

Plumbing the depths: extracellular water storage in specialized leaf structures and its functional expression in a three-domain pressure-volume relationship

Running title: Three-domain pressure-volume relationship reflects leaf structure

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Abstract:

A three-domain pressure – volume relationship (PV curve) was studied in relation to leaf anatomical structure during dehydration in the grey mangrove, *Avicennia marina*. In domain 1, relative water content (RWC) declined 13% with 0.85 MPa decrease in leaf water potential, reflecting a decrease in extracellular water stored primarily in trichomes and petiolar cisternae. In domain 2, RWC decreased by another 12% with further reduction in leaf water potential to -5.1 MPa, the turgor loss point. Given the osmotic potential at full turgor (-4.2 MPa) and the effective modulus of elasticity (~40 MPa), domain 2 emphasized the role of cell wall elasticity in conserving cellular hydration during leaf water loss. Domain 3 was dominated by osmotic effects and characterized by plasmolysis in most tissues and cell types without cell wall collapse. Extracellular and cellular water storage could support an evaporation rate of $1 \text{ mmol m}^{-2}\text{s}^{-1}$ for up to 54 and 50 min, respectively, before turgor loss was reached. This study emphasizes the importance of leaf anatomy for the interpretation of PV curves, and identifies extracellular water storage sites that enable transient water use without substantive turgor loss when other factors, such as high soil salinity, constrain rates of water transport.

Key words: mangrove, PV curve, leaf structure, dehydration, plasmolysis, extracellular water, cisternae, trichomes, leaf water capacitance.

Introduction

Increasing vulnerability to drought is a major global concern as rise in average temperature is associated with increase in the frequency and intensity of drought in many locations (Reichstein *et al.*, 2013). Drought - induced tree mortality has been recorded worldwide in diverse ecosystems and climatic zones in the past few decades (Allen *et al.*, 2015). These reports range across a continuum of drought tolerance from species as drought sensitive as those growing in tropical rainforests (Rowland *et al.*, 2015) to the highly drought and salinity tolerant species of mangrove forests (Lovelock *et al.*, 2009). Drought-induced mortality has been linked with stem hydraulic deterioration (Anderegg *et al.*, 2012, Rowland *et al.*, 2015). Determination of stem vulnerability to drought can be difficult, especially in trees. However, Zimmermann (1983) suggested that leaves should be more vulnerable to hydraulic dysfunction than stems, as shown, for example, in sugar maple (Choat *et al.*, 2005). Indeed, leaf traits, such as the turgor loss point, can provide a powerful means of identifying species that are most vulnerable to drought (Bartlett *et al.*, 2012).

There is an urgent need to better understand the interplay of leaf water relations and leaf structure. Most recent studies have focussed on correlations between hydraulic anatomy and hydraulic conductance that inform our understanding of morphological constraints on carbon gain in relation to water loss by leaves under different environmental conditions (Sack & Scoffoni, 2013). For example, interspecific differences in vein and stomatal densities are associated with differences in leaf hydraulic conductance, and hence also in capacities for photosynthetic gas exchange under conditions of both high and low water availability (Brodribb & Holbrook, 2003). However, much less is known about the influence of other aspects of leaf anatomy on leaf water relations as measured by pressure/volume relationships.

The pressure-volume method is used to analyse the water content of a leaf in terms of water potential and its components (Scholander *et al.*, 1964, Tyree & Hammel, 1972, Cheung *et al.*, 1975). For a leaf or tissue in a pressure chamber, with the petiole protruding through a seal into air at atmospheric pressure, a plot of the pressure in the chamber vs. the volume of sap expressed from the petiole or the residual weight of the leaf (hereafter called the PV curve) yields a relationship which is usually reported as having two domains with differing behaviours. In these two-domain examples, when the

leaf water potentials are relatively high, decline in water content with increasing pressure in the chamber is dominated by quasi-elastic shrinkage of the cells as the turgor pressure is reduced. This first domain ends when turgor falls to near zero (i.e. intracellular osmotic pressure approximately equals the air pressure in the chamber). Further reduction in cell water content is determined mainly by the osmotic behaviour of the flaccid cell and is often approximated by the van't Hoff law. Thus the PV curve is information-rich, enabling the calculation and functional evaluation of parameters such as leaf capacitance for water storage (Q) (Tyree & Ewers, 1991), the osmotic potentials at full turgor (Ψ_{π}^{100}) and at the turgor loss point (Ψ_{π}^0), and an effective volumetric modulus of elasticity (ϵ), all of which are strongly associated with interspecific variation in drought tolerance (Bartlett *et al.*, 2012).

The theory underpinning interpretation of PV curves was developed with the approximation that leaves behave like osmometers (Scholander *et al.*, 1965) and that an average behaviour can be described for all cells in a leaf. However, two aspects of leaf structural complexity affect interpretation of PV curves. First, leaves are composed of a diverse range of cells specialised in different aspects of function. These cell types are structurally distinct, displaying, for example, differences in cell size and in the thickness and composition of cell walls, each of which could affect their modulus of elasticity, and hence maintenance of turgor during desiccation. In addition, even within a given cell type, variation in solute composition among adjacent hydrated cells (McCully *et al.*, 2010) could affect the osmotic potentials of individual cells at full turgor, and hence also affect the water potentials at which turgor is lost (Bartlett *et al.*, 2012). Such differences between cell types and between cells of a given type could explain the curvilinear transition between the linear elastic and osmotic domains of the PV curve (Cheung *et al.*, 1975). Indeed, Melkonian *et al.* (1982) suggested that the departure from linearity of the elastic domain of the PV curve signals the onset of turgor loss in the most vulnerable cells within a leaf; similarly, Cheung *et al.* (1975) suggested that the turgor loss point estimated from the onset of osmotic behaviour in the PV curve represents the extreme condition when all cells have lost turgor.

A second complication to interpretation of PV curves arises from the distribution of water in symplastic and apoplastic spaces within leaves. The symplastic space consists of the intracellular components of living cells bounded by the plasma membranes. The apoplastic space is more difficult to define.

Canny (1995) recognized four different apoplastic spaces: the lumina of xylem conduits and fibers, the gaseous intercellular space, and the water and Donnan free spaces of cell walls. However, more spaces may be identified as functional leaf anatomy becomes better understood. Differentiation of symplastic and apoplastic water is important in the estimation of PV parameters, such as elasticity.

In some species, PV curves with more than two domains have also been reported. The temporary accumulation of water in apoplastic spaces has given rise in some studies to a third domain at the highest levels of hydration in a PV curve. For example, in sphagnum moss, an initial domain largely corresponded to the loss of water stored in the specialised, dead, hollow hyaline cells (Hajek & Beckett, 2008). In this domain, sphagnum moss shoots lost up to 70% of water content without a measurable change in shoot water potential. Once this extracellular water was depleted, then the PV curves of drying shoots showed the two successive domains dominated by turgor reduction and osmotic relationships (Hajek & Beckett, 2008). Similar patterns were observed in other poikilohydric species of moss, lichen, liverworts, and a filmy fern (Beckett, 1997). A similar domain has also been reported in more complex homoiohydric species, such as white oak (Parker & Pallardy, 1987), *Pseudotsuga menziesii* (Mirb.) Franco (Kubiske & Abrams, 1991), and olive (Dichio *et al.*, 2003), but interpreted as an artefact of rehydration, also known as “the plateau effect” (Parker & Pallardy, 1987).

In the present study, the nature of the PV curve was related to leaf anatomy in *Avicennia marina* subsp. *australasica* (Walp.) J. Everett, a highly salt-tolerant mangrove species (Naidoo *et al.*, 2011, Nguyen *et al.*, 2015). Leaves of *A. marina* naturally endure enormous variability in hydration with leaf water potentials ranging from -0.1 MPa during rainfall events to -6.0 MPa during low tides under hot, dry weather (Constable, 2014, Walker, 2014). The leaf structure of *A. marina* is typical of C₃ species in having layers of palisade and spongy mesophyll. In addition, *A. marina* has salt secretion glands, a trichome layer on the abaxial surface, and a hypodermal water storage layer, similar to many other halophytes (Hutchings & Saenger, 1987). Thus *A. marina* is ideal for our study because it is a widely distributed species with leaves that are both highly tolerant of drought and possess complex cellular structures.

Materials and Methods

Plant materials

One branch was collected from each of the five co-occurring trees of *A. marina* subsp. *australasica* growing naturally along the Clyde River, Batemans Bay, New South Wales, Australia, and brought back to the lab in a black plastic bag stored in an insulated box to prevent water loss. A hand-held refractometer (A.S.T. Co. Ltd., Japan) was used to measure salinity of surface water collected at high tide and soil water collected at low tide from a soil depth of 30 cm. Soil water was extracted with a suction device (McKee, 1993) from three locations around each tree and measurements of these three salinities were averaged to give one value per tree (n = 5 trees).

PV curve

Upon return to the lab, the cut ends of all branches were recut under a solution of 25 mM NaCl, transferred to a bucket, keeping the cut ends under solution and enclosing the bucket to keep the humidity high around the leaves. The branches were allowed to rehydrate overnight in the dark in a cold room at 5°C. The composition of this perfusion solution matched average salt concentrations measured in the xylem sap of stems and leaves from *Avicennia marina* grown in seawater salinity under laboratory (Ball, 1988) and natural field conditions (Stuart *et al.*, 2007). Before PV curves were constructed, leaves were cut from branches near the petiole – stem junctions while the petioles were submerged in perfusion solution. Leaves were then transferred to a beaker, keeping the petioles under solution. The beaker was covered with plastic wrap to maintain a high humidity around the leaves and they were allowed to fully hydrate in the dark at room temperature for at least 30 min. The leaves were then dabbed dry with tissue paper to remove excess water before measuring leaf fresh mass (XP 205 Mettler Toledo balance, Mettler - Toledo Ltd., Greifensee, Switzerland) and leaf water potential (Pressure chamber model 1050D, PMS Instrument, Albany, USA).

Five PV curves were constructed, each made with one leaf from one tree. Leaf water potential was measured at intervals corresponding to decreases in leaf fresh mass of 5-10 mg as the leaves air-dried at room temperature. Time intervals between measurements ranged from 1 min at the start of dehydration to 40 mins for the final measurements, made when leaf water potentials reached values between -5 and -6 MPa, corresponding to the minimal values Constable (2014) measured during diurnal periods under field conditions in the same population of trees as in the present study. The leaf

perimeter with petiole excluded was traced on paper for leaf area measurement with a LI 3100 area meter (LICOR Inc., Lincoln, Nebraska, USA). Leaf dry mass was recorded after 48 h oven-drying to constant mass at 70°C. PV curve parameters were calculated as in Table 2 and other particulars are given in the results.

Leaf anatomy

Light microscopy

Two opposite, mature leaves were selected for anatomical analyses from the same branches that provided leaves for measurement of PV curves. One leaf in each pair was used for measurements of the tissue composition and trichome volume. Trichomes were approximated as cylinders, and trichome volumes were estimated based on height and internal lumen diameter. Transverse sections (20 - 30 µm thickness) were cut from the petiole and leaf blade with a sledge microtome (GSL 1, S. Lucchinetti, Schenkung Dapples, Zürich, Switzerland) and stained with either Toluidine blue (0.05% w/v in water) or a 50:50 mixture of Alcian Blue (1% w/v in 50% v/v alcohol) and Safranin O (1% w/v in water). The matching leaf in each pair was used to estimate trichome density, which required an inside-out epidermal preparation. The leaf epidermis was loosened by incubating the leaf in maceration solution (five parts 30% v/v hydrogen peroxide to one part glacial acetic acid) at 70°C for at least two hrs. Then the abaxial epidermis was removed with forceps, washed in water, and stained as above. Micrographs were taken at a range of magnifications using an upright microscope (DM 6000, Leica, Wetzlar, Germany) equipped with a digital camera (SPOT Flex 64MP Color FireWire 15.2, Diagnostic instruments.inc, USA) and analysed using ImageJ (Rasband, 1997 - 2014).

Cryo- Scanning Electron Microscopy (cryo-SEM)

PV curves were measured, and then leaf anatomical features, including the distribution of gas, liquid water, and cell structure, were visualized at different states of hydration using cryo – Scanning Electron Microscopy (cryo-SEM) according to McCully *et al.* (2009). One branch with four twigs similar in size was selected from each of three of the five study trees, then cut under perfusion solution (25 mM NaCl) and left for a few hours until fully hydrated. Then the four twigs, each with at least three pairs of fully expanded leaves, were cut under perfusion solution from each branch. One twig from each branch was sampled immediately upon removal from perfusion solution and the

remaining three twigs of each branch were then air-dried for differing periods of time before sampling to capture change in leaf structure associated with key features of the previously measured PV curves. At each sampling time, one leaf in each pair was used to measure leaf water potential while the other leaf was frozen rapidly (less than 0.1 s) with cryo-pliers cooled with liquid N₂ (LN2) (McCully *et al.*, 2010). Frozen pieces (1 - 2 x 0.5 cm) were cut from the desired region under LN2, placed in cryo-vials and stored in LN2 until processed for cryo-SEM. Then, frozen leaf segments were trimmed to smaller pieces (3 mm length) under LN2, planed with a cryo-microtome (Leica EM FC7, Leica, Wetzlar, Germany) and visualised by cryo-SEM (Hitachi 4300SE/N, Hitachi High Technologies, Japan) following McCully *et al.* (2010).

Mucilage Analysis

Sample preparation

Gel extruded from petioles of *A. marina* was collected and dispersed in 5% EtOH/water. The solution was centrifuged to remove insoluble extraneous matter and the supernatant freeze-dried.

Polysaccharide analysis (% composition)

The reductions of the constituent uronic acid methyl esters and the free uronic acids were carried out following the protocol of Kim and Carpita (1992) and of Pettolino *et al.* (2012). The reduced polysaccharides were then hydrolysed, reduced, acetylated and subject to gas chromatography mass spectrometry (GC/MS) analysis as described by Peng *et al.* (2000).

Water uptake by trichomes

Three twigs, each with a fully mature leaf pair, were harvested from three saplings of *A. marina* that had been hydroponically-grown (nutrient solution salinized with addition of 250 mM NaCl) from propagules that originated from the same field populations used for PV curve analyses. Leaves were rehydrated and then air-dried, with water potential determined at intervals during drying as described above for PV curves. Following each measurement of leaf water potential, leaves were tested for uptake of water by the trichome layer that covered the abaxial leaf surface. Upon removal from the pressure chamber, a leaf was positioned to view the trichome layer under an epi-fluorescence microscope (Zeiss Axiostar plus, Carl Zeiss, Germany). A drop of 0.1% fluorescein sodium salt

solution (British Drug Houses, Poole, England) was applied to the trichome surface and fluorescence from the dye was observed by standard methods under blue-exciting light. A threshold for uptake of surface water was determined by the water potential at which dye applied to the surface disappeared into the trichomes.

Data analysis

Data analyses using simple linear regression or one-way ANOVA were performed in Genstat version 16 (Payne, 2014). Data did not require transformation prior to analysis. Where relationships were significant, differences between treatment means were tested *post-hoc* for significance (here considered $P \leq 0.05$) using Fisher's Least Significant Difference test. Unless otherwise stated, all results given in the text are mean values $\pm$ standard error (se), $n = 5$.

Results

PV curve analysis

All measurements were made on leaves collected from mature trees that grew naturally where soil water salinity at a depth of 30 cm averaged 49 ± 0.5 ppt (parts per thousand by weight) and surface water at high tide averaged 35 ppt. For reference, standard seawater has a salinity of 35 ppt, and an osmotic potential of -2.4 MPa. Thus the roots were exposed to soil and surface water with an osmotic potential ranging from -3.4 to -2.4 MPa, respectively. However, surface water salinity can become higher during low tide when drying concentrates salt at the soil surface.

Characteristics of the leaves used for PV curve measurements are summarised in Table 3. Fully expanded, sun leaves of *A. marina* had an average area of 14.8 ± 1.1 cm² and contained 0.66 ± 0.05 g of water when fully hydrated. Relative water content (RWC) was measured on these leaves as a function of leaf water potential (Ψ_{leaf}) during air-drying. Variation in RWC with Ψ_{leaf} followed a complicated relationship with three domains as shown in Fig. 1a.

Water relations parameters of *A. marina* leaves were calculated from plots relating water content to water potential (commonly called PV curves) and are summarised in Table 4. Domain 1 of the curve

was characterised by rapid decline in RWC produced by relatively small application of pressure in the chamber. On average, RWC declined $13 \pm 1\%$ in this domain, for which there was less than 1 MPa decrease in the inferred leaf water potential. Water potential at the transition from the first to the second domain (Ψ_x) averaged -0.85 ± 0.06 MPa (point **B**, Fig. 1a). Domain 2 covered the largest variation in Ψ_{leaf} : a decrease from -0.85 to -5.1 MPa, five times larger than that of domain 1. Over the second domain, RWC decreased by an average of $12 \pm 3\%$, almost as much as that of domain 1. Finally, domain 3 was characterized by more rapid rate of leaf water loss for a given decrease in leaf water potential than the previous domain, domain 2. The transition between domains 2 and 3 (point **C**, Fig. 1a) averaged -5.1 ± 0.1 MPa. Given the salinities of pore and surface water, Ψ_{leaf} at this transition was inferred to be the leaf water potential at turgor loss point (Ψ_{π}^0), which was supported by calculations from the fitted straight line (dashes and points) in Fig. 1b as shown below.

The data set used in Fig. 1a was replotted in Fig. 1b with the reciprocal of Ψ_{leaf} , ($|1/\Psi_{\text{leaf}}|$) plotted as a function of relative water content deficit, calculated with total leaf water content (RWD+, %). The starting point, **c**, of the linear relationship between $|1/\Psi_{\text{leaf}}|$ and RWD+ indicated the reciprocal of leaf water potential at the turgor loss point ($|1/\Psi_{\pi}^0|$); further, the intercept **d_x** is given by the coordinates $\text{RWD+} = \text{RWD}_x+$, i.e. the percentage of water content decrease in domain 1, and $|1/\Psi_{\text{leaf}}| = |1/\Psi_{\pi}^{100}|$, i.e. the reciprocal of the osmotic potential at full turgor. According to these calculations, leaf water potential at the turgor loss point and osmotic potential at full turgor averaged -5.1 ± 0.1 MPa and -4.2 ± 0.1 MPa, respectively, in *A. marina* leaves. These calculations excluded extracellular water implied in domain 1. If that water was included, then Ψ_{π}^{100} would be calculated from the $1/\Psi_{\text{leaf}}$ intercept, **d_o**, as shown in Fig. 1b and would average -3.5 ± 0.1 MPa.

The PV curves were replotted without domain 1, as suggested by Beckett (1997) and Dichio *et al.* (2003) to account for effects of extracellular water (the plateau effect) on calculation of leaf water relations parameters from the PV curve (Supplement S1). Calculation of Ψ_{π}^0 was unaffected by removal of domain 1, but Ψ_{π}^{100} was changed because the method of Beckett (1997) and Dichio *et al.* (2003) redefined the status of a fully hydrated leaf. Specifically, calculation of maximum leaf water content (WC_{max}) changed from $(\text{FM}_{\text{max}} - \text{DM})$ to $(\text{FM}_{\text{max}} - \text{WC}_{\text{ex}} - \text{DM})$, where FM_{max} is the maximum leaf fresh mass, DM is the leaf dry mass, and WC_{ex} is the extracellular water content. As WC_{max}

decreased, RWC increased accordingly (see Table 2 for RWC calculation). Upon the removal of domain 1, Ψ_{π}^{100} was calculated at the $1/\Psi_{\text{leaf}}$ intercept of the linear regression between inverse leaf water potential ($|1/\Psi_{\text{leaf}}|$, MPa^{-1}) and relative water deficit, calculated without extracellular water, (RWD-, %), i.e. point **d** (Supplement S1b), and averaged -4.2 ± 0.1 MPa. This value was equal to that calculated at **d_x** (-4.2 ± 0.1 MPa, $P = 0.968$) but significantly different from that calculated at **d_o** (-3.5 ± 0.1 MPa, $P < 0.001$).

Calculation of water storage capacitance and bulk modulus of elasticity

The area-specific water storage capacitance (called water storage capacitance hereafter) was calculated as the mass of water lost per unit area per unit decrease in water potential (Table 2). Two values of water storage capacitance were calculated from different points on the PV curve (Table 4). Water storage capacitance of domain 1 (**Q_x**) was calculated from points **A** to **B** (Fig. 1a) and the capacitance of domain 2 (**Q_e**) was calculated from points **B** to **C** (Fig. 1a). Q_x averaged $69 \pm 6 \text{ g m}^{-2} \text{ MPa}^{-1}$, and Q_e averaged $13 \pm 4 \text{ g m}^{-2} \text{ MPa}^{-1}$. In other words, application of a chamber pressure of 1.0 MPa during dehydration in domain 1 would release approximately 69 g water per m^2 leaf area whereas similar application of 1.0 MPa in domain 2 would release about 13 g water per m^2 leaf area. The average storage capacitance over both domains (**Q_g**, points **A** to **C**, Fig. 1a) was $22 \pm 4.8 \text{ g m}^{-2} \text{ MPa}^{-1}$.

The effective bulk modulus of elasticity, **ε**, is defined as the change in chamber pressure or water potential required for a fractional decrease in a cell's water volume ($\epsilon = \Delta P / (\Delta V/V)$). The compressibility of water over this range of pressures is negligible, so $\epsilon = \Delta P / \Delta \text{RWC}$. The values of **ε** thus estimated (Table 4) averaged 7 ± 1 and 37 ± 10 MPa for domains 1 (**ε_x**) and 2 (**ε_e**), respectively, with an average value, **ε_g**, of 21 ± 2 MPa calculated over both domains.

Water storage capacitance and elasticity calculated upon the removal of domain 1 averaged $13 \pm 2 \text{ g m}^{-2} \text{ MPa}^{-1}$ and $38 \pm 4 \text{ MPa}$, respectively, and were not significantly different from those calculated from domain 2, i.e. Q_e and ϵ_e , ($P = 0.383$, and $P = 0.841$, respectively).

Leaf structure

Petiole

The basic features of petioles and leaves were characterised by bright field microscopy (Fig. 2).

Petioles (Fig. 2a) of *A. marina* leaves were covered with two types of multicellular trichomes.

Trichomes of the first type were clustered together with mucus secretion glands in a groove at the petiole - stem junction. Trichomes of the second type covered the rest of the petiole and leaf abaxial surfaces where the trichomes coexisted with salt secretion glands. These trichomes had a rivet-like shape with a cap cell on top, one to two stalk cells in the middle, and a basal cell at the bottom. The cap and stalk cells were dead with a hollow lumen. Trichomes of both types were transparent; however, they appeared black if cyanobacteria were present.

The cortex was the largest tissue within the petiole and contained two distinct layers. A layer of collenchyma (6 -7 cells thick) occurred beneath the epidermis, and was underlain by a layer of parenchyma (15 - 16 cells thick). Substantial gas spaces occurred between parenchymal cells. The gas spaces in the petiole cortex extended into the bundle sheath extension of the midvein but not to the higher vein orders. The remaining space in the petiole was occupied by the two fimbrial veins and the central vascular cylinder which contained a circular array of vascular bundles as commonly found in eudicots. However, xylem vessels were not evenly distributed among the vascular bundles, with more vessels occurring in bundles near the abaxial than adaxial sides of the petiole. Finally, the pith occupied the most central space, surrounded by vascular tissue. The pith consisted mainly of tightly packed parenchymal cells interspersed with small phloem bundles.

Leaf lamina

Leaves had a reticulate venation system with a prominent midvein as shown in Fig. 2b. A thick cuticle covered the adaxial leaf surface except where salt secretion glands occurred at the base of scattered depressions in the leaf surface. These salt secretion glands were in contact with underlying epidermal

and hypodermal cells. The midvein vascular structure was similar to that of the petiole. Nevertheless, a ring of fibers surrounding the central vascular cylinder was more developed in the midvein than in the petiole. In place of the cortex, the midvein was bounded by a bundle sheath extension (BSE) that connected the vascular tissue with the upper and lower epidermis. Leaves of *A. marina* were heterobaric but bundle sheath extensions only occurred at vein orders ranging from 1st to 4th. The midvein was also linked with adjacent mesophyll and hypodermal tissues (Fig. 2c).

Four major tissues contributed most of the leaf lamina thickness (Table 3). The structure of *A. marina* leaves was typical of C₃ species, with veins embedded between the palisade and spongy mesophyll (Fig. 2c). Spongy mesophyll thickness was about two thirds that of palisade mesophyll. Together, they accounted for 43 ± 1% of total leaf thickness. *Avicennia marina* leaves had an additional hypodermal layer located below the upper epidermis, accounting for about 36 ± 1% of leaf thickness. Trichomes on the abaxial leaf surface were similar in structure to the rivet-like trichomes on the petiole but longer, and accounted for 19 ± 1% of total leaf thickness. Salt secretion glands occurred on both leaf surfaces, but stomata were only on the abaxial surface.

Change in leaf structures during drying

Leaves were cryo-preserved during drying to fix the cell structure and the spatial distribution of gas and liquid. Changes in these three components of leaf structure were assessed in relation to the three domains of the PV curve using cryo-SEM. Domain 1 was characterized by the occurrence of extracellular water in the gas spaces of the rivet-shaped trichomes, petiole, bundle sheath extension, and leaf lamina. Extracellular water was not observed in samples taken in domain 2. Changes in cell RWC could not be determined from the images because of variation in cell size and shape between leaves. However, during domain 3, plasmolysis was apparent in most cells, but no cell wall collapse was observed.

Trichome water status changed with leaf water status. For reference, general trichome structure is shown in Fig. 3a. Trichome lumina were full of liquid water when leaves were fully hydrated (Fig. 3b). During domain 1, this extracellular water was replaced with gas (Fig. 3d) while the stalk cells of

trichomes retained their shape; however, the cap cells were collapsed (Fig. 3c, d). In domains 2 and 3, both cap and upper stalk cells were shrivelled and collapsed (Fig. 3e).

Additional measurements using fluorescence microscopy on fresh materials demonstrated the onset of trichome drainage with dehydration (Fig. 4). When viewed under white light the cap cells of the trichomes formed a continuous surface (Fig. 4a). The hollow stalk cells appeared like dark circular areas centred beneath the cap cells. No structure was visible under blue light showing the absence of detectable auto-fluorescence (Fig. 4b). In contrast, the presence of fluorescein was detected on the leaf surface by its bright green fluorescence under blue light (Fig. 4c - f). When trichomes were filled with water ($\Psi_{\text{leaf}} > 0.1$ MPa), fluorescein quickly spread over the leaf surface without loss in fluorescence (Fig. 4c, d, Supplement movie 1). Drainage of water-filled trichomes was detected when Ψ_{leaf} declined to -0.25 ± 0.03 MPa ($n = 3$). Under these conditions, fluorescein drops remained where applied for approximately a minute before rapidly disappearing into the trichomes (Fig. 4e, f, Supplement movie 2). The estimated density and volume of trichomes (Table 3) indicated that they could potentially hold up to 10% of total leaf water.

Petiole gas spaces also functioned in temporary water storage of well-hydrated leaves, and, therefore, were named cisternae. Due to the complex three-dimensional structure, it was not possible to obtain a reliable estimate of water storage in cisternae from the micrographs. When viewed by bright-field microscopy, longitudinal sections through the petiole revealed greater surface structure delimiting these cisternae than expected (Fig. 5a, b). Closer inspection under cryo-SEM revealed that the walls of these cisternae had a rough surface that appeared coated with a mucus-like substance that formed globular structure, hereafter called droplets, with drying. Analysis of a gel extruded naturally from petioles revealed a composition consistent with mucilaginous polysaccharides (Table 5). When leaves were well hydrated ($\Psi_{\text{leaf}} = -0.1$ MPa), most cisternae were full of water but some contained large droplets with diameters averaging 6.8 ± 0.8 μm ($n = 20$) (Fig. 5c, d). Where the planing knife cut through the ice of a water-filled cistern, the eutectic domains of the ice indicated water of high solute content. At the end of domain 1, no cisternae were filled with water and droplet diameter averaged 4.9 ± 0.3 μm ($n = 20$) (Fig. 5e), smaller than that observed at higher leaf water

potentials. As leaf water content decreased over domain 2, very few droplets were found and their average diameter was $2.2 \pm 0.3 \mu\text{m}$ ($n = 20$) (Fig. 5f).

In the leaf lamina and midvein, small gas spaces between pith parenchyma and between mesophyll cells were also filled with liquid water but only when leaf water potential was less negative than -0.1 MPa (Fig. 6). Where the planing knife cut through the ice of a water-filled intercellular gas space, the general absence of eutectic domains in the ice indicated water of very low solute content, in contrast to that of the cisternae. The boundary of the lamina spaces was not specially coated like that of the cisternae. No gas space or external water was found between hypodermal cells. Due to variability in cell sizes within and between leaves, it was not possible to quantify changes in size of the hypodermal cells over domains 1 and 2.

In domain 3, a wide variety of cell types, including collenchyma and parenchyma cells in the petiole and the hypodermal, mesophyll cells, and epidermal cells in the leaf lamina, showed similar responses to decreases in leaf water potential below the turgor loss point. Gaps appeared between cell walls and the enclosed cells, as is characteristic of plasmolysis (Fig. 7).

Discussion

The results of the present study revealed a new twist in an old curve and novel insights into the interpretation of the PV curve in *Avicennia marina*. This PV curve exhibited three domains over the range of hydration in which the leaves naturally function under field conditions, even in a single day (Constable, 2014, Walker, 2014). The three domains of leaf dehydration were characterized by (1) the presence and consumption of extracellular water, (2) decline in turgor, and (3) the occurrence of plasmolysis. Each domain, as illustrated in the summary diagram (Fig. 8), and its implications for leaf function, are discussed in detail below.

Domain 1: dominated by variation in extracellular water

The domain of highest hydration was characterised by the presence of extracellular water and its disappearance as RWC decreased by 13% while leaf water potential only declined from -0.1 to -0.85

MPa (Fig. 1a, Fig. 8). At the highest hydration level, water filled extracellular spaces, including those between mesophyll cells (Fig. 6c), the hollow cores of trichomes (Fig. 3b), and the petiolar cisternae (Fig. 5c, d). As the leaf dried, water was replaced by gas in these distinctive spaces (Fig. 3d, 5e, f, 6d) over different ranges of water potentials, implying that filling of the spaces occurred under different conditions and depended on different processes.

Liquid water was only observed in some mesophyll extracellular gas spaces when water potential was less negative than -0.1 MPa. Water storage between mesophyll cells would have a negative impact on gas exchange because diffusion of CO₂ is about 10,000 times slower in water than in air, but the extent of this storage was small and short-lived. The lack of eutectic domains in this extracellular water that had been cryo-preserved indicated that the water had very low solute concentrations. How can we account for movement of liquid water from cells to extracellular gas spaces in the absence of an osmotic gradient? The answer may be similar to processes driving refilling of embolized protoxylem vessels in well-hydrated tissues (Rolland *et al.*, 2015). Once the cells had achieved high water potentials, capillary forces due to the combination of hydrophilic walls and close contact between cells may have driven extracellular water accumulation in confined spaces. This limited filling of extracellular gas spaces in excised leaves was not an artefact as similar observations have been made on *A. marina* leaves under natural conditions when they were collected predawn following exposure to nocturnal wetting events (Constable, 2014). Such accumulation of extracellular water in these well-hydrated leaves presumably occurred through foliar absorption of atmospheric water, even though roots grew in seawater; in other words, the water potential gradient during leaf wetting events was reversed from the atmosphere to the leaves to the soil.

The presence of low solute concentrations in the extracellular water has important implications for salt tolerance of halophytes. During symplastic water uptake at the roots (Moon *et al.*, 1986), *A. marina* excludes approximately 90 - 95% of salt in soil water from entry to the transpiration stream (Ball, 1988). When grown in seawater, *A. marina* typically has 25 mM NaCl in the transpiration stream (Ball, 1988, Stuart *et al.*, 2007). In *A. marina*, water and associated salt are distributed across a leaf lamina through the vascular system to vein endings and subsequently dispersed to client cells by symplastic pathways (Fitzgerald & Allaway, 1991). This symplastic control prevents catastrophic salt

accumulation in apoplastic spaces and facilitates salt management within a leaf. In *A. marina*, part of the salt transported in the transpiration stream is accommodated in growing cells for osmotic adjustment of the vacuoles while the remainder is exported from the leaves via either the phloem or epidermal salt secretion glands, thereby maintaining a favourable salt balance (Ball, 1988). Thus the observation of low solute concentrations in the extracellular water (Fig 5d) is consistent with strong symplastic control of both water and associated salt distribution within the leaves (Fitzgerald & Allaway, 1991).

Water-filled trichomes began to empty when Ψ_{leaf} declined below -0.25 MPa (Fig. 4e, f, Supplement movie 2). In contrast to intercellular spaces in the mesophyll, the trichome lumina appeared to contain a mucilaginous substance. This was suggested by the similarity of the ice texture in water-filled trichomes that had been cryo-preserved (Fig 1b) to that observed in cryo-preserved mucilaginous secretions of root tips (McCully *et al.*, 2009). Such a hydrophilic substance in combination with the highly hydrophilic walls and the small diameter of the hairs could have facilitated water uptake at relatively high water potentials, as might occur with exposure to rain or dew. Indeed, under natural field conditions, Constable (2014) observed trichomes to be full of water when leaves of *A. marina* were wet in the morning. Trichomes seemed to serve as one of the main water storage sites, which could hold up to 10% of total leaf RWC. This storage water could be released to the leaf as leaf water potentials declined with dehydration if water transport into the leaf followed a symplastic pathway through the living basal cells of the trichomes to the underlying cells (Fig. 3a). Similarly, water absorbed by trichomes contributed to the water status of underlying leaf cells in oak leaves (Fernández *et al.*, 2014). Thus, identification of trichomes as a major water storage site in *A. marina* also implies that the trichomes may provide a means for rehydration via foliar water uptake.

Finally, the present study identified a novel water storage structure, here named the cisternae, which occurred mainly in the petiole and extended into the bundle sheath extension of the midvein near its junction with the petiole. Extracellular water occurred in cisternae until leaf water potentials declined to approximately -0.8 MPa. Unlike the extracellular spaces in the mesophyll and the trichomes, the cisternae were relatively large, completely bounded by the walls of cells comprising the surrounding tissues (Fig. 5a,b), and appeared to contain a combination of mucilage, mostly arabinose, and a

relatively high concentration of solutes (Table 5, Fig. 5d, f). The latter must have been sufficient to attract water from the surrounding cells into the cisternae when leaf water potentials rose above -0.8 MPa. Extension of the cisternae into BSE of the midvein would enhance the reach of their influence on leaf water relations as the BSEs are known to play a role in extravascular water dispersal (Canny, 1986, Buckley *et al.*, 2011, Sommerville *et al.*, 2012, Zsogon *et al.*, 2015). Conversely, because the osmotic potential of the cisternae would vary with water content, differential drainage of the cisternae during leaf drying could contribute to the curvature linking domains 1 and 2 in the PV curve.

Domain 2: Dominated by variable turgor

The second domain was characterized by 12% loss in leaf RWC as leaf water potentials declined from -0.85 to -5.1 MPa. Decrease in cell volume, except for that in trichomes, was not obvious in our micrographs, possibly due to the small scale of total loss in RWC (i.e. 12% across domain 2, Fig. 1a), combined with high variability in cell sizes (Fig. 8). For example, a 12% change in leaf water content would be equivalent to a 4% change in each dimension of an isotropic cell.

Moreover, there were no obvious changes in gas spaces between mesophyll cells with decreasing Ψ_{leaf} , consistent with observations of desiccating sunflower leaves (Fellows & Boyer, 1978). Tissue connections in the leaf lamina were maintained as cell walls and membranes also maintained contact during shrinkage (i.e. cytorrhysis). Given the large osmotic potential at full turgor ($\Psi_{\pi}^{100} = -4.2$ MPa) and the high modulus of elasticity, most of the decline in leaf water potential with decrease in RWC in the second domain was associated with decreasing turgor pressure.

The curvature between domains 2 and 3 may reflect differential turgor loss in different cell types with the turgor loss point representing the extreme case where all cells have lost turgor (Cheung *et al.*, 1975). Some cells might lose water more rapidly than others due to their properties or their positions within the leaf. In addition, ϵ is likely to be itself a function of turgor. For example, the high and constant ϵ region could represent cell wall stretching, and the lower and variable modulus region could represent change in shape. Thus, the curvature shown linking domains 2 and 3 (Fig. 1) likely reflects the large contribution of different cell types to water relations in *A. marina*. Similar curvature was found in many studies where PV curves were constructed from repeated measurements on a

single leaf or shoot (Cheung *et al.*, 1975, Meinzer *et al.*, 1986, Parker & Pallardy, 1987). In contrast, a sharp transition between domains 1 and 2 has been observed when the PV curve is constructed from a composite of measurements made on many leaves of a species, such as those from woody tropical rainforest (Brodribb & Holbrook, 2003) or used in theoretical analyses, e.g. Suarez and Sobrado (2000). The convexity of the transition may make a useful tool for comparing hydraulic complexity in leaves grown under different conditions or between different species.

Domain 3: Dominated by osmotic behaviour of flaccid cells

Domain 3 was characterised by plasmolysis (Fig. 7, 8) as leaf water potentials declined below the turgor loss point once RWC decreased below approximately 75% (or below about 86% if the extracellular water was not included in the accounting) (Table 4). In most living cell types, connections between tissues or between cells comprising a tissue were maintained during desiccation-induced shrinkage. No collapse of cell walls was observed. However, separation between cell walls and plasma membranes developed when Ψ_{leaf} was about 1 MPa lower than Ψ_{π}^0 . Plasmolysis in *A. marina* was also noticed in the field when Ψ_{leaf} was as low as -6 MPa, and the cells recovered following nocturnal rehydration (Constable, 2014). The fact that *A. marina* leaves could recover after reaching the turgor loss point was consistent with a study by Brodribb and Holbrook (2003) which showed that irreversible damage to PSII system only occurred in woody tropical rainforest species at Ψ_{leaf} much lower than Ψ_{π}^0 .

Ecophysiological implications of the new PV curve to *A. marina* function

The results of the current study emphasized the importance of leaf anatomy to leaf water relations with far reaching implications for leaf function under field conditions. Previous studies have referred to the first domain as “the plateau effect” (Parker & Pallardy, 1987, Dichio *et al.*, 2003), attributed to artefactual rehydration. The presence of domain 1 due to extracellular water does not change values of PV curve parameters, including Ψ_{π}^0 , Ψ_{π}^{100} , ϵ , and Q, as long as those parameters are calculated using data from appropriate domains as shown in Fig. 1 and indicated in Table 4, consistent with recommendations to account for “the plateau effect” (Parker & Pallardy, 1987). However, dismissal of

domain 1 as an artefact of rehydration would lead to false assumptions about leaf functions in *A. marina* that are inconsistent with field observations and leaf anatomy.

The PV curves revealed a high modulus of elasticity in leaves of *A. marina*, which plays a major role in cell function over the whole range of water potentials naturally experienced by these leaves. At maximum hydration, leaf water potentials were very high (i.e. -0.1 MPa) while osmotic potential at full turgor was -4.2 MPa (Fig. 1b). The turgor pressure would have been around 4.1 MPa, but there was no evidence that cells burst under these conditions. Presumably, the high modulus of elasticity, i.e. 37 MPa (Table 4), contributed to cell survival when the leaves were fully hydrated.

Previous studies have interpreted the role of a high ϵ on leaf function under low water potentials in two ways. First, high ϵ was proposed to enhance water uptake into a transpiring leaf by increasing the water potential gradient between the leaf and the soil (Bowman & Roberts, 1985). However, Bartlett *et al.* (2012) argued that this proposal disregards the role of hydraulic conductance in determining the water potential gradient. Indeed, studies of diurnal gas exchange in the same population of *A. marina* as the present study showed that reduction in leaf hydraulic conductance began when leaf water potentials declined below -3 MPa (Walker, 2014). Consequently, decline in Ψ_{leaf} with decreasing turgor would not necessarily enhance water uptake into leaves as they dried. Indeed, a low ϵ would allow the concentration of the cytoplasm to vary significantly over the range of positive turgor, whereas a high ϵ would keep the cytoplasmic concentrations similar, and also reduce the possibly deleterious effects of changes in cell geometry. Thus, we agree with the second suggestion that a high modulus of elasticity coupled with a low solute potential enables leaves to maintain the levels of hydration required for cellular function during drying to the turgor loss point (Cheung *et al.*, 1975, Bartlett *et al.*, 2012).

The extent of water storage in domains 1 and 2 revealed by PV curve could play an important role in the maintenance of diurnal photosynthetic activities in leaves of *A. marina*. Specifically, extracellular water storage capacitance of domain 1 (Q_e) was about five times greater than the intracellular water storage capacitance of domain 2 (Q_i). However, Q_e was functional over a range of Ψ_{leaf} approximately

five times less than that of Q_i . Consequently, extra and intracellular water storage was about equal. In other words, storage of water in extracellular spaces doubled the amount of water available for use in gas exchange without loss in turgor. For example, area-specific evaporation rates measured for *A. marina* grown at the same location as those in the present study ranged from 1 to 2 mmol H₂O m⁻² s⁻¹ (Martin *et al.*, 2010). So a leaf with extracellular water storage capacitance of 69 g H₂O m⁻² MPa⁻¹ (Table 4) or 3800 mM H₂O m⁻² MPa⁻¹ as estimated from domain 1, when fully charged, could alone supply the water loss needed to support photosynthesis at the above evaporation rates for 3230 to 1615 s, that is for 54 to 27 min with only 0.85 MPa decrease in Ψ_{leaf} . Once extracellular water is exhausted, then the water storage capacitance of domain 2 (i.e. 13 g H₂O m⁻² MPa⁻¹ or 700 mM H₂O m⁻² MPa⁻¹) could alone support the same rates of gas exchange for another 50 to 25 min until the turgor loss point (i.e. -5.1 MPa) is reached. Given the relative proportions of space occupied by different tissue layers in the leaf lamina (Table 3), the hypodermis alone could have buffered water loss from the mesophyll by contributing almost half of the water available for photosynthesis during dehydration over domain 2 (all in the absence of water flux from the roots). Thus, water stored within a leaf could supplement that provided by the roots, thereby extending the duration of gas exchange activities for perhaps about two hours longer than otherwise, which could be critically important for a species when soil salinity constrains the supply of water to the leaves.

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724

Table 1. Abbreviations

Parameter	Symbol	Unit
Calculation with extracellular water included	+	
Calculation without extracellular water	-	
Water mass difference	Δw	g
Leaf water potential gradient	$\Delta \Psi$	MPa
Leaf dry mass	DM	g
Leaf fresh mass	FM	g
Maximum leaf fresh mass	$FM_{\max}$	g
Water storage capacitance	Q	$g\ MP^{-1}m^{-2}$
Water storage capacitance at domain 2	Q_e	$g\ MP^{-1}m^{-2}$
General water storage capacitance	Q_g	$g\ MP^{-1}m^{-2}$
Water storage capacitance at domain 1	Q_x	$g\ MP^{-1}m^{-2}$
Relative water content	RWC	%
RWC at turgor loss point	RWC_{π}^0	%
RWC at full turgor	RWC_{π}^{100}	%
RWC at the transition between domains 1 and 2	RWC_x	%
Relative water deficit	RWD	%
RWD at turgor loss point	RWD_{π}^0	%
RWD during domain 2	RWD_e	%
RWD during domain 1	RWD_x	%
Leaf area	S	cm^2, m^2
Maximum leaf water content	$WC_{\max}$	g
Extracellular water content	WC_x	g
Pressure gradient	ΔP	MPa
RWC difference	ΔRWC	%
Fractional decrease in a cell's water volume	$\Delta V/V$	
Volumetric modulus of elasticity	ϵ	MPa
Bulk modulus of elasticity at domain 2	ϵ_e	MPa
General bulk modulus of elasticity	ϵ_g	MPa
Bulk modulus of elasticity at domain 1	ϵ_x	MPa
Leaf water potential	Ψ_{leaf}	MPa
Leaf water potential at the transition between domains 1 and 2	Ψ_x	MPa
Leaf water potential at full hydration	Ψ_{100}	MPa
Leaf water potential at turgor loss point	Ψ_{π}^0	MPa
Osmotic potential at full turgor	Ψ_{π}^{100}	MPa

Table 2. Summary of PV curve components and their calculations as previously described (Scholander *et al.*, 1964, Turner, 1988, Tyree & Ewers, 1991, Bartlett *et al.*, 2012).

Parameter	Symbol	Unit	Calculation
Water storage capacitance	Q	g MP ⁻¹ m ⁻²	$Q = \frac{\Delta w}{\Delta \Psi} \frac{1}{S}$ $= \frac{\Delta RWC \cdot WC_{\max}}{\Delta \Psi} \frac{1}{S}$
Bulk modulus of elasticity	ε	MPa	$\epsilon = \frac{\Delta P}{\frac{\Delta V}{V}} = \frac{\Delta P}{\Delta RWC}$
Maximum water content	WC_{max}	g	WC _{max} = FM _{max} - DM
Relative water content	RWC	%	RWC = (FM - DM)/WC _{max}
Leaf water potential gradient	ΔΨ	MPa	ΔΨ = Ψ _{tip} - Ψ ₁₀₀
Relative water content deficit	RWD	%	RWD = 100 - RWC

Table 3. Properties of fully expanded, field-grown, sun leaves of *A. marina* used for measurements of PV curves and anatomical analyses. Values are mean $\pm$ se (n = 5, except for * where n = 15)

Parameter		Symbol	Unit	Mean	se (n = 5)
Leaf area		S	cm ²	14.8	1.1
Maximum fresh mass		FM_{max}	g	1.04	0.07
Dry mass		DM	g	0.38	0.02
Maximum water content		WC_{max}	g	0.66	0.05
Leaf mass per area		LMA	g m ⁻²	256.3	13.9
Maximum water content per dry mass		WC_{max} DM⁻¹	g g ⁻¹	1.76	0.08
Leaf thickness			μm	588	10
Contribution to leaf thickness	Upper epidermis		%	3	0
	Hypodermis		%	36	1
	Palisade mesophyll		%	30	1
	Spongy mesophyll		%	13	0
	Trichome		%	19	1
Trichome	Density		cm ⁻²	218,758	8,783
	Volume*		μm ³	20,460	3,288 (n = 15)

Table 4. Leaf water relations of *A. marina* derived from three – domain PV curves as shown in Fig. 1. Values are mean $\pm$ se, n = 5. Note that symbols + or – denote whether extracellular water was included or excluded, respectively, in the calculations.

Parameter	Symbol	Unit	Mean	se (n = 5)
Relatively water deficit during domain 1	RWD_{x+}	%	13	1
Leaf water potential at RWC_{x+}	Ψ_x	MPa	-0.85	-0.03
Relative water deficit during domain 2	RWD_{e+}	%	12	1
Osmotic potential at full turgor	Ψ_{π}^{100}	MPa	-4.2	-0.1
Water potential at turgor loss point	Ψ_{π}^0	MPa	-5.1	-0.1
RWC at full turgor	RWC_{π}^{100}	%	87	1
RWC at turgor loss point, including extracellular water	RWC_{π}^{0+}	%	75	3
RWC at turgor loss point, excluding extracellular water	RWC_{π}^{0-}	%	86	2
General bulk modulus of elasticity	ϵ_g	MPa	21	2
Modulus of elasticity for domain 1	ϵ_e	MPa	7	1
Modulus of elasticity for domain 2	ϵ_x	MPa	37	4
General water capacitance	Q_g	$g\ m^{-2}\ MPa^{-1}$	22	2
Water storage capacitance for domain 1	Q_x	$g\ m^{-2}\ MPa^{-1}$	69	6
Water storage capacitance for domain 2	Q_e	$g\ m^{-2}\ MPa^{-1}$	13	2

Table 5. Composition of *A. marina* petiolar gel. Values are mean percent dry mass of the total dry mass of the polysaccharides named in the table below $\pm$ se, n = 3. Abbreviations: Rha: Rhamnose, Ara: Arabinose, Xyl: Xylose, Man: Mannose, ManUA: Mannuronic acid, Gal: Galactose, GalUA: Galacturonic acid, Glc: Glucose, GlcUA: Glucuronic acid. A: esterified uronic acid analysis, B: unesterified uronic acid analysis. NA: not applicable.

	Rha	Ara	Xyl	Man	ManUA	Gal	GalUA	Glc	GlcUA
A (%)	2.07	83.47	0.97	0.23	NA	10.60	NA	2.67	NA
se	0.19	0.32	0.09	0.03	NA	0.29	NA	0.22	NA
B (%)	2.23	83.17	0.80	0.23	0.00	10.53	0.00	0.53	2.50
se	0.19	0.50	0.06	0.03	NA	0.20	NA	0.15	0.15

Figure legends:

Fig. 1. A representative pressure volume (PV) curve of an *Avicennia marina* leaf grown at Batemans Bay, New South Wales, Australia: (a) Relative water content, calculated with total leaf water content, (RWC+, %) as a function of leaf water potential in absolute value ($|\Psi_{\text{leaf}}|$, MPa). Key points and their x, y coordinates are indicated by open symbols. In domain 1, **A** (open circle) shows the point of full hydration at which $|\Psi_{\text{leaf}}|$ was 0.1 MPa and leaf RWC was set at 100%; the transition between domains 1 and 2 is indicated by **B** (open diamond); **C** (open triangle) is the turgor loss point at the transition between domains 2 and 3. (b) Inverse leaf water potential in absolute value ($|1/\Psi_{\text{leaf}}|$, MPa⁻¹) as a function of relative water deficit, calculated with total leaf water content, (RWD+, %). The full data set is plotted in the inset graph, whereas points representing extracellular water from points **A** to **B** in panel A were excluded from the data set plotted in the main graph. The main graph shows relationships among calculated values of Ψ_{leaf} at the turgor loss point (point **c**, open circle), the y intercept (**d₀**), and the intercept (**d_x**) at RWD+ = RWD_x+, i.e. relative water deficit during domain 1. At point c, $|1/\Psi_{\text{leaf}}^0| = 0.2$ so $|\Psi_{\text{leaf}}^0| = 5$ MPa. The linear regression between $|1/\Psi_{\text{leaf}}|$ and RWD+ after the turgor loss point was reached followed the equation: $y = -0.0034x + 0.2682$ ($r^2 = 0.98$). Therefore, $|1/\Psi_{\text{leaf}}|$ at **d₀** = 0.2682, and at **d_x** = 0.2376 as RWD+ at **dx** = 11. Accordingly, $|\Psi_{\text{leaf}}|$ at **d₀** = 3.7 MPa and at **d_x** (Ψ_{π}^{100}) = 4.3 MPa.

Fig. 2. Transverse sections of (a) petiole, (b) leaf midvein, and (c) leaf lamina of *A. marina*. Symbols: B: bundle sheath extension; C: cisternae; F: fimbrial vein; H: hypodermis; P: palisade mesophyll; S: spongy mesophyll; T: trichomes; V: vascular bundle. Yellow arrows indicate mucus secretion glands in (a) and salt secretion glands in (b) and (c). Black arrow indicates stomate. Bars are 100 μm .

Fig. 3. Trichomes from the abaxial surface of leaves of *A. marina* at different hydration states viewed with bright-field microscopy (a) and cryo-SEM (b-e). (a) Trichome general structure. Symbols: ca: cap cell, st: stalk cell, ba: basal cell. (b) Both cap and stalk cells were filled with water at $\Psi_{\text{leaf}} = -0.1$ MPa. (c, d) Cap cells were collapsed but stalk cells maintained their shapes and were filled with air at $\Psi_{\text{leaf}} = -0.7$ MPa. (e) The lower stalk cell maintained its shape while the cap and upper stalk cell were shrivelled and collapsed at $\Psi_{\text{leaf}} = -4.3$ MPa. Bars are 20 μm . (f) Diagrammatic representation of changes in trichomes with dehydration, showing a trichome fully expanded and filled with water (blue) at $\Psi_{\text{leaf}} = -0.1$ MPa, shrunken, filled with gas (white) and with a collapsed cap cell at $\Psi_{\text{leaf}} = -0.25$ MPa, and shrunken, filled with gas, and with the cap and upper stalk cells collapsed at $\Psi_{\text{leaf}} = -4.3$ MPa.

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Fig. 5. Petiolar cisternae in leaves of *A. marina*. Distribution of cisternae (★) in transverse (a) and longitudinal (b) sections as visualized by bright- field microscopy. Cryo-SEM micrographs of cisternae

filled with water when $\Psi_{\text{leaf}} = -0.1$ MPa (c), or air and droplets when $\Psi_{\text{leaf}} = -0.1$ MPa (c, d), $\Psi_{\text{leaf}} = -0.7$ MPa (e) and $\Psi_{\text{leaf}} = -6$ MPa (f). Bars are 50 μm in (a, b); and 25 μm in (c-f).

Fig. 6. Cryo-SEM micrographs of transverse sections through (a, b) pith parenchyma in the midvein and (c, d) paradermal sections through spongy mesophyll cells in the lamina of leaves of *A. marina* differing in leaf water status. Arrows point at extracellular spaces filled with ice, indicating the presence of liquid water (a, c) at $\Psi_{\text{leaf}} = -0.06$ MPa and with gas (b,d) at $\Psi_{\text{leaf}} = -1$ MPa. Bars equal 20 μm in (a, b) and 10 μm in (c, d).

Fig. 7. Plasmolysis in different cell types of leaves of *A. marina* after turgor had been lost ($\Psi_{\text{leaf}} \approx -6$ MPa). (a) Petiolar collenchyma, (b) petiolar parenchyma surrounding gas-filled cisternae, (c) hypodermis, and (d) palisade mesophyll. Arrows indicate gaps between cell walls and cell membranes. Bars are 25 μm in (a, b), and 20 μm in (c, d).

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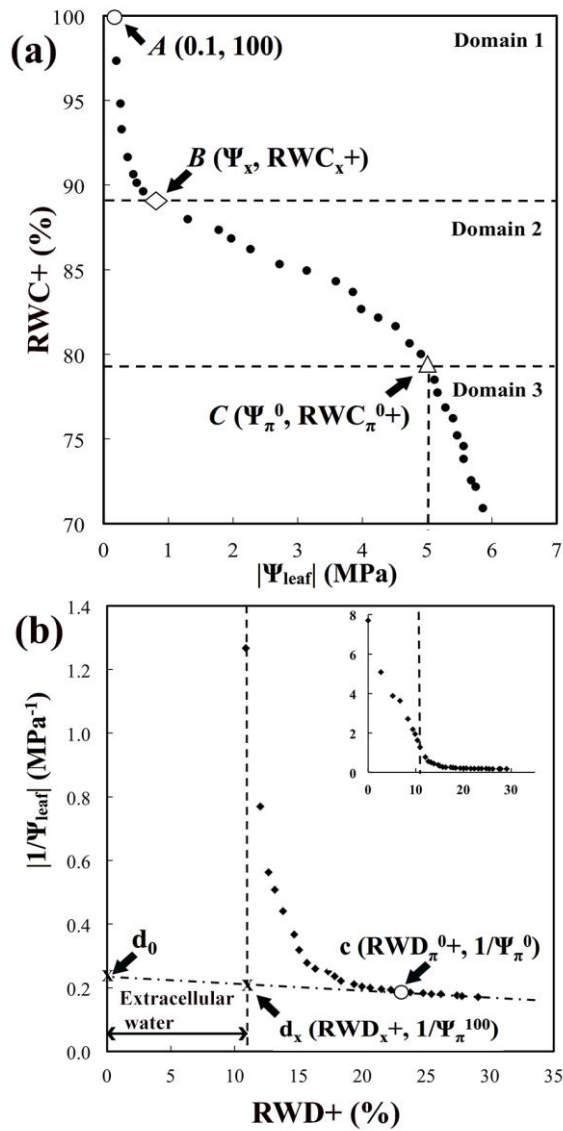


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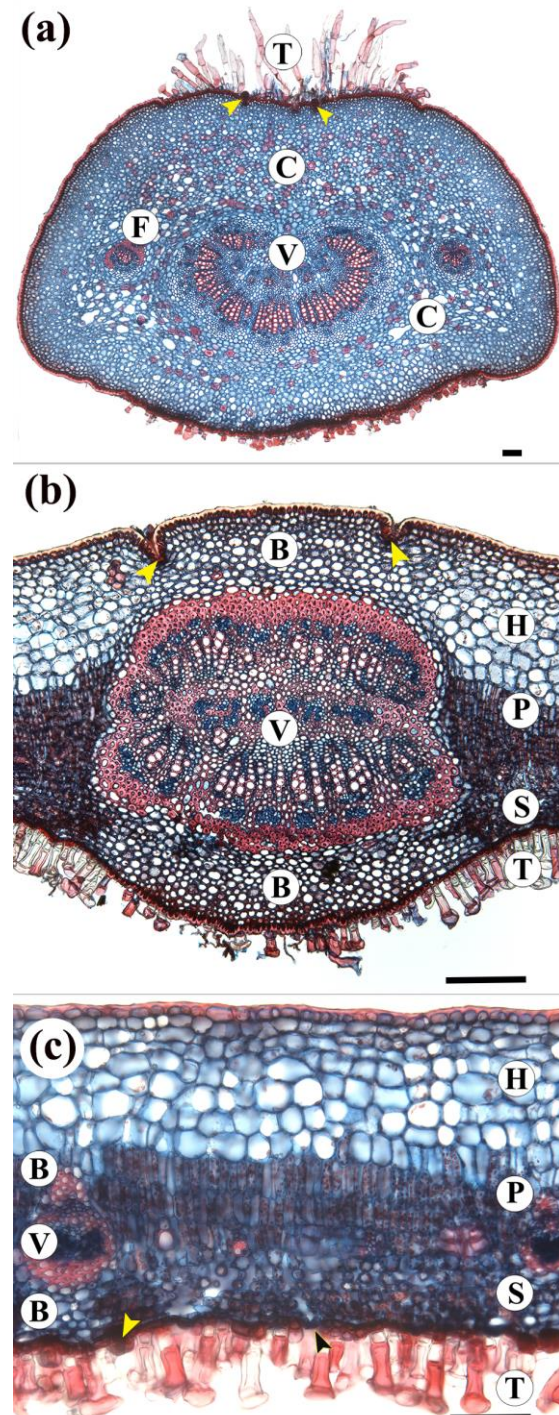


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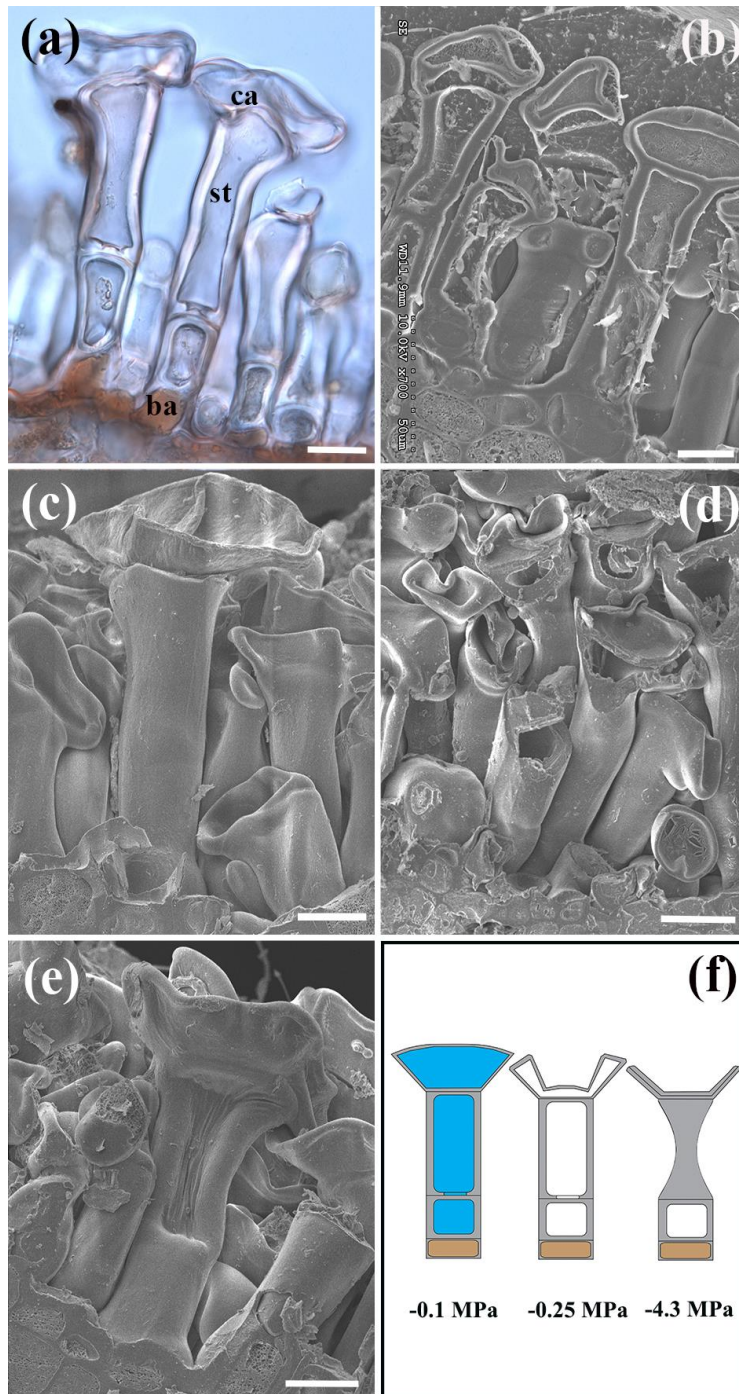


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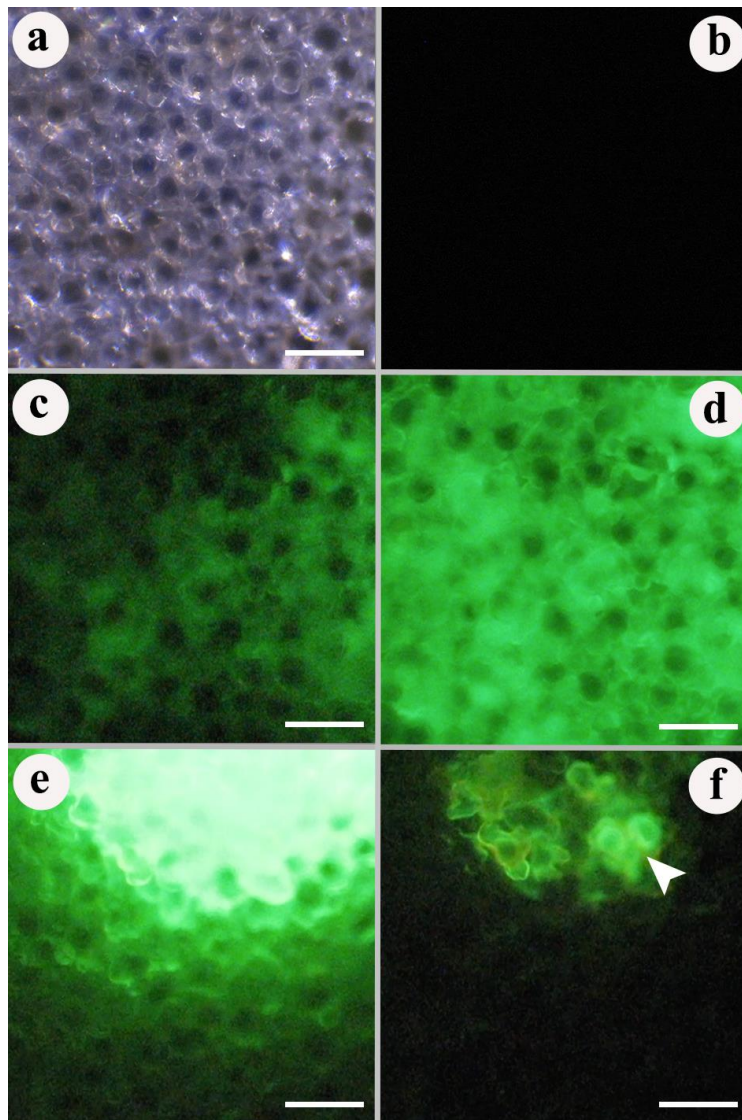


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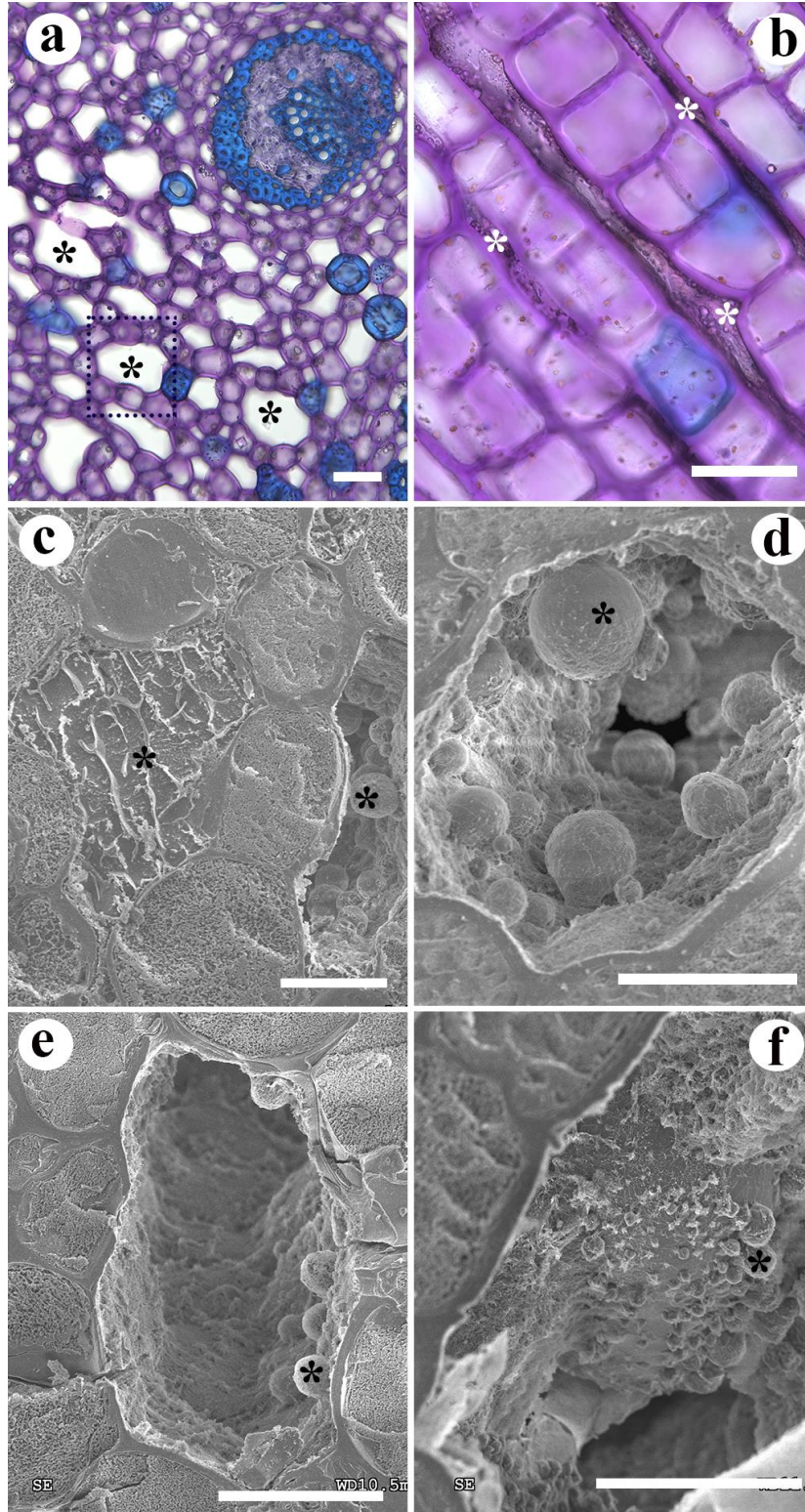


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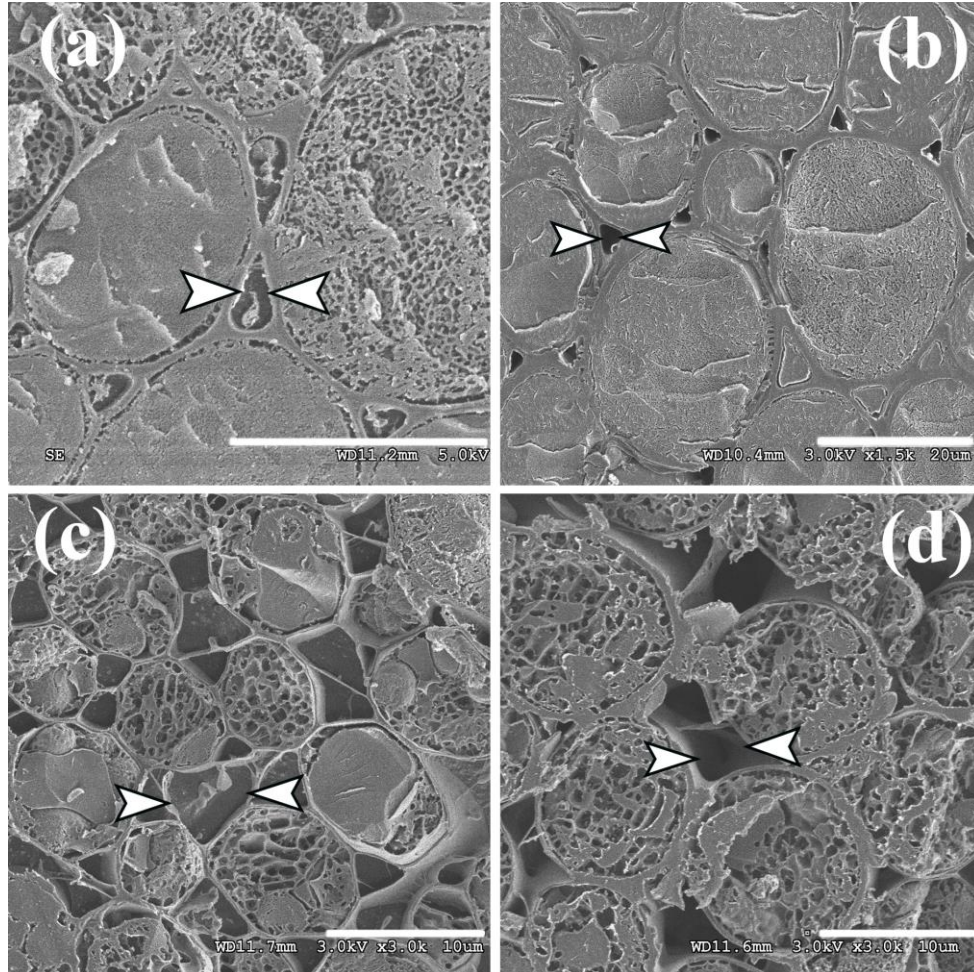


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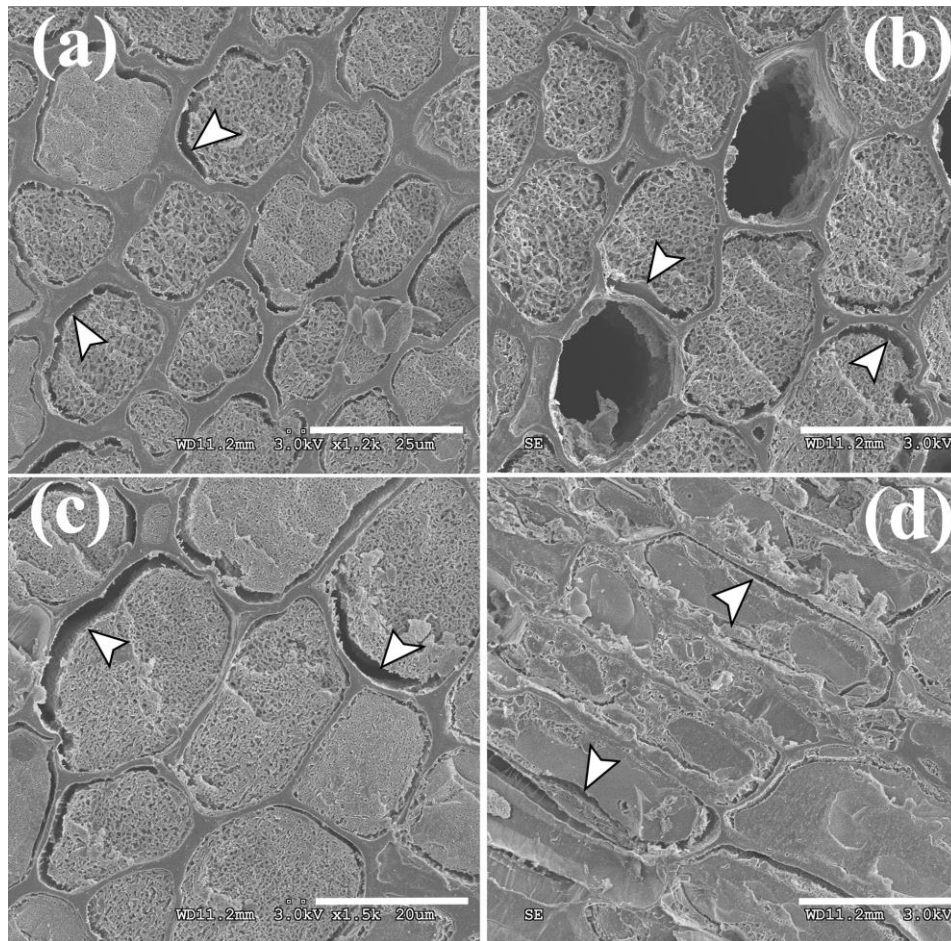
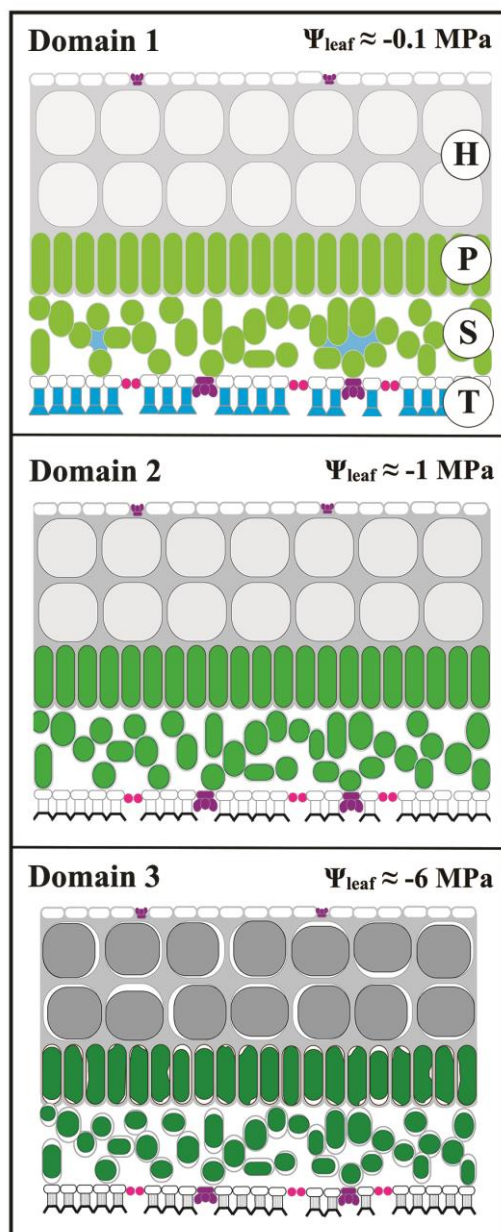


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