sections from two separate leaves. For deeply curved adaxial surfaces of the remaining species, fragments of three leaves were sectioned longitudinally between ridges and stomata were counted on five to seven micrographs of lateral surfaces inside invaginations. Micrographs of adaxial sides of leaves were made at 115× under an Olympus SZX16 dissection microscope for estimation of the number of veins per mm^2. Thus, total stomata were calculated per mm^2 of planar surface area, without taking into account depth of invaginations. For *Spartina argentinensis*, *Spartina bakeri*, *Spartina densiflora*, *Spartina patens*, *Spartina gracilis*, and *Spartina spartinae*, which all have multiple sizes of ridges, numbers of stomata were calculated separately for each ridge size and a mean value was taken.

Stomatal counts were also performed from scanning electron microscopy (SEM) micrographs on adaxial sides of leaves of *S. argentinensis*, *S. cynosuroides*, *S. gracilis*, *S. densiflora*, and *S. pectinata*. Longitudinal breaks of leaves in SEM micrographs allowed counts of stomata to be made within grooves. Polymer microspheres, with 19 μm diameter (SPI Supplies), were included next to samples to allow standardization to mm^2.

Statistical analysis

Where indicated, analysis of variance (ANOVA) was performed with Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA). Tukey's HSD (honest significant difference) tests were used to analyse differences between leaf parameters and species. All analyses were performed at $\alpha = 0.05$.

Table 2 Thickness of the leaf blades between veins, from the abaxial side to the bottom of the furrows (minimal), and to the top of the largest ridges (maximal)

Spartina species	Natural habitat	Leaf thickness (μm)		Distance between veins (μm)
		Maximal	Minimal	
S. alterniflora	Low marsh	343.0 ± 33.9 (d)	146.3 ± 16.9 (ab)	37.4 ± 4.1 (ab)
S. anglica	Low marsh	342.3 ± 13.1 (d)	118.5 ± 13.1 (bc)	45.1 ± 10.2 (a)
S. argentinensis	High marsh	672.3 ± 41.0 (a)	168.0 ± 18.6 (a)	35.4 ± 4.2 (ab)
S. bakeri	High marsh	455.0 ± 45.4 (c)	90.3 ± 12.1 (cd)	26.4 ± 4.6 (bc)
S. densiflora	High marsh	594.3 ± 24.9 (ab)	135.3 ± 14.1 (ab)	37.6 ± 4.5 (ab)
S. patens	High marsh	524.8 ± 76.7 (bc)	74.9 ± 8.1 (d)	16.9 ± 1.2 (c)
S. spartinae	High marsh	578.5 ± 30.3 (ab)	153.8 ± 9.5 (ab)	24.6 ± 3.8 (bc)
S. cynosuroides	Freshwater	207.0 ± 8.6 (e)	140.7 ± 8.6 (ab)	30.0 ± 7.8 (abc)
S. gracilis	Freshwater	264.0 ± 21.2 (de)	113.9 ± 4.3 (bc)	19.8 ± 2.1 (c)
S. pectinata	Freshwater	232.3 ± 20.9 (e)	113.7 ± 14.6 (bc)	35.3 ± 4.5 (ab)

Distance between veins was measured as the shortest distance between the closest bundle sheath cells of the adjoining bundles. Means are from leaves from four individuals ± SE. Letters indicate significant differences between species at $\alpha = 0.05$.

Results

Leaf anatomy

There is a large degree of variation in the general leaf anatomy of *Spartina* species (Fig. 1). In all species studied, the abaxial side of the leaf is flat; the adaxial side is characterized by ridges. The size and shape of leaf ridges was quite variable across *Spartina* species. In all species, irrespective of their anatomy, the height of ridges is maximal in the central part of the leaf blade and decreases towards the lateral margins.

The low marsh species *Spartina alterniflora* and *Spartina anglica* have moderately thick leaves, up to approx. 340 μm (Table 2). The ridges are uniform (no distinction between ridges over major and minor leaf veins) with flat tops (Fig. 1). Leaf thickness was not significantly different for both species (Table 2).

The second group of species occurs mostly in high marsh zones, and includes *S. argentinensis*, *S. bakeri*, *S. densiflora*, *S. patens*, and *S. spartinae*. These species have much thicker leaves than low marsh species, with the highest maximal and minimal leaf thickness in *S. argentinensis*, *S. densiflora*, and *S. spartinae* (maximal is up to 672 μm and minimal is up to 168 μm; Table 2). High marsh species have much larger leaf ridges over major vascular bundles compared with low marsh species. Large leaf ridges alternate with ridges of small and medium size in different ways in different species. Distal balloon-like ends of major leaf ridges on adaxial leaf surfaces are filled with large colorless parenchyma cells that have direct contact with xylem between extensions of chlorenchyma. Vascular bundles located near the abaxial epidermis are separated from it by groups of mechanical cells. But in *S. argentinensis* and *S. spartinae*, all vascular bundles are separated from the abaxial epidermis by one or two layers of colorless parenchyma cells (Fig. 1). Thus, in all high marsh species, vascular bundles have direct contact with colorless parenchyma on their xylem, and often also with strands of parenchyma cells on their phloem. Minimal leaf thickness in high marsh species is from 14 to 25% of the total leaf thickness.

The thinnest leaves were from the three freshwater species, *S. gracilis*, *S. cynosuroides*, and *S. pectinata* (maximal thickness is up to 264 μm, Table 2). In addition, these three species have the least undulated adaxial leaf surfaces. Groups of bulliform cells located at the bottom of furrows are well-developed in these three species (and in the high marsh *S. bakeri*), while in all other species they are not very prominent. In *S. cynosuroides*, a layer of flattened, large parenchyma cells underlie groups of bulliform cells. Minimal leaf thickness in freshwater species ranges from *c.* 50% (*S. bakeri* and *S. pectinata*) to 75% (*S. cynosuroides*) of the total leaf thickness.

All the *Spartina* species studied have Kranz type leaf anatomy. However, only *S. gracilis* has layers of mesophyll and bundle sheath chlorenchyma arranged in the classical way, by surrounding vascular bundles (Fig. 1i), whereas others have bundle sheath extensions. The most impressive forms of bundle sheath extensions are in *S. alterniflora* and *S. anglica*, the low marsh species (Fig. 1a,b), where specialized bundle sheath cells surround vascular bundles located near the abaxial epidermis and extend, usually in two rows, up to the adaxial epidermis in each ridge. In the other species, the chlorenchymatous bundle sheath creates different forms of extensions, especially in major veins, while some of the minor veins are the classical Kranz type. In all high marsh species having large ridges over the major veins, bundle sheath cells also surround vascular bundles on the phloem side and form different shapes of extensions above their xylem towards the colorless parenchyma. In all species, vascular bundles have an internal mestome sheath and outer Kranz sheath with randomly or centrifugally arranged chloroplasts, except for *S. densiflora* which has peripheral, or sometimes centripetal, chloroplast positioning in bundle sheath cells.

The difference between vein density in the species studied (the minimal distance between the closest bundle sheath cells

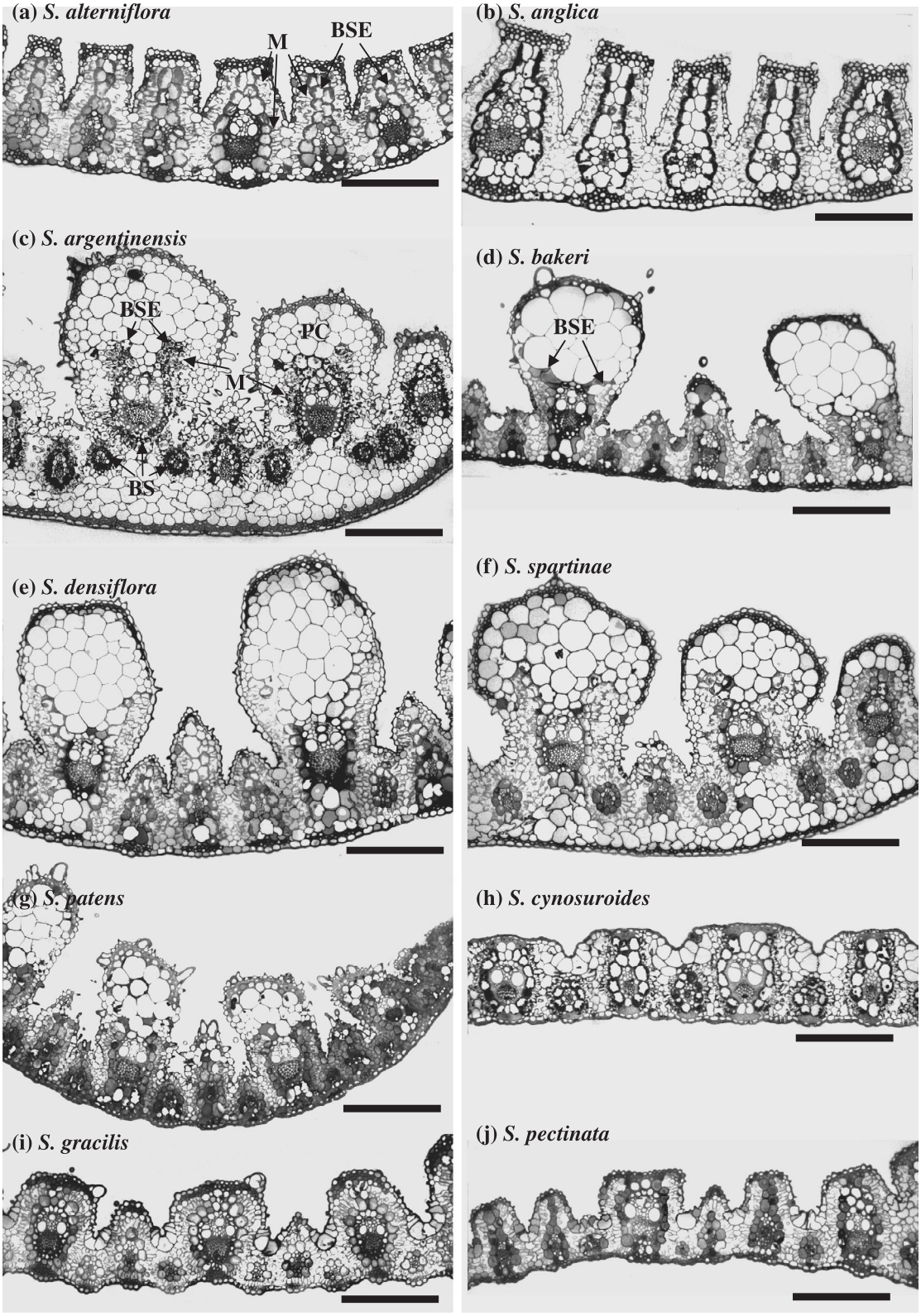

Fig. 1 Light micrographs showing cross-sections of *Spartina* leaves. (a, b) low marsh species, (c–g) high marsh species, and (h–j) freshwater *Spartina* species. M, mesophyll; BS, bundle sheath; BSE, bundle sheath extension; PC, parenchyma cells. Bars, 200 μm.

of adjoining vascular bundles) varies from 16.9 μm in high marsh *S. patens* to 45.1 μm in low marsh *S. anglica* (Table 2); there was no significant relationship to different habitats.

Species also differed in the distribution of sclerenchyma in leaves. The low marsh species, *S. anglica* and *S. alterniflora*, have groups of mechanical fibers under the phloem of veins while the flat tops of ridges are fully lined by one or two layers of mechanical cells (Fig. 1a,b). In high marsh species, the tops of major ridges and some smaller veins are also lined with one layer of mechanical cells, and on the abaxial side mechanical fibers are distributed in groups under the phloem or, as in *S. argentinensis* and *S. spartinae* (Fig. 1c,f), one layer of mechanical cells underlie the abaxial epidermis with rare gaps. In the freshwater species *S. cynosuroides*, *S. gracilis*, and *S. pectinata* (Fig. 1h–j), small groups of mechanical fibers are located under the phloem of most veins near the abaxial epidermis. On the adaxial side, fibers underlie the epidermis on top of the ridges in *S. gracilis* and *S. pectinata* and are distributed only in small groups above xylem in large veins of *S. cynosuroides*.

Cuticle thicknesses are approx. 1.7- to 4-fold higher on the abaxial side than on the adaxial side of the leaf (Fig. 2, Table 3). The highest mean values for cuticle thickness on the abaxial side was 0.63 μm for epidermal cells over mechanical tissue and 0.51 μm for cells between veins in high marsh species, while on the adaxial side the mean values were similar (0.13–0.22 μm) in all species. Thickness of the outer epidermal cell wall is rather uniform across the adaxial side of leaf blades (measured on top of ridges and close to the bottom of furrows) and across the abaxial side (over mechanical tissue and between veins). However, mean thickness of the outer epidermal cell wall was much higher (1.4- to 2.7-fold) on the abaxial than on the adaxial side of the leaf.

The freshwater species *S. cynosuroides* and *S. pectinata*, and the high marsh species *S. bakeri*, had the lowest cuticle thickness (*c*. 0.21 μm) on the abaxial side of the leaf. Otherwise, high marsh species had significantly thicker cuticles, ranging from 0.53 to 0.91 μm (ANOVA, $P < 0.05$). In the freshwater species *S. gracilis* the value was approx. 0.4 μm, an intermediate value that was comparable with the low marsh species *S. alterniflora* and *S. anglica*, and the high marsh species *S. argentinensis*. Also, cuticle thickness on the abaxial side of leaves is usually higher in cells located above mechanical tissue compared with cells adjoining parenchyma cells, and this difference is very pronounced in all high and low marsh species. By contrast, cuticle thickness is similar across the leaf in freshwater species. On the adaxial side, the thickness of the cuticle is less near the bottom of furrows compared with the top of ridges (from 1.2 times in *S. gracilis* up to 1.9 times in *S. spartinae*).

Total thickness of the cell wall + cuticle complex for salt marsh species is, at a minimum, twofold higher on the abaxial compared with the adaxial side of the leaf. This trend also exists in one freshwater species, *S. pectinata*, while in the freshwater species *S. cynosuroides* and *S. gracilis* total thickness is similar on both sides of the leaf. In freshwater species, cuticle + cell wall thickness on adaxial leaf surfaces is greater than in salt water species, both at tops of ridges and in furrows between ridges (ANOVA, $P < 0.05$; Table 3).

Analyses by TEM show that the main cuticle structural types could be subdivided in two groups. Species of the first group have a homogenous-reticulate type cuticle on both sides of leaves (*S. anglica*, *S. alterniflora*, *S. patens*, and *S. cynosuroides*; Fig. 2a,b,i–l). In this type, the outermost part consists of a homogeneous cuticle proper while the inner layer is reticulate; the net of microfibrils is mostly uniformly distributed across the reticulate layer but their thickness and density vary between species (e.g. Fig. 2a,k). Species of the second group (the remainder) have different cuticle structures on adaxial and abaxial sides of the leaf (Fig. 2c–h). The abaxial side of the leaf is covered by a homogeneous-lamellate–reticulate type cuticle (Fig. 2c,e,g) with a homogeneous cuticle proper and a polylamellated external part of the inner reticulated layer. Density and positioning of lamellae vary between species: some species have primarily periclinal lamellation (which is dense in *S. argentinensis* and *S. spartinae* in comparison with more loosely arranged lamellae in *S. densiflora*), or they have more anticlinally or chaotically orientated rare lamellae (*S. bakeri*, *S. pectinata*, and *S. gracilis*). These six species have mostly a homogeneous-reticulate type of cuticle on the adaxial side, but sometimes cells located above mechanical tissue have a cuticle with very rare single lamellae in the reticulate layer (Fig. 2d,f).

External leaf morphology

Different leaf ridge morphologies seen in Fig. 1 lead to different dimensions between ridge and furrow when viewed by SEM from the adaxial side (Fig. 3). Terminology for characters follows those used for grasses and other species of *Spartina* (Ellis, 1979, 1986; Watson & Dallwitz, 1992; Koyro & Huchzermeyer, 2004). Presence/absence of specific micromorphological features on the adaxial and abaxial epidermis of species of *Spartina*, as well as descriptions of prickles, silica cells, salt glands, papillae, and stomata, are summarized in Table 4.

Morphological descriptions of the epidermis in grasses are commonly organized into costal (over veins) and intercostal (between veins) areas. All species of *Spartina* examined in this study had ridges on adaxial costal areas that alternate with furrows. In species with uniformly-sized ridges, such as *S. alterniflora* (Figs 1a, 3a) and *S. anglica* (Figs 1b, 3b), each furrow includes a distinguishable intercostal area, but in species with both major and minor ridges, such as *S. gracilis* (Figs 1i, 3i), adaxial intercostal areas are obscure.

Ridges were absent from the abaxial surface in all species, and distinctions between costal and intercostal areas were often not clear (Fig. 4). In some species, such as *S. gracilis* and *S. pectinata*, rows of stomata and salt glands could be used to determine margins of veins (Fig. 4i,j).

Several cell types and micromorphological features were observed on adaxial and abaxial leaf surfaces (Figs 3, 4). Long

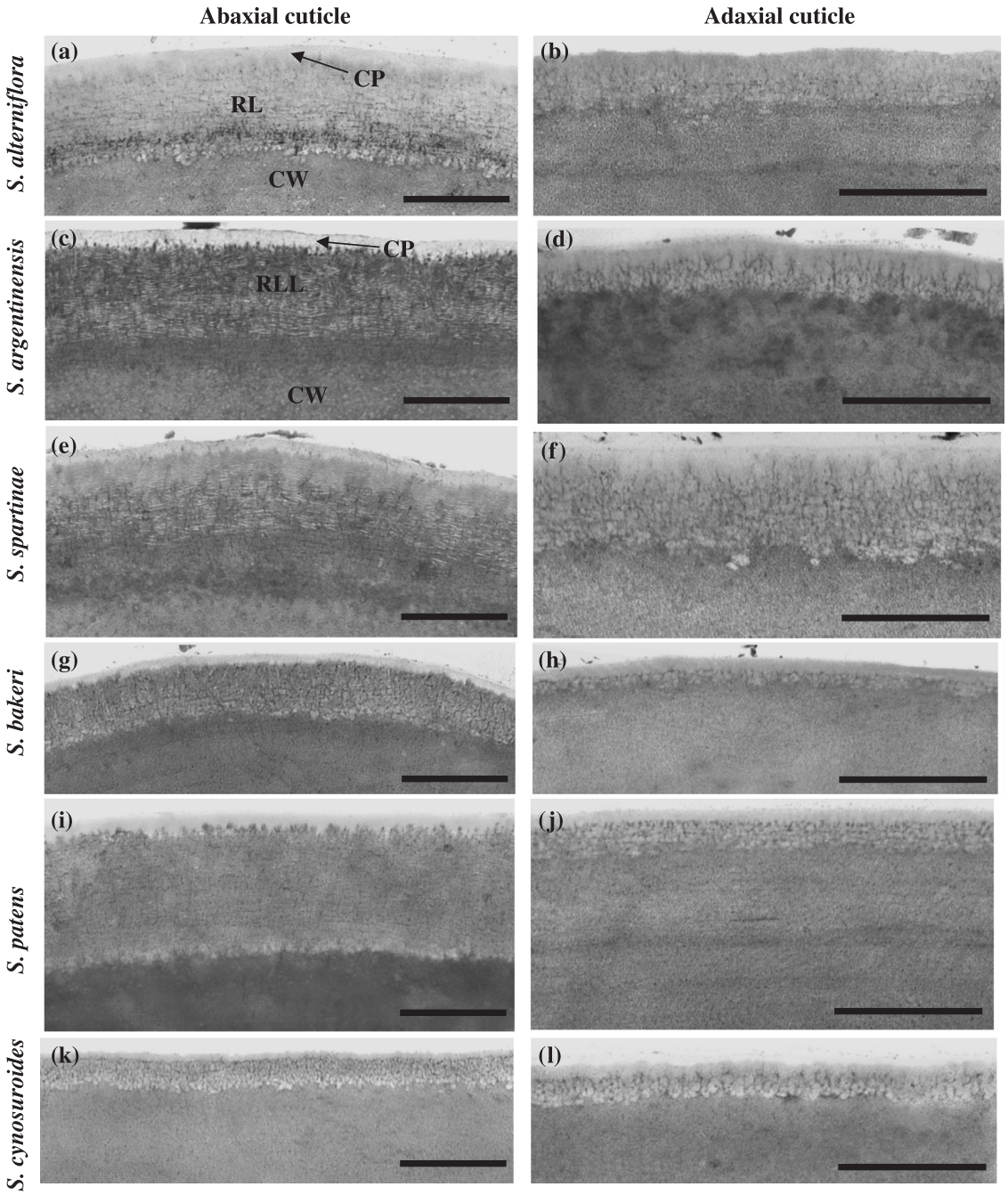

Fig. 2 The ultrastructure of the leaf cuticle in representative species. On the abaxial side, in all species except for *Spartina cynosuroides*, the cuticle of the epidermal cell is over the mechanical tissue. On the adaxial side of the leaf, the cuticle of the epidermal cell is on the top of the ridges (over the mechanical tissue) in (b), (f), and (h), and epidermal cells of the lateral side of the furrow closer to the bottom on (d), (j), and (l). CP, cuticle proper; CW, cell wall; RL, reticulate layer; RLL, reticulate-lamellate layer. Bars, 0.5 μm.

cells with sinuous edges were the most common type of cell in the epidermis of both surfaces, but were often obscured on the abaxial surface by the cuticle or by an abundance of papillae on the adaxial surface. Papillae were only found on adaxial surfaces as outgrowths from long cells and subsidiary cells (Figs 3, 5a–d) and were seen commonly overarching stomata and salt glands (Fig. 5b–d). Papillae were generally simple, but in *S. argentinensis* were consistently bilobed (Fig. 3c). Unicellar prickles with dilated bases and sharp apices were found on all taxa except *S. alterniflora* and *S. anglica.* The largest prickles were often at margins of major ridges (Fig. 3d,g,h,i,j) or along tops of minor ridges as in *S. gracilis* (Fig. 3i). Salt glands were observed on adaxial surfaces of *S. alterniflora* (Fig. 5c,d), *S. anglica*, *S. gracilis*, and *S. pectinata*, and abaxial surfaces of

Table 3 Thickness of the epidermal cell wall and cuticle complex

Spartina species	Abaxial side						Adaxial side					
	Over mechanical tissue (over veins)			Over parenchyma tissue (between veins)			Over mechanical tissue (at the top of the ridge)			Over parenchyma tissue (near bottom of furrows)		
	C	CW (μm)	C + CW	C	CW (μm)	C + CW	C	CW (μm)	C + CW	C	CW (μm)	C + CW
S. alterniflora	0.53 ± 0.01 (cd) (A)	1.61 ± 0.07 (d) (B)	2.14 ± 0.08 (g) (A)	0.36 ± 0.02 (c) (B)	1.98 ± 0.06 (d) (A)	2.33 ± 0.07 (e) (A)	0.23 ± 0.01 (bc) (C)	0.83 ± 0.03 (e) (D)	1.06 ± 0.03 (e) (C)	0.13 ± 0.02 (bd) (D)	1.35 ± 0.04 (ef) (C)	1.48 ± 0.05 (ef) (B)
S. anglica	0.46 ± 0.04 (cd) (A)	2.23 ± 0.16 (c) (A)	2.70 ± 0.20 (ef) (A)	0.33 ± 0.01 (c) (B)	2.30 ± 0.09 (cd) (A)	2.63 ± 0.10 (ce) (A)	0.20 ± 0.01 (bcd) (C)	0.86 ± 0.11 (de) (B)	1.06 ± 0.13 (e) (B)	0.13 ± 0.01 (bd) (C)	1.02 ± 0.05 (gh) (B)	1.16 ± 0.06 (gh) (B)
S. argentinensis	0.53 ± 0.01 (c) (A)	3.07 ± 0.05 (b) (A)	3.62 ± 0.05 (bd) (A)	–			0.15 ± 0.01 (de) (B)	1.67 ± 0.13 (c) (B)	1.80 ± 0.13 (c) (B)	0.13 ± 0.003 (cd) (B)	1.62 ± 0.16 (de) (B)	1.75 ± 0.16 (de) (B)
S. bakeri	0.22 ± 0.01 (e) (A)	2.41 ± 0.09 (c) (A)	2.64 ± 0.10 (f) (A)	0.16 ± 0.01 (d) (B)	2.46 ± 0.09 (cd) (A)	2.62 ± 0.09 (de) (A)	0.10 ± 0.01 (f) (C)	1.19 ± 0.04 (d) (B)	1.29 ± 0.04 (de) (B)	0.07 ± 0.003 (e) (D)	0.97 ± 0.02 (h) (C)	1.04 ± 0.01 (h) (C)
S. densiflora	0.91 ± 0.06 (a) (A)	2.40 ± 0.11 (c) (A)	3.32 ± 0.10 (cd) (A)	0.75 ± 0.06 (a) (B)	2.51 ± 0.02 (cd) (A)	3.26 ± 0.06 (be) (A)	0.25 ± 0.01 (b) (C)	1.56 ± 0.06 (c) (B)	1.81 ± 0.06 (c) (B)	0.14 ± 0.01 (bd) (C)	1.81 ± 0.06 (cd) (B)	1.95 ± 0.07 (cd) (B)
S. patens	0.84 ± 0.03 (a) (A)	4.35 ± 0.08 (a) (B)	5.18 ± 0.11 (a) (A)	0.59 ± 0.03 (b) (B)	4.68 ± 0.06 (a) (A)	5.24 ± 0.08 (a) (A)	0.22 ± 0.01 (bc) (C)	1.55 ± 0.04 (c) (C)	1.66 ± 0.04 (c) (B)	0.15 ± 0.003 (abc) (C)	1.26 ± 0.09 (fg) (D)	1.41 ± 0.09 (fg) (B)
S. spartinae	0.67 ± 0.02 (b) (A)	3.08 ± 0.10 (b) (A)	3.75 ± 0.10 (b) (A)	0.55 ± 0.02 (b) (B)	3.34 ± 0.05 (bc) (A)	3.89 ± 0.06 (bcd) (A)	0.29 ± 0.01 (a) (C)	1.27 ± 0.20 (cd) (B)	1.58 ± 0.22 (cd) (B)	0.15 ± 0.01 (ab) (D)	0.76 ± 0.02 (h) (C)	0.92 ± 0.02 (h) (C)
S. cynosuroides	0.19 ± 0.01 (e) (A)	3.36 ± 0.09 (b) (A)	3.55 ± 0.09 (bd) (A)	0.16 ± 0.01 (d) (B)	3.73 ± 0.72 (b) (A)	3.89 ± 0.74 (b) (A)	0.14 ± 0.01 (ef) (BC)	3.29 ± 0.06 (a) (A)	3.42 ± 0.06 (a) (A)	0.11 ± 0.01 (d) (C)	3.54 ± 0.12 (a) (A)	3.65 ± 0.12 (a) (A)
S. gracilis	0.42 ± 0.02 (d) (A)	2.63 ± 0.08 (c) (B)	3.06 ± 0.08 (ce) (B)	0.38 ± 0.02 (c) (B)	3.20 ± 0.05 (bc) (A)	3.58 ± 0.06 (bc) (A)	0.21 ± 0.01 (c) (C)	2.24 ± 0.04 (b) (C)	2.45 ± 0.04 (b) (D)	0.17 ± 0.01 (a) (D)	2.53 ± 0.03 (b) (B)	2.70 ± 0.03 (b) (C)
S. pectinata	0.21 ± 0.01 (e) (A)	4.73 ± 0.06 (a) (A)	4.94 ± 0.06 (a) (A)	0.20 ± 0.01 (d) (A)	4.68 ± 0.11 (a) (A)	4.89 ± 0.11 (a) (A)	0.15 ± 0.01 (de) (B)	2.30 ± 0.08 (b) (B)	2.45 ± 0.08 (b) (B)	0.12 ± 0.002 (d) (C)	1.97 ± 0.07 (c) (C)	2.09 ± 0.07 (c) (C)
Averages												
Low marsh	0.50	1.92	2.42	0.35	2.14	2.48	0.22	0.85	1.06	0.13	1.19	1.32
High marsh	0.63	3.06	3.70	0.51	3.25	3.75	0.20	1.45	1.63	0.13	1.28	1.41
Freshwater	0.27	3.57	3.85	0.25	3.87	4.12	0.16	2.61	2.77	0.13	2.68	2.81

C, cuticle; CW, cell wall; C + CW, thickness of cuticle + cell wall. Means are from 10 to 25 individual measurements ± SE. Lower case letters indicate significant differences between species in column; upper case letters indicate significant differences in rows between C, CW, and C + CW thicknesses separately, at $P < 0.05$.

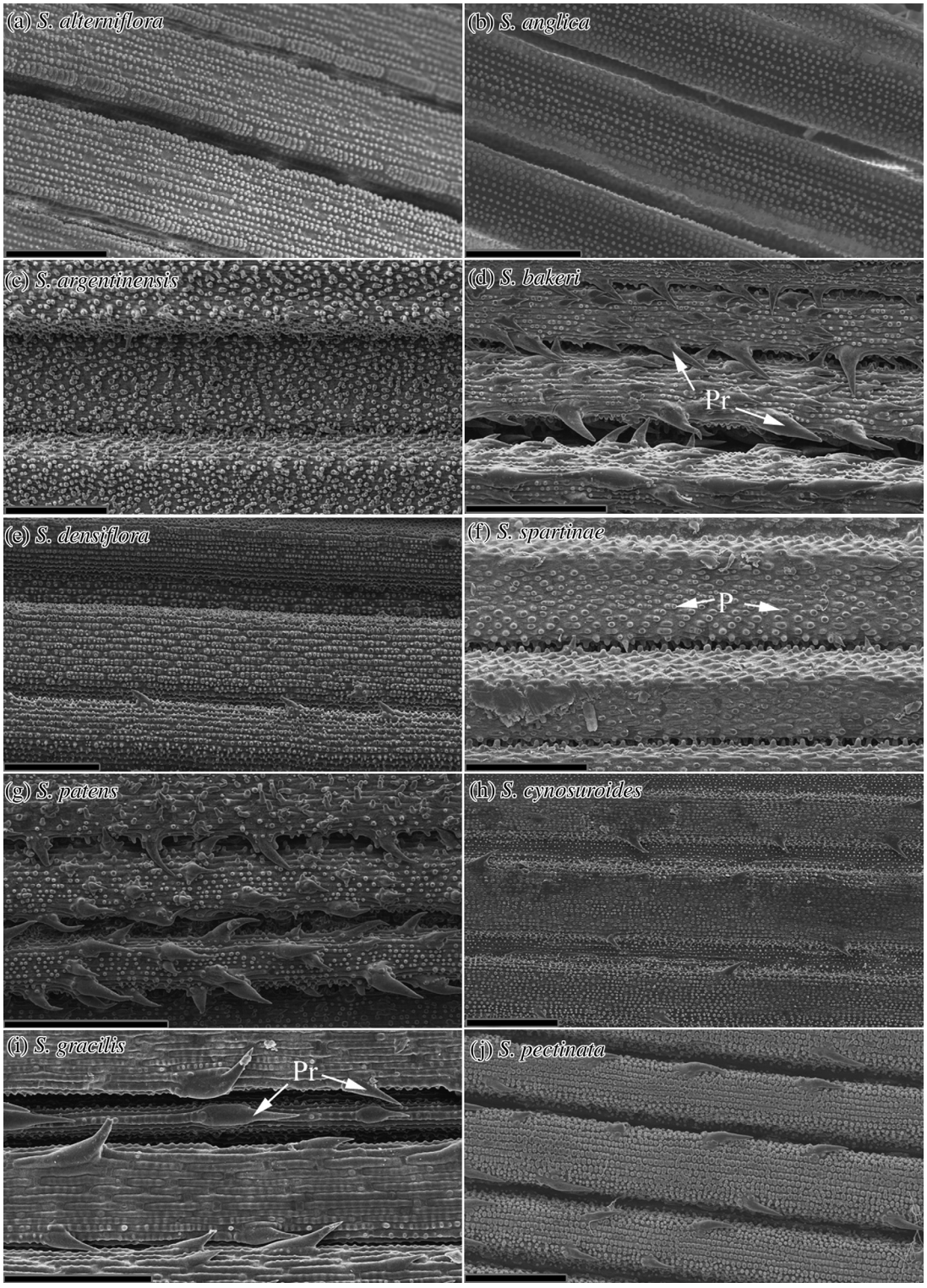

Fig. 3 Scanning electron micrographs showing adaxial surfaces of *Spartina* leaves. Bars, 150 μm. Micrograph of *Spartina pectinata* by Jessica L. Casey. P, papillae; Pr, prickle.

Table 4 Characteristics of the adaxial and abaxial epidermis of *Spartina* species examined with SEM for this study

	Prickles		Silica cells		Salt glands		Papillae		Stomata		
Spartina species	a	b	a	b	a	b	a	b	a	b	Other features/remarks
S. alterniflora	0	0	r	r	+	+	s	0	+	+	Papillae overarching stomata
S. anglica	0	0	r	s	+	+	s	0	+	[1]	Papillae overarching stomata
S. argentinensis	+	0	r	r	0	0	s, l	0	+	0	Abundant lobed papillae
S. bakeri	+	0	0	r	0	0	s	0	+	0	Abundant large prickles
S. densiflora	+	0	0	0	0	+	s	0	+	[1]	
S. patens	+	0	r	s, r	0	0	s	0	+	0	Abundant large prickles
S. spartinae	+	0	c, r	c, s	0	0	s	0	+	+	
S. cynosuroides	+	0	r, s	r, s	0	+	s	0	+	+	Adaxial subsidiary cells with papillae
S. pectinata	+	0	0	c, s	+	+	s, l	0	+	+	
S. gracilis	+	0	s	s, r	+	+	s	0	+	+	Adaxial subsidiary cells with papillae

a, adaxial; b, abaxial; 0, not observed; +, present; shape of silica cells (c, crecentric; r, rounded; s, saddle-shaped); common type of papillae (s, simple; l, lobed). Densities of stomata are presented in Table 5.
[1]Observed with light microscopy.

Table 5 Densities of stomata on adaxial and abaxial leaf surfaces in *Spartina* grasses

Spartina species	Natural habitat	Adaxial stomata (mm^{-2})	Abaxial stomata (mm^{-2})
S. alterniflora	Low marsh	191.0 ± 21.4 (8); a	0.58 ± 0.58 (5); c
S. anglica	Low marsh	168.4 ± 9.4 (5); a	0.72 ± 0.61 (9); c
S. argentinensis	High marsh	129.9 ± 17.9 (4); a	0.0 ± 0.0 (4); c
S. bakeri	High marsh	136.1 ± 6.7 (3); a	0.0 ± 0.0 (6); c
S. densiflora	High marsh	176.7 ± 23.7 (3); a	2.4 ± 1.7 (7); c
S. patens	High marsh	194.5 ± 59.2 (3); a	0.0 ± 0.0 (3); c
S. spartinae	High marsh	151.4 ± 30.6 (3); a	2.8 ± 1.6 (4); c
S. cynosuroides	Freshwater	114.1 ± 11.8 (6); a	99.7 ± 16.0 (5); ab
S. pectinata	Freshwater	209.2 ± 47.3 (5); a	74.6 ± 19.1 (6); b
S. gracilis	Freshwater	135.9 ± 6.4 (3); a	103.1 ± 5.0 (4); a

The mean ± SE (*n*) is shown for each species and leaf surface. Letters indicate significant differences between species at $\alpha = 0.05$.

S. alterniflora, *S. anglica*, *S. cynosuroides*, *S. densiflora*, *S. gracilis* (Figs 4i, 5f), and *S. pectinata* (Fig. 4j). Adaxial salt glands were located in rows above the rows of stomata on walls of ridges. On abaxial surfaces salt glands were also found in rows, often alternating with stomata, as in *S. gracilis* and *S. pectinata* (Fig. 4i,j). Stomata with dome-shaped subsidiary cells were observed in rows near the base of ridges (i.e., close to the bottom of furrows; Fig. 5a,b) on adaxial surfaces on all taxa, but on the abaxial surface were only seen at margins of veins on *S. cynosuroides* (Fig. 4h), *S. gracilis* (Fig. 4i), and *S. pectinata* (Fig. 4j). Except for *S. bakeri* (abaxial only), *S. densiflora* (neither surface), and *S. pectinata* (abaxial only), crecentric, rounded, or saddle-shaped silica cells were seen on both leaf surfaces of the remaining species. On both leaf surfaces silica cells generally alternated with long cells over the veins. Densities of cork cells were less than 8 mm^{-2}. Cork cells were crescent shaped; they are apparently restricted to abaxial leaf surfaces in *Spartina* and were only observed on *S. alterniflora*, *S. anglica*, *S. argentinensis*, *S. spartinae*, *S. pectinata*, and *S. gracilis*.

Leaf stomatal densities on abaxial surfaces ranged from 0 to 103 mm^{-2} across species in the study (Table 5). On the abaxial side of leaves, freshwater species had high stomatal densities, while salt marsh species had significantly lower stomatal densities (ANOVA, $P < 0.0001$). In all species there were higher numbers of stomata on adaxial surfaces, with values ranging from 114 to 209 mm^{-2} (Table 5). However, there were no reliable differences in adaxial stomatal densities between species (ANOVA, $P = 0.204$).

Gas exchange and carbon isotope composition

Photosynthesis rates and leaf conductance (g_v) were measured on adaxial and abaxial leaf surfaces in *S. alterniflora*, *S. anglica*, and *S. cynosuroides*. Leaves of *S. cynosuroides* had similar g_v and

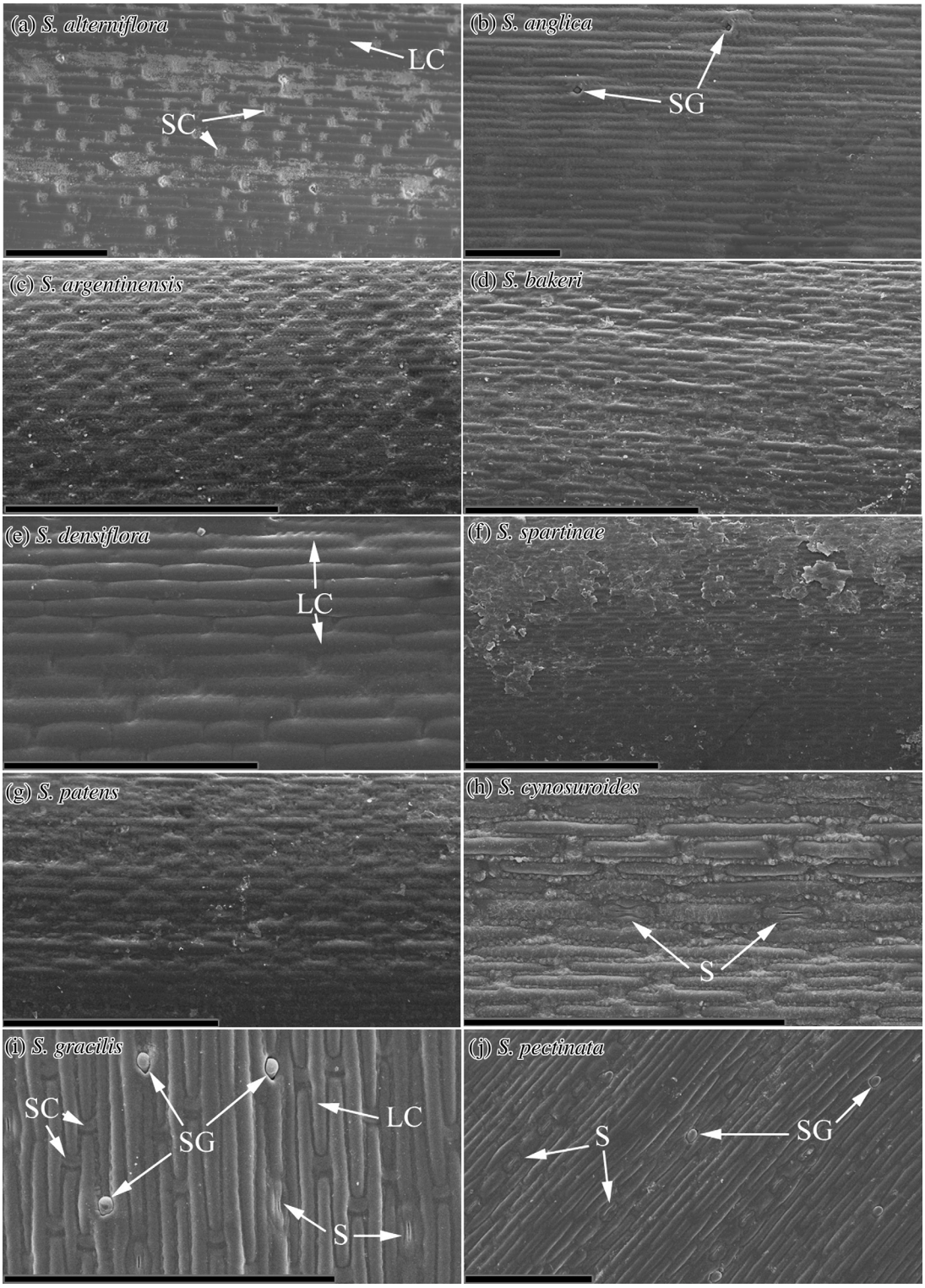

Fig. 4 Scanning electron micrographs showing abaxial surfaces of *Spartina* leaves. Micrographs by Claudia M. Dasilva-Carvalho (*Spartina patens*) and Jessica L. Casey (*Spartina pectinata*). LC, long cell; S, stomata; SC, short cell; SG, salt gland. Bars, 150 μm.

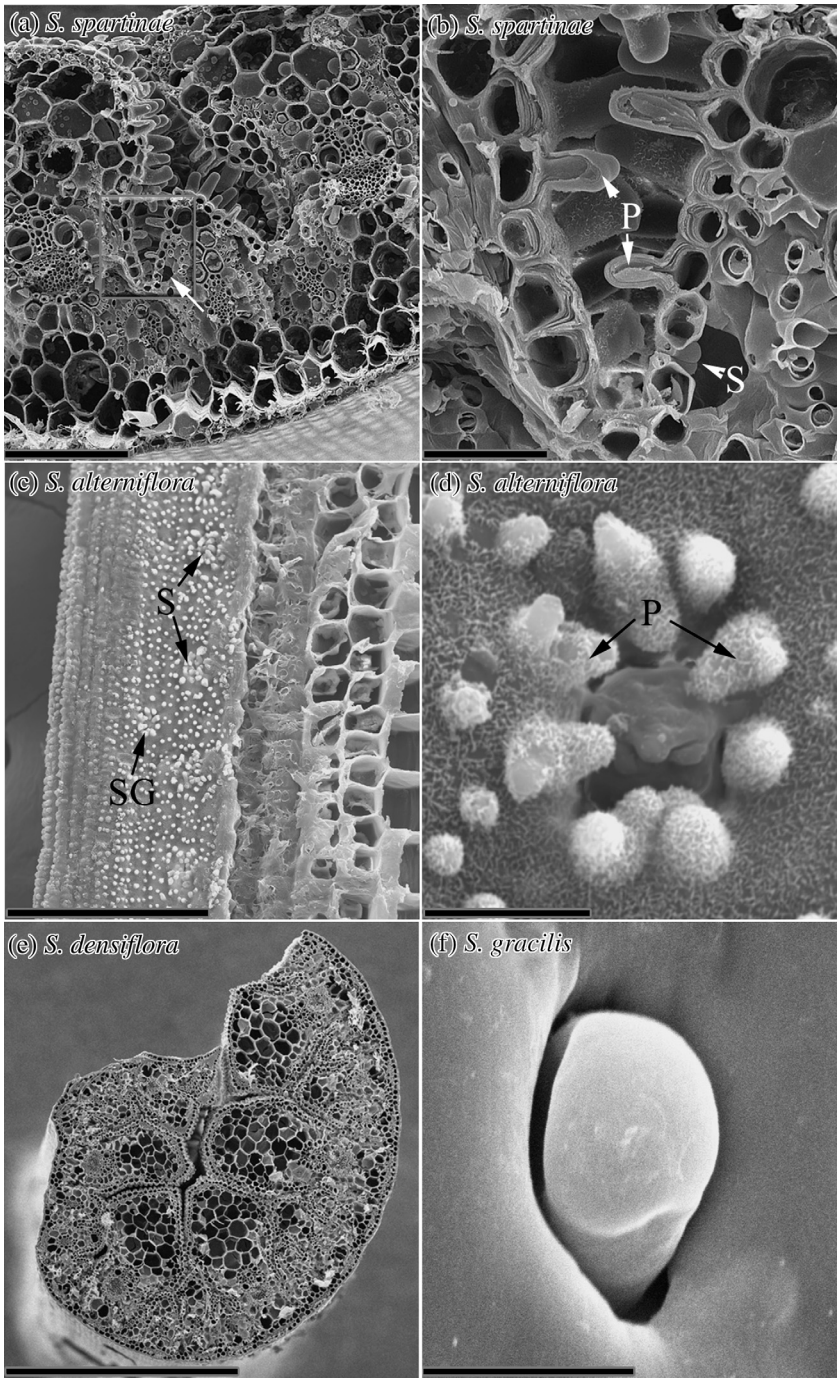

Fig. 5 Illustration of some structural features of leaf ridges, leaf curling, stomata, and salt glands in *Spartina* species. (a) *Spartina spartinae*. Stomata are located near the bottoms of leaf furrows between ridges on the adaxial leaf surface (arrow). (b) Enlargement of box region in (a). A cross section of a stomate can be seen. (c) *Spartina alterniflora*. Frequently papillae are densely congregated near stomata and salt glands. (d) *Spartina alterniflora*. Higher magnification of an adaxial salt gland surrounded by papillae. (e) *Spartina densiflora.* Illustration of leaf ridges fitting together tightly as the leaf is rolled. (f) *Spartina gracilis*. Enlargement of abaxial salt gland. Micrographs by Jessica J. Bitner (*S. spartinae*) and Jerad L. Gorney (*S. densiflora*). P, papillae; S, stomata; SG, salt gland. Bar, (a,c) 150 μm, (b) 25 μm, (d,f) 10 μm, (e) 1000 μm.

rates of CO_2 uptake from both surfaces, while in *S. alterniflora* and *S. anglica* CO_2 uptake by leaves only occurred on the adaxial side, which correlated with g_v (Figs 6, 7). The CO_2 compensation points for *S. alterniflora*, *S. anglica*, and *S. cynosuroides* were 4.7, 5.0, and 4.1 μmol mol^{-1}, respectively.

Leaf $\delta^{13}C$ ranged from −14.5 to −13.5‰ across species (Table 6). Although there were significant differences in $\delta^{13}C$ between species (ANOVA, $P = 0.034$), there were no relationships to habitat type (ANOVA, $P = 0.328$).

Leaf porometer measurements demonstrate a greater leaf surface conductance on adaxial surfaces compared with abaxial surfaces across species (Fig. 8). The ratio $g_{vad} : g_{vab}$ ranged from 2.5 in freshwater *S. gracilis* to 8.2 in low marsh *S. anglica*. There were significant differences between species (ANOVA, $P < 0.0001$), with salt marsh species *S. alterniflora*, *S. anglica*, *S. argentinensis*, *S. bakeri*, and *S. densiflora* having the highest $g_{vad} : g_{vab}$ ratios, and freshwater species *S. gracilis*, *S. cynosuroides*, and *S. pectinata* having the lowest ratios. In addition, salt

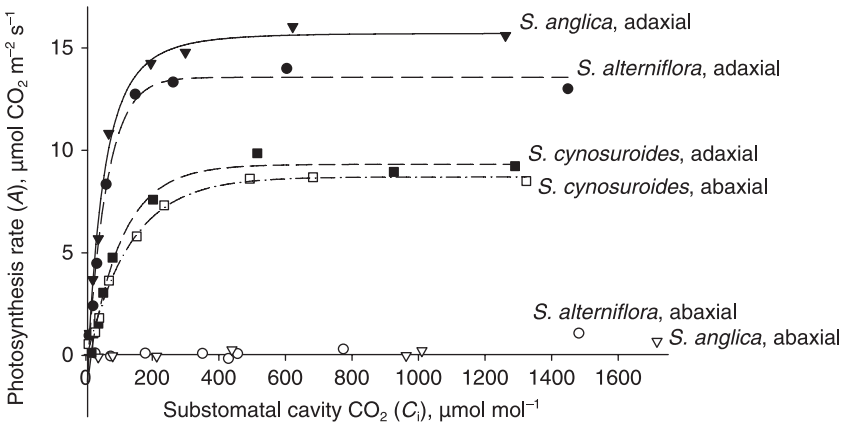

Fig. 6 Relationship of leaf photosynthesis rates (*A*) to intercellular CO_2 concentrations (C_i) in *Spartina alterniflora*, *Spartina anglica*, and *Spartina cynosuroides*. Closed symbols, measurements on adaxial leaf surfaces; open symbols, measurements on abaxial leaf surfaces. The results represent the average values for two experiments.

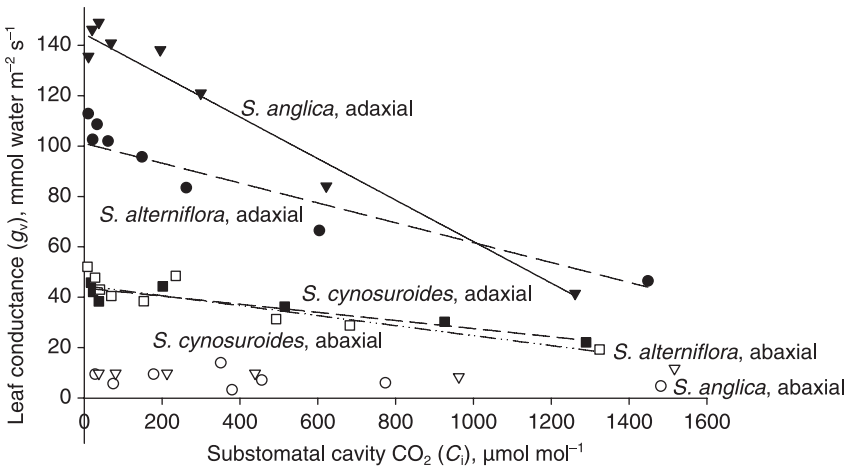

Fig. 7 Relationship between leaf conductance (g_v) and intercellular CO_2 concentrations (C_i) in *Spartina alterniflora*, *Spartina anglica*, and *Spartina cynosuroides*. Closed symbols, measurements on adaxial leaf surfaces; open symbols, measurements on abaxial leaf surfaces. The results represent the average values for two experiments.

marsh species *S. patens* and *S. spartinae* had similarly low values for $g_{vad} : g_{vab}$ (Fig. 8).

Discussion

General anatomy and type of photosynthesis

There is considerable diversity in leaf anatomy in *Spartina*. All species studied in this genus are C_4 plants with Kranz type leaf anatomy, C_4 type carbon isotope ratios, and a phosphoenolpyruvate (PEP)-carboxykinase (CK) type C_4 cycle. 'Classical PEP-CK type Kranz anatomy' is characterized by the presence of an internal mestome sheath and an outer Kranz sheath with randomly or centrifugally arranged chloroplasts (Dengler & Nelson, 1999; Voznesenskaya *et al.*, 2006). Among the species studied, only *S. gracilis* has classical PEP-CK type anatomy. All other species have different forms of chlorenchyma extensions towards the top of the ridges, increasing the partial volume of chlorenchyma (both mesophyll and bundle sheath) in leaves. The volume of colorless parenchymatous tissue is highest in the high marsh species *S. argentinensis*, *S. densiflora*, and *S. spartinae*, having layers of colorless parenchyma cells on both sides of leaves. These features may be important, since large vacuolar space for storage of salts is one of the main mechanisms in salinity tolerance (Munns & Tester, 2008).

Phylogeny

There are two major clades (I and II) within *Spartina* (Baumel *et al.*, 2002). Clade I contains the high marsh species *S. argentinensis* as sister to the hexaploid species, and includes the low marsh species *S. alterniflora*. The allopolyploid species *S. anglica* formed from species in Clade I. North American *S. alterniflora* hybridized with European *S. maritima*, forming infertile hybrids (*S.* × *neyrautii* and *S.* × *townsendii*). A later chromosome doubling event in *S.* × *townsendii* resulted in the fertile dodecaploid *S. anglica* (Ainouche *et al.*, 2004). Thus, similar appearances of *S. alterniflora* and *S. anglica* can be attributed to a parental relationship. Clade II contains tetraploid species (Baumel *et al.*, 2002), including high marsh *S. patens* and *S. bakeri*, and freshwater *S. cynosuroides* and *S. pectinata*. The placement of *S. densiflora* was somewhat unclear in the phylogeny (Baumel *et al.*, 2002) because material was collected in California and is likely of hybrid origin (Ayres *et al.*, 2008).

There are clear differences between leaves of saltwater and freshwater species. Saltwater species have more prominent ridges

Table 6 Leaf carbon isotope composition ($\delta^{13}C$) of the *Spartina* species in this study

Spartina species	Natural habitat	Leaf $\delta^{13}C$ (‰)
S. alterniflora	Low marsh	−14.50 ± 0.19 (a)
S. anglica	Low marsh	−13.70 ± 0.15 (bc)
S. argentinensis	High marsh	−13.53 ± 0.14 (c)
S. bakeri	High marsh	−13.70 ± 0.22 (bc)
S. densiflora	High marsh	−14.31 ± 0.05 (a)
S. patens	High marsh	−14.10 ± 0.33 (abc)
S. spartinae	High marsh	−14.22 ± 0.06 (ab)
S. cynosuroides	Freshwater	−13.61 ± 0.09 (c)
S. pectinata	Freshwater	−13.91 ± 0.17 (abc)
S. gracilis	Freshwater	−13.68 ± 0.32 (bc)

Means are from three individuals ± SE. Letters indicate significant differences at $\alpha = 0.05$.

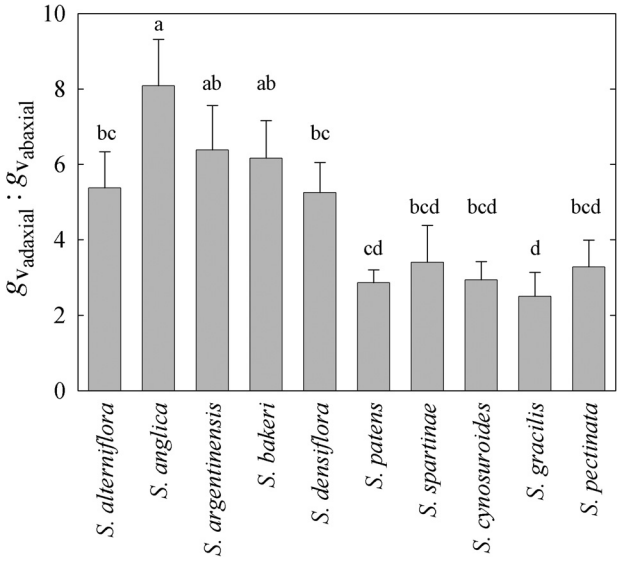

Fig. 8 Ratio of leaf conductance on adaxial surfaces (g_{vad}) to abaxial surfaces (g_{vab}) of *Spartina* species. Bars indicate ± SE ($n = 6$–22). Letters indicate significant differences at $\alpha = 0.05$.

on adaxial surfaces, thicker leaves, and stomata located on the adaxial side of the leaf. These do not follow phylogenetic relationships (both freshwater and some high marsh species occur in Clade II, while the low marsh species *S. alterniflora* and the high marsh species *S. argentinensis* appear in Clade I).

Stresses imposed by salt marsh environments influence leaf structure to a great degree in *Spartina*. In most cases, micromorphological (Table 4), anatomical (Fig. 1), and stomatal (Table 5) features appear to correlate to a much greater degree with environmental conditions (Giurgevich & Dunn, 1979) rather than phylogenetic relationships (Baumel *et al.*, 2002). Leaves in some closely-related species such as *S. patens* and *S. bakeri*, or *S. alterniflora* and *S. foliosa* (own pers. obs.) appear similar. But other similar-looking species (e.g., *S. argentinensis* and *S. bakeri*) are not closely related. Similarly, cuticle structure in leaf cells does not appear to be related to phylogeny.

Stomata

Freshwater species (*S. gracilis*, *S. pectinata*, and *S. cynosuroides*), which are less prone to being exposed to water stress, have stomata on both adaxial and abaxial sides of leaves (amphistomatous leaves). By contrast, salt marsh species have stomata almost exclusively on adaxial leaf surfaces (epistomatous leaves).

Measurements of leaf conductance with a porometer showed ratios of $g_{vad} : g_{vab}$ in salt marsh species (*S. anglica*, *S. alterniflora*, *S. argentinensis*, *S. bakeri*, and *S. densiflora*) was on average 6.3, about twofold higher than in freshwater species (*S. cynosuroides*, *S. pectinata*, and *S. gracilis*), having an average $g_{vad} : g_{vab}$ ratio of 2.9 (Fig. 8). Salt marsh species *S. patens* and *S. spartinae* had similar ratios to freshwater species (*c.* 3.1). Conductance from intercellular air space to atmosphere is dependent on both conductance through stomata and a boundary layer of unstirred air. In salt marsh grasses, there is a long boundary layer path owing to the location of stomata deep in furrows on the adaxial side of the leaf, which will reduce leaf conductance and water loss. In measurements of leaf conductance during gas exchange, the boundary layer is reduced by air flow in the cuvette, while in measurements with the porometer the boundary layer will be higher because of the unstirred layer of air.

CO_2 exchange and carbon isotope composition

Amphistomatous freshwater species took up CO_2 equally from both sides of the leaf while there was no net uptake of CO_2 from the abaxial side in epistomatous salt marsh species. Lack of CO_2 uptake from the cuvette indicates that Scotch tape is a simple but effective way to isolate gas exchange measures to one side of a leaf.

The low marsh species *S. alterniflora* and *S. anglica* and freshwater *S. cynosuroides* exhibit a C_4 type response to varying CO_2, including a low compensation point and saturation of photosynthesis at relatively low CO_2 levels (*c.* 300 µbar substomatal levels). Measured CO_2 assimilation rates were similar to measurements of short-form *S. alterniflora* in its natural habitat (Giurgevich & Dunn, 1979, 1982) and glasshouse-grown *Spartina* species by Pezeshki (1991), Ewing *et al.* (1995), Nieva *et al.* (1999, 2003), and Maricle *et al.* (2007b), but lower than those reported in *S.* × *townsendii* (Long & Woolhouse, 1978) or *S. cynosuroides* (Giurgevich & Dunn, 1981). Rates of photosynthesis can be influenced by growth and measurement conditions, and adaptations for conserving water. C_4 photosynthesis is especially advantageous when leaf conductance is low, as a high affinity of PEP carboxylase for inorganic carbon and low photorespiration maintains a low $C_i : C_a$, and a steep, inward CO_2 gradient. This may be important in *Spartina* plants with restricted access to stomata on the adaxial side of leaves, and especially with some curling of leaves.

All species fell into ranges of carbon isotope discrimination typical for C_4 species (O'Leary, 1988) with $\delta^{13}C$ values between −13.5‰ and −14.5‰; there were no trends relative to habitat

types or distribution of stomata. Carbon isotope discrimination that occurs during photosynthesis in C_4 plants is dependent on $C_i : C_a$ and on the amount of leakage of CO_2 from bundle sheath cells (Farquhar, 1983). To evaluate whether there are differences in carbon isotope discrimination between *Spartina* species or habitat types, online measurements of discrimination during photosynthesis will be required, since there are nonphotosynthetic processes during synthesis of biomass that cause additional discrimination (by 1 or 2‰) (Henderson *et al.*, 1992, 1998; Kubasek *et al.*, 2007).

Epidermal features

The cuticle of plants is important as it is the outermost layer that separates and protects plant tissues from the outer environment. Its main functions are to reduce water loss, reflect and attenuate radiation, and protect tissue from mechanical, fungal, or insect damage (Kerstiens, 1996). Its effectiveness in these roles may be affected by both its thickness and structure.

In most *Spartina* species the cuticle, cell wall, and cell wall + cuticle complex were at least twice as thick on the abaxial side compared with the adaxial side of the leaf. Average cuticle thickness on the abaxial side of leaves of salt marsh species was about twofold higher than freshwater species. This feature may be related to leaf rolling under unfavorable conditions in salt marsh species, where the thin adaxial cuticle is hidden inside the leaf, and the thicker abaxial cuticle is on the outside of the leaf where more protection is needed. It is not clear why in salt marsh species of *Spartina* the cuticle is thicker in epidermal tissue that is located over mechanical tissue than between veins (see Table 3). The thickness of cuticle in *Spartina* (maximum up to 0.9 μm) is comparable to, or lower than, some desert or halophytic Chenopodiaceae (e.g., from 0.65 μm and up to 2.18 μm in different *Tecticornia* species; see Voznesenskaya *et al.*, 2008; or *Anabasis* species having thickness of the cuticle *c.* 1 μm; Lyshede, 1977). In contrast to *Spartina*, with a thicker cuticle on the abaxial side, nearly all dicot species studied by Kravkina (2000) had a thicker cuticle on the adaxial side of the leaf, while in representative desert grass species (*Aristida purpurea* and *Stipagrostis pennata*), and six representative temperate and tropical grass species, the cuticle has equal thickness on both sides of the leaf (N. K. Koteyeva & E. V. Voznesenskaya, unpublished).

The cuticle usually consists of several layers, most often a homogeneous or lamellated outer cuticle proper and a reticulated (containing polysaccharide microfibrils sometimes referred to as dendrites) inner layer (Holloway, 1982; Jeffree, 1996). Many investigators believe the outermost layer (cuticle proper) plays the most important role in water permeability (Riederer & Schreiber, 2001). Most often the polylamellate structure was found in the outermost cuticle layer but also some species have lamellate structure of all cuticle regions (Holloway, 1982; Miroslavov *et al.*, 1998). The significance of lamellated structure, with alternating layers of cuticle and waxes, is not clear (Hallam, 1982). A homogenous-lamellate-reticulate type of cuticle structure with the highest density of lamellae was only found on the abaxial side of the leaf in three high marsh plants, *S. argentinensis*, *S. densiflora*, and *S. spartinae*, which show the greatest structural adaptations to water stress. This kind of cuticle structural type has not been previously described, but this structure is very close to *Pyrus communis* or *Hedera helix*, with a lamellated cuticle proper and a rather thick lamellated zone between it and the inner reticulated layer (Holloway, 1982; Jeffree, 1996).

Adaptation to saline conditions

In contrast to freshwater species, salt marsh species experience water stress associated with sediment salinity. The high marsh is inundated with tides infrequently, so soils dry between tides and sediment salinities are elevated to high levels. Therefore, species that grow in high marshes are expected to have adaptations related to high salinity. High marsh species of *Spartina* have the largest ridges on adaxial leaf surfaces (Fig. 1). These ridges help to increase boundary layer resistance to water loss and therefore lower total leaf conductance to water vapor. In addition, leaf ridges fit together tightly as leaves roll (Fig. 5e). In salt marsh grasses, stomata are found near the bottom of furrows (Fig. 5a,b). As the leaf curls, furrows 'close' above the stomata, which helps reduce transpiration. These species have very few stomata on the abaxial side of leaves (Fig. 4, Table 5), which can restrict water loss in times of high water stress. In addition, leaf furrows contain abundant papillae (especially near stomata; Fig. 5a,b). These adaptations further help conserve water, most likely by restricting or impeding air flow in furrows.

Many of the features observed in *Spartina* leaves can be viewed as mechanisms to prevent water loss. Similar adaptations have been observed in the grasses *Festuca novae-zelandiae* in response to water deficit (Abernethy *et al.*, 1998) and *Deschampsia antarctica* in response to cold temperatures (Gielwanowska *et al.*, 2005). In these studies, stomata were preferentially located on adaxial leaf surfaces and leaves rolled to conceal adaxial surfaces in response to water deficit. Variations in stomatal distribution also occur among species without identified adaptations. For example, some grasses locate stomata almost exclusively on abaxial leaf surfaces (Anderson & Briske, 1990) or without an obvious pattern relating to the environment (Redmann, 1985).

In addition to water stress, the presence of salt can also be damaging because of the introduction of harmful inorganic ions (Zhu, 2001). Many halophyte species have therefore developed salt-secreting glands to reduce internal salt concentration. Modified, bicellular microhairs variously called hydathodes (Sutherland & Eastwood, 1916; Skelding & Winterbotham, 1939; Metcalfe, 1960) or salt glands (Levering & Thomson, 1971, 1972; Koyro & Huchzermeyer, 2004) were observed on several *Spartina* species in the present study. As in *S.* × *townsendii* (Koyro & Huchzermeyer, 2004), salt glands on

adaxial surfaces of these taxa were located in rows above the stomata on walls of ridges. Salt glands in *Spartina* consist of two cells, specifically a large basal cell and a smaller cap cell (Levering & Thomson, 1971; cf. Fig. 1 in Long *et al.*, 1975). Salt glands in dicot species normally have more than two cells (Levering & Thomson, 1971), suggesting a different evolutionary origin from salt glands in *Spartina*. Most members of the subfamily Chloridoideae are characterized by clavate bicellular microhairs in which the distal cell is inflated and rounded (Ellis, 1979, 1986, Thomasson *et al.*, 1986, Watson & Dallwitz, 1992). In *Spartina* these hairs appear to be modified into salt-secreting glands that occur on abaxial and adaxial leaf surfaces (Skelding & Winterbotham, 1939; Metcalfe, 1960; Levering & Thomson, 1971, 1972). There were no differences in the occurrence of salt glands between salt marsh versus freshwater species, indicating this may be an ancestral trait in *Spartina*. Another means of excluding salt from the cytoplasm in leaves is storage in vacuoles; most high marsh species have large water storage parenchyma cells which can help protect against salt toxicity and dehydration.

Leaf rolling in response to stress

Leaves of many grass species are known to roll during water stress (Redmann, 1985; Alvarez *et al.*, 2008). In the present study, many differences between species and habitat types can be explained by the ability of *Spartina* leaves to curl (Heckathorn & DeLucia, 1991). The extent of leaf rolling and unrolling depends on the presence of bulliform cells, the width of furrows and the shape of the ridges. *Spartina alterniflora* has a very limited ability to roll leaves (Hester *et al.*, 1998). Examination of leaf anatomy at the microscopic level supports this observation, where leaf ridges are shaped such that leaf rolling is less pronounced (Fig. 1a,b). During leaf curling, leaf ridges fit together tightly (Fig. 5e). Curling of leaves in salt marsh species can greatly reduce transpiration, since slight leaf curvature could close adaxial furrows and reduce boundary layer conductance, and stomata are nearly absent on abaxial leaf surfaces.

Despite being adapted for life in an environment that is regularly inundated with tides, salt in estuarine water necessitates numerous adaptations for conserving water. These adaptations are especially notable in high marsh species, which experience the highest salinities in their natural habitat.

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