

# Function and structure of leaves contributing to increasing water storage with height in the tallest *Cryptomeria japonica* trees of Japan

Wakana Azuma<sup>1</sup> · H. Roaki Ishii<sup>1</sup> · Katsushi Kuroda<sup>2</sup> · Keiko Kuroda<sup>1</sup>

Received: 20 June 2015 / Revised: 21 August 2015 / Accepted: 26 August 2015 / Published online: 4 September 2015  
© Springer-Verlag Berlin Heidelberg 2015

## Abstract

**Key message** In *Cryptomeria japonica*, transfusion tissue in leaves may have functions of water storage and supply, which could compensate for hydraulic constraints with increasing height.

**Abstract** The tallest trees of *Cryptomeria japonica* occur in climatic regions similar to the world's tallest trees. We hypothesized that tall *C. japonica* trees would have evolved adaptive mechanisms to overcome height growth limitation. Here, we focused on foliar water storage, a mechanism recently discovered in *Sequoia sempervirens*. In *C. japonica*, leaf water potential at turgor loss did not change with height or light availability, while leaf hydraulic capacitance and succulence (water content per leaf surface area) increased, suggesting hydraulic compensation. Plasticity of leaf morphology could contribute to avoiding negative effects of height on photosynthesis. We also focused on the structure and function of transfusion tissue in leaves and its role in water storage and supply. Cross-sectional area of transfusion tissue increased with height, whereas that of xylem was constant. We confirmed that water flowed from vascular bundle to mesophyll via the transfusion tissue. Cryo-scanning electron microscopy images of leaf cross sections showed that transfusion cells were flattened, but not fully dehydrated when leaf water potential decreased

in situ and by experimental dehydration, and cell deformation was more marked for treetop leaves than for lower-crown leaves. The shape of transfusion cells recovered at predawn as well as after experimental rehydration. As in *S. sempervirens*, transfusion tissue of *C. japonica* may function as a hydraulic buffer, absorbing and releasing water according to leaf water status. Anatomical and hydraulic properties contributing to foliar water storage may be an adaptive mechanism acquired by tall Cupressaceae trees to overcome the hydraulic constraints on physiological function with increasing height.

**Keywords** Hydraulic limitation · Capacitance · Leaf morphology · Leaf anatomy · Transfusion tissue · Vertical gradient

## Introduction

The hydraulic limitation hypothesis (HLH, Ryan and Yoder 1997) proposed that as tree height increases, available water decreases due to increasing hydrostatic pressure and distance of water transport, while water demand for transpiration and photosynthesis increases (Cowan 1982; Zimmermann 1983; Midgley 2003). The resulting water stress constrains various physiological processes such as photosynthetic production (Ambrose et al. 2010), turgor needed for cell expansion (Woodruff et al. 2004), etc., ultimately limiting height growth (Ryan et al. 2006). However, HLH alone cannot explain growth decline with increasing height (McDowell et al. 2002; Niinemets 2002). Hydraulic limitation could be compensated in various ways, including decreasing leaf water potential (Barnard and Ryan 2003), osmotic adjustment (Woodruff et al. 2004) and water storage (Phillips et al. 2003; Scholz et al.

Communicated by M. Shane.

✉ H. Roaki Ishii  
hishii@alumni.washington.edu

<sup>1</sup> Graduate School of Agricultural Science, Kobe University, 2-411 Science and Technology Bldg., Kobe 657-8501, Japan

<sup>2</sup> Department of Wood Properties, Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan

2011). While height growth of normal-statured trees may indeed be hydraulically limited (e.g., Nabeshima and Hiura 2008; Renninger et al. 2009; Meinzer et al. 2010), the prominent tall trees are likely to have evolved adaptive mechanisms to overcome hydraulic limitation (Ishii et al. 2014), which would explain why they grow so much taller than other species. In tall and large trees of the world, growth rates continue to increase with increasing tree size (Ambrose et al. 2010; Stephenson et al. 2014; Sillett et al. 2015) suggesting that, despite their great size, these trees are not carbon limited as HLH would predict.

The hydraulic limitation hypothesis is also based on the assumption that the only source of water for trees is from the roots. However, in *Sequoia sempervirens*, the tallest species, absorption of moisture from leaf surfaces and water storage in leaves alleviates water stress in treetop leaves, decoupling leaf water status from soil moisture content and decreasing reliance on long-distance water transport from roots (Burgess and Dawson 2004; Simonin et al. 2009; Ishii et al. 2014). In *S. sempervirens*, hydraulic capacitance and succulence of leaves increase with height, and treetop leaves have capacity to store more than five times the daily transpiration demand (Ishii et al. 2014). As a result, despite decreasing leaf water potential with increasing height (Koch et al. 2004; Ishii et al. 2008), leaf osmotic potential at turgor loss, which is expected to decrease when leaves experience constant drought conditions (Pallardy 2007; Rodriguez et al. 2012; Negret et al. 2013), remains constant within the crown of *S. sempervirens* (Ishii et al. 2014). Although these results indicate the importance of foliar water storage for maintaining the water status of treetop leaves in tall trees, the detailed hydraulic mechanism and anatomical features involved are not fully understood.

Here, we focused on anatomical features of leaves that could contribute to foliar water storage. As part of the hydraulic system in addition to the xylem, transfusion tissue, specific to gymnosperms and composed of tracheids and parenchyma cells, is suggested to function as a pipeline for water and nutrients between the vascular bundle and leaf mesophyll (Takeda 1931; Hu and Yao 1981; Brodribb et al. 2010; Aloni et al. 2013). In some gymnosperms, transfusion cells lacking endodermis or bundle sheath collapse easily under experimentally induced negative pressure (Brodribb and Holbrook 2005; Zhang et al. 2014). This is associated with declines in leaf hydraulic conductance ( $K_{\text{leaf}}$ ), but is easily reversible (Zhang et al. 2014). In *S. sempervirens*, transverse-sectional area of transfusion tissue increases with height (Ishii et al. 2014) and collapsed transfusion cells were observed close to vascular cells in the upper-crown leaves (Oldham et al. 2010). These observations suggest that transfusion tissue serves to protect the xylem from embolism by releasing stored water,

temporarily relieving tension in the xylem, which could compensate for the negative effects of hydraulic limitation with increasing height (Oldham et al. 2010). However, there are no investigations of the hydraulic function of transfusion tissue in tall trees.

The world's tallest trees appear to grow in thermally similar climates (Larjavaara 2014). The tallest trees in Japan, *Cryptomeria japonica* (Cupressaceae), are found in the northwest coast of Honshu Island in humid continental climate, which is a localized temperate, maritime climate similar to Mediterranean and maritime west coast climates (climate classification according to Peel et al. 2007), where the tallest gymnosperm (*S. sempervirens*) and angiosperm (*Eucalyptus regnans*) occur, respectively. *C. japonica* is also the major plantation tree in Japan and a valuable forest resource. Because *C. japonica* and *S. sempervirens* belong to the same family and grow tall in similar climates, we hypothesized that the tallest *C. japonica* trees would have evolved adaptive mechanisms to overcome hydraulic limitation, similar to *S. sempervirens*. To test this, we investigated vertical changes in leaf hydraulic properties focusing on foliar water storage, the compensating mechanism recently discovered in *S. sempervirens* (Ishii et al. 2014). We also observed microscopic structures of leaves both in situ and in the laboratory to elucidate the role of transfusion tissue in leaf hydraulic functioning.

## Materials and methods

The study was conducted at Nibuna-Mizusawa Forest Reserve (NMFR), Tashirozawa National Forest in Akita Prefecture, Japan (40.08°N, 140.25°E, and 200 m ASL). The site is a mixed forest composed of ca. 250-year-old *C. japonica* trees and deciduous broadleaved trees, such as *Aesculus turbinata* and *Pterocarya rhoifolia*. The mean annual precipitation was 1671.1 mm and the mean annual temperature 0.2 °C in 1981–2010. Snow covers the ground from early December to late March and the maximum snow depth in 2013 was 131 cm.

The NMFR was designated to protect the tallest *C. japonica* forest in Japan. Tree height and diameter at breast height (DBH, 1.3 m above ground level) of the 159 *C. japonica* trees in the 1-ha research plot established by Akita Pref. Univ. were  $49.8 \pm 1.8$  m and  $111.6 \pm 5.5$  cm (mean  $\pm$  SD), respectively, in 2012. In May 2013, we accessed the crown of four study trees, 48–52 m tall and 105–118 cm DBH, using single-rope climbing technique. We collected small branches (30–50 cm long) with attached foliage from the outer crown of each tree at 5–10 m intervals from just below treetop to the lowest living branches in the four trees (height range 19–52 m). We took hemispherical photographs directly above each

sampling location and calculated light availability expressed as canopy openness (%) using Gap Light Analyzer (ver 3.1, Simon Fraser University, Burnaby, BC, Canada). The sampled branches were immediately re-cut under water, sealed in black plastic bags, and fully rehydrated in the laboratory overnight.

### Leaf water relations

We obtained the pressure–volume curve of three small, foliated shoots from each sampled branch comprising second- and current-year internodes, using the bench-drying approach to the pressure–volume technique (Tyree and Hammel 1972; Schulte and Hinckley 1985). We repeatedly measured bulk leaf water potential ( $\Psi_L$ , MPa) with a pressure chamber (Model 1000, PMS Instruments, Corvallis, USA) and fresh weight of the sample shoots before and after each water potential reading. Care was taken to increase and decrease the pressure in the chamber very slowly (less than  $0.01 \text{ MPa s}^{-1}$ ), so as not to damage the sample shoots (repeat pressurization method, Hinckley et al. 1980; Ritchie and Roden 1985; Parker and Colombo 1995).

After the pressure–volume measurement, all the sample shoots were photographed for measurement of total leaf surface area ( $A_L$ ,  $\text{m}^2$ ) as described below and then oven dried to constant weight to obtain leaf dry mass ( $M_D$ , g). To estimate drought tolerance of shoots, we calculated fresh weight at saturation ( $M_F$ , g), osmotic potential at saturation ( $\Psi_{\text{sat}}$ , MPa), osmotic potential at turgor loss ( $\Psi_{\text{tlp}}$ , MPa), and relative water content at turgor loss ( $\text{RWC}_{\text{tlp}}$ ) at the bulk shoot level.  $\Psi_{\text{tlp}}$  is a physiological measure of plant water stress (Bartlett et al. 2012), which decreases if leaves experience constant drought conditions (e.g., Pallardy 2007; Rodriguez et al. 2012; Negret et al. 2013). The saturated leaf water content ( $M_W = M_F - M_D$ ) was used for calculating leaf hydraulic capacitance ( $C_L$ ,  $\text{mol m}^{-2} \text{MPa}^{-1}$ ), and succulence ( $S_L$ ,  $\text{g H}_2\text{O m}^{-2}$ ).

$$C_L = \delta\text{RWC}/\delta\Psi_L(M_D/A_L) (M_W/M_D)/M,$$

$$S_L = M_W/A_L,$$

where  $\delta\text{RWC}/\delta\Psi_L$  is the slope of the  $\Psi_L$ –RWC relationship calculated from the pressure–volume curve and  $M$  is the molecular weight of water.

### Leaf morphology and anatomy

After pressure–volume measurement, each sample shoot was placed on a slide viewer, illuminated from below, and photographed to obtain the shoot silhouette image. Then all leaves were detached from the shoot axis, laid on the slide viewer without overlap, and photographed. Photographed

images of shoots and leaves were analyzed using image analysis software (Image-J ver. 1.48; National Institute of Health, USA) to quantify shoot silhouette area ( $A_S$ ,  $\text{m}^2$ ) and projected leaf area ( $A_P$ ,  $\text{m}^2$ ). To obtain  $A_L$ , perimeter-to-width ratios obtained from leaf transverse sections were multiplied by  $A_P$  (Barclay and Goodman 2000). We calculated leaf mass per area ( $\text{LMA} = M_D/A_P$ ,  $\text{g m}^{-2}$ ) and shoot silhouette area to projected leaf area ratio ( $\text{SPAR} = A_S/A_P$ ), a measure of leaf overlap within the shoot.

Second-year leaves of a different set of sample shoots from the same branches were fixed with FAA (formalin, acetic acid, 50 % ethyl alcohol; 5:5:90 v/v) and washed under tap water overnight. For all heights, transverse sections ( $24 \mu\text{m}$  thickness) were taken consistently from the same part of the leaf (at the midpoint between leaf tip and its attachment to the stem) using a sliding microtome installed with a holder for frozen sectioning (REM-710, Yamato Kohki Industrial Co., LTD., Japan). Leaf sections were double stained with safranin-fast green to differentiate lignified cells and living cells. Three leaf transverse sections from each sampled branch were observed under an optical microscope (Nikon, Eclipse 80i, Nikon, Tokyo) and photographed with a digital camera (E-620, Olympus, Tokyo). Then, we quantified the cross-sectional area of xylem ( $A_X$ ,  $\text{mm}^2$ ) and transfusion tissue ( $A_{\text{TT}}$ ,  $\text{mm}^2$ ) using Image-J.

### Structure and function of transfusion tissue

To roughly detect the pathway of water flow through leaf tissues, the cut end of foliated shoots comprising third-, second- and current-year internodes sampled from 50 to 26 m height were immersed in an aqueous solution of 0.5 % (w/v) acid fuchsin. After 1 and 2 h, we observed by eye that the leaf apices were stained red, indicating that the shoots had absorbed the acid fuchsin. Three leaves from each shoot were transversely sectioned at midpoint to  $24 \mu\text{m}$  thickness, using the same methods as described above. The leaf sections were observed under the optical microscope.

To observe diurnal changes in leaf anatomy and water status, intact foliated shoots at treetop (52 and 51 m) and lowest (26 and 19 m) branches in two of the study trees were flash frozen in situ in liquid nitrogen ( $\text{LN}_2$ ) at pre-dawn and midday on a clear day in September 2014. The frozen shoots were then cut and kept immersed in  $\text{LN}_2$ , transported to the laboratory, and stored in a freezer at  $-80 \text{ }^\circ\text{C}$ . A fresh transverse surface was made at the midpoint of second-year leaves from each frozen shoot using an electronic microtome cryostat (HM 505 E, Microm International, Walldorf, Germany) at  $-35 \text{ }^\circ\text{C}$ . The specimen was attached to the holder with Tissue-Tek

Embedding Compound (Sakura FinetekUSA, Torrance, CA, USA) and then transferred to a cryo-scanning electron microscopy system (cryo-SEM; JSM6510, JEOL, Tokyo, Japan). Secondary electron images were obtained at an accelerating voltage of 3 kV with shallow sublimation to observe water within the leaf structure. After obtaining images, samples were sublimated deeply for approximately 30 min to remove water from the sample surfaces to observe the leaf structure clearly.

Along with the frozen samples, we collected a pair of small branches (30–50 cm long) from each of the sampling locations at midday. Using one set of these samples, we measured bulk leaf water potential ( $\Psi_L$ ) with a pressure chamber as reference of in situ  $\Psi_L$  at midday. The remaining set of sample branches were immediately re-cut under water, sealed in black plastic bags, and fully rehydrated in the laboratory overnight. Three to five foliated shoots on the rehydrated branches were flash frozen in LN<sub>2</sub> before removal from the branch. Three to five additional shoots were bench dried until  $\Psi_L$  reached values similar to that at midday in the field, then flash frozen in LN<sub>2</sub>. We obtained cryo-SEM images of the rehydrated and bench-dried leaves before and after deep sublimation using the same methods as described above.

We analyzed the cryo-SEM images to compare the shape of transfusion cells under different conditions. In each image, we randomly chose ten transfusion cells and measured the cross-sectional area ( $A_{TC}$ ,  $\mu\text{m}^2$ ) and perimeter ( $P_{TC}$ ,  $\mu\text{m}$ ) using Image-J to calculate circularity ( $C_{TC} = 4\pi A_{TC}/P_{TC}^2$ ) of each transfusion cell.

### Statistical analyses

For each variable of leaf water relations, leaf morphology and leaf anatomy, intercepts and slopes of the relationships among study trees were compared by analysis of covariance (ANCOVA) with individual study trees as the random effect and height and canopy openness as the covariate. Although the random effect of the study trees was significant for some variables, the relationships with height and canopy openness was similar for all study trees. Thus to examine species characteristics, we pooled the data for all study trees and analyzed each variable in relation to height and canopy openness using regressions analysis.

We compared  $A_{TC}$  and  $C_{TC}$  between different water statuses and heights by three-way analysis of variance (ANOVA) with water status and height as the main effects and study trees as a random effect. Although the random effect of the study tree was marginally significant ( $F > 4.8$ ,  $P < 0.048$ ) for some variables, the trends were similar for all study trees. All statistical analyses were done using JMP 11 (SAS Inc., Cary, NC, USA).

## Results

### Leaf water relations

Osmotic potential at turgor loss ( $\Psi_{\text{tip}}$ ) did not change with either height or canopy openness (Fig. 1a, b;  $P = 0.89$  and  $P = 0.23$ , respectively). The osmotic potential at saturation ( $\Psi_{\text{sat}}$ ) did not change with height or canopy openness (Fig. 1c, d;  $P = 0.39$  and  $P = 0.54$ , respectively). Relative water content at turgor loss ( $\text{RWC}_{\text{tip}}$ ) increased with both height and canopy openness ( $R^2 = 0.18$ ,  $P = 0.002$  and  $R^2 = 0.18$ ,  $P = 0.002$ , respectively). Leaf hydraulic capacitance ( $C_L$ ) and succulence ( $S_L$ ) both increased with height (Fig. 1e, g;  $P = 0.04$  and  $P < 0.0001$ , respectively).  $C_L$  and  $S_L$  of treetop leaves were almost twice that of lower-crown leaves. In relation to canopy openness,  $C_L$  did not change, while  $S_L$  increased consistently (Fig. 1f, h;  $P = 0.26$  and  $P < 0.0001$ , respectively).

### Leaf morphology and anatomy

Leaf area of the sample shoots was constant within the crown ( $R^2 = 0.006$ ,  $P = 0.58$ ), but leaf dry mass increased with height ( $R^2 = 0.21$ ,  $P = 0.0006$ ). As a result, Leaf mass per area (LMA) increased with both height and canopy openness (Fig. 2, b;  $P < 0.0001$  and  $P < 0.0001$ , respectively). Shoot silhouette area to projected leaf area ratio (SPAR) decreased with height and canopy openness (Fig. 2c, d;  $P < 0.0001$  and  $P = 0.0004$ , respectively); that is to say, treetop leaves are arranged spherically along the shoot axis with more overlap than lower-crown leaves.

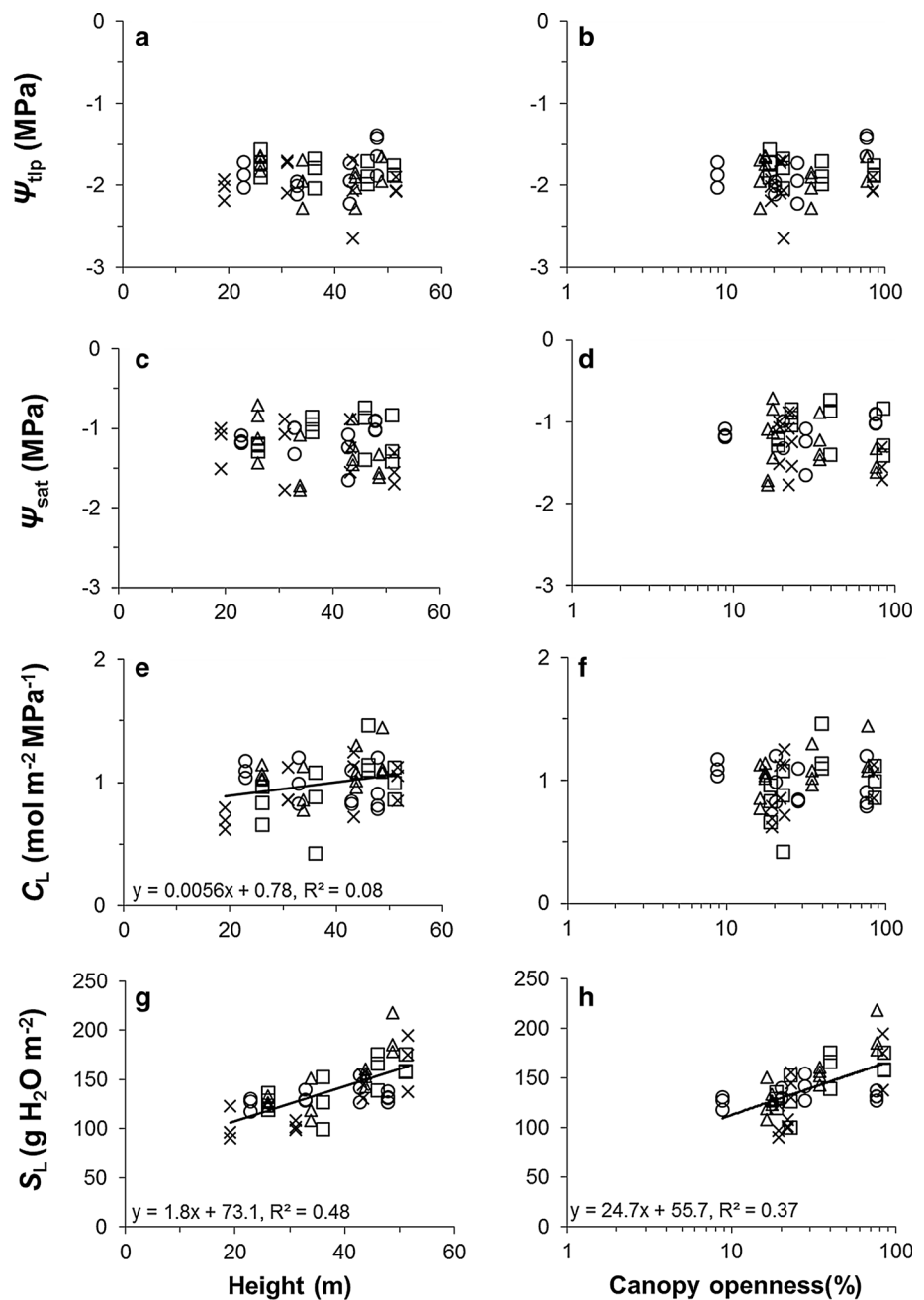
Cross-sectional area of xylem ( $A_X$ ) did not change with either height or canopy openness (Fig. 2e, f;  $P = 0.58$  and  $P = 0.52$ , respectively), whereas that of transfusion tissue ( $A_{TT}$ ) increased with both height and canopy openness (Fig. 2g, h;  $P < 0.0001$  and  $P < 0.0001$ , respectively), such that  $A_{TT}$  of treetop leaves was three times that of lower-crown leaves.

### Structure and function of transfusion tissue

Transfusion tissue was located on both sides of the vascular bundle surrounded with bundle sheath (Fig. 3). One hour after the absorption of acid fuchsin solution, xylem, phloem and transfusion tissues of both samples at 50 and 26 m were stained (white arrows in Fig. 3b). Two hours after the absorption, cells in the mesophyll were also stained (white arrows in Fig. 3c), indicating that water flowed from xylem to mesophyll via transfusion tissue.

The cryo-SEM images of leaves flash frozen in situ are shown in Fig. 4, while the images of leaves flash frozen in the laboratory are shown in Fig. 5. The explanation

**Fig. 1** Leaf water relations of *Cryptomeria japonica* trees. Osmotic potential at turgor loss (a, b), osmotic potential at saturation (c, d), leaf hydraulic capacitance (e, f), and succulence (g, h) shown in relation to height and light availability (canopy openness). Lines indicate significant linear regressions ( $P < 0.01$ , except for the relationship between  $C_L$  and height where  $P = 0.04$ ). Symbol shapes denote different trees



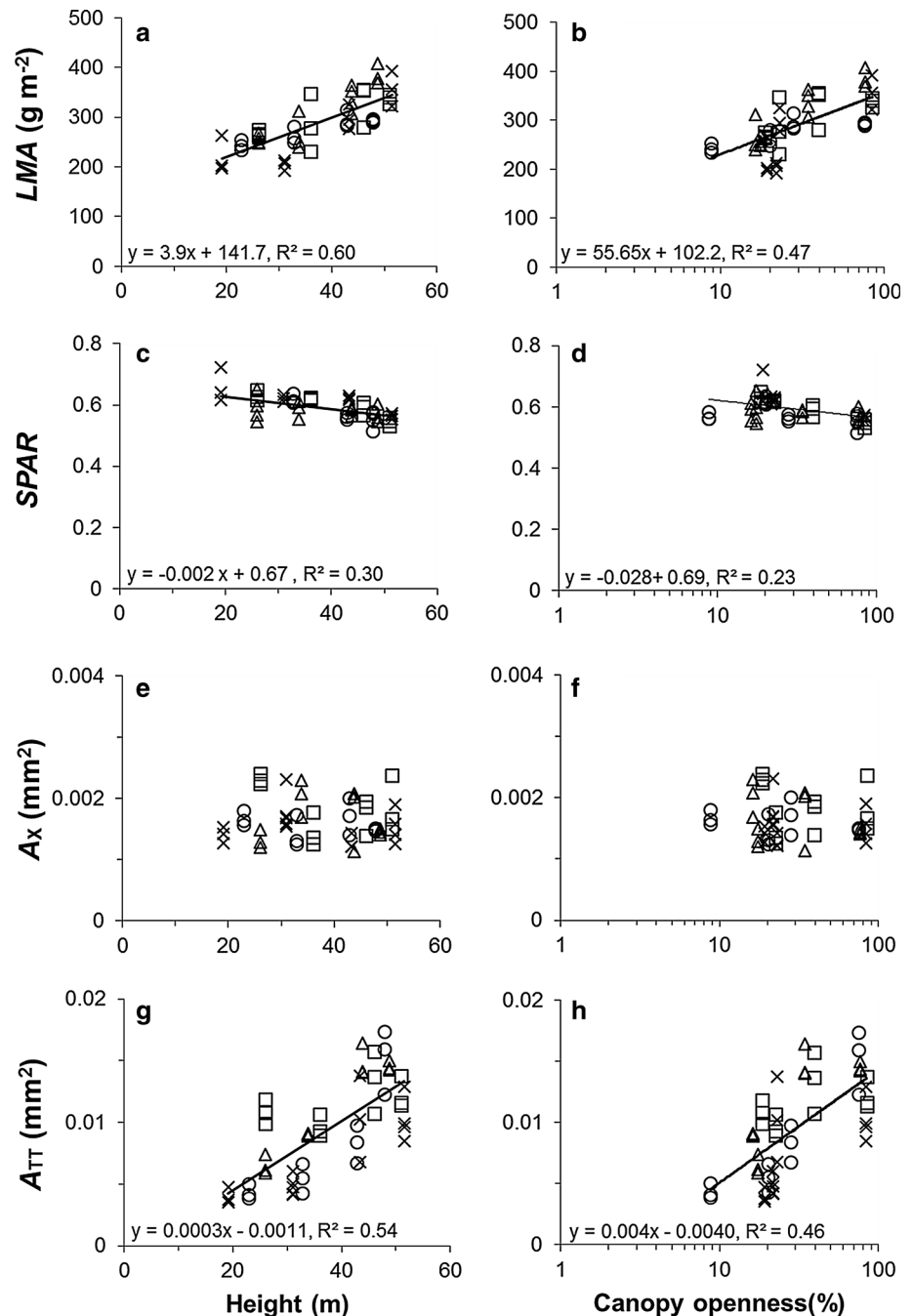
below is common to all observed leaves of the two study trees.

In leaves that were flash frozen in situ at predawn, all transfusion cells and xylem tracheids of both the top (Fig. 4b) and lower-crown leaves (Fig. 4c) were filled with water and fully expanded. In leaves that were flash frozen in situ at midday, all transfusion cells of both the top- (Fig. 4d;  $\Psi_L = -18.6$  MPa, for both study trees) and lower-crown (Fig. 4e;  $\Psi_L = -15.5$  and  $-12.2$  MPa, for the two study trees) leaves were filled with water, but some transfusion cells were flattened and/or concave (arrows in

Fig. 4d, e), while xylem cells were filled with water and their shapes were maintained. We did not observe xylem embolism in any of the cryo-SEM images of the top and lower-crown leaves, regardless of conditions. The shapes of bundle sheath and phloem were also maintained (Fig. 4d, e). The shape of the deformed transfusion cells were more clearly observed in the cryo-SEM images after deep sublimation (arrows in Fig. 4f, g).

In cryo-SEM images of leaves after rehydration in the laboratory, all transfusion cells and xylem tracheids of both the top- (Fig. 5a) and lower-crown leaves (Fig. 5b) were

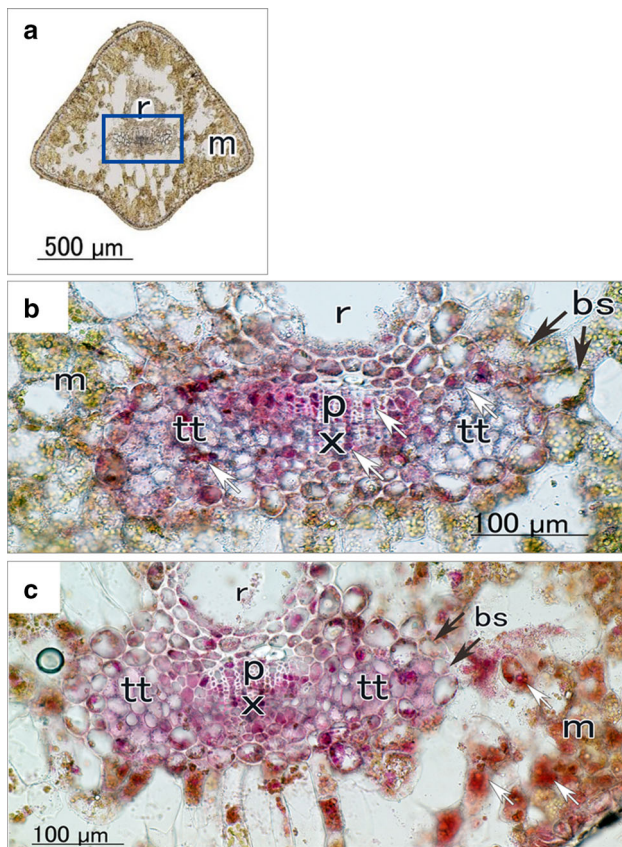
**Fig. 2** Leaf morphology and anatomy of *C. japonica* trees. Leaf mass per area (**a**, **b**), shoot silhouette area to projected leaf area ratio (**c**, **d**), and cross-sectional area of xylem (**e**, **f**) and that of transfusion tissue (**g**, **h**) shown in relation to height and light availability (canopy openness). Lines indicate significant linear regressions ( $P < 0.01$ ) and symbols shapes denote different trees



filled with water and fully expanded. This was similar to the leaves that were flash frozen in situ at predawn (Fig. 4b, c). After bench drying to  $\Psi_L$  values equal to those observed at midday in the field, all transfusion cells of both the top- (Fig. 5c) and lower-crown leaves (Fig. 5d) were filled with water, but some transfusion cells were flattened and/or concave (arrows in Fig. 5c, d), while xylem cells were filled with water and their shapes maintained. We did not observe xylem embolism in any of the cryo-SEM images of the top and lower-crown leaves, regardless of

treatments. The shape of bundle sheath and phloem was also maintained (Fig. 5c, d). The shape of the deformed transfusion cells were more clearly observed in the cryo-SEM images after deep sublimation (arrows in Fig. 5e, f).

In leaves that were flash frozen in situ,  $C_{TC}$  at midday was lower (more flattened) than that at predawn (Table 1,  $F = 89.8$ ,  $P < 0.001$ ) and the difference was greater for treetop leaves than for lower-crown leaves ( $F = 12.5$ ,  $P = 0.007$ ). For both the treetop and lower-crown leaves,  $A_{TC}$  at midday was smaller than that at predawn ( $F = 38.1$ ,



**Fig. 3** **a** Optical microscope image of the transverse section a treetop leaf (50 m) of *C. japonica*. **b** Magnified image of the rectangular region in **a**, 1 h after absorption of acid fuchsin solution, where phloem, xylem, and transfusion tissue were stained by acid fuchsin (e.g., white arrows). **c** Two hours after absorption, the mesophyll was also stained (e.g., white arrows). *r* resin duct, *m* mesophyll, *p* phloem, *x* xylem, *tt* transfusion tissue, *bs* bundle sheath. We also observed same phenomenon in the lower-crown leaves

$P < 0.001$ ). For both treetop and lower-crown leaves frozen in the laboratory,  $C_{TC}$  and  $A_{TC}$  of bench-dried leaves were lower and smaller, respectively, than that of rehydrated leaves ( $F = 49.6$ ,  $P < 0.001$  and  $F = 20.0$ ,  $P < 0.001$ , respectively).

## Discussion

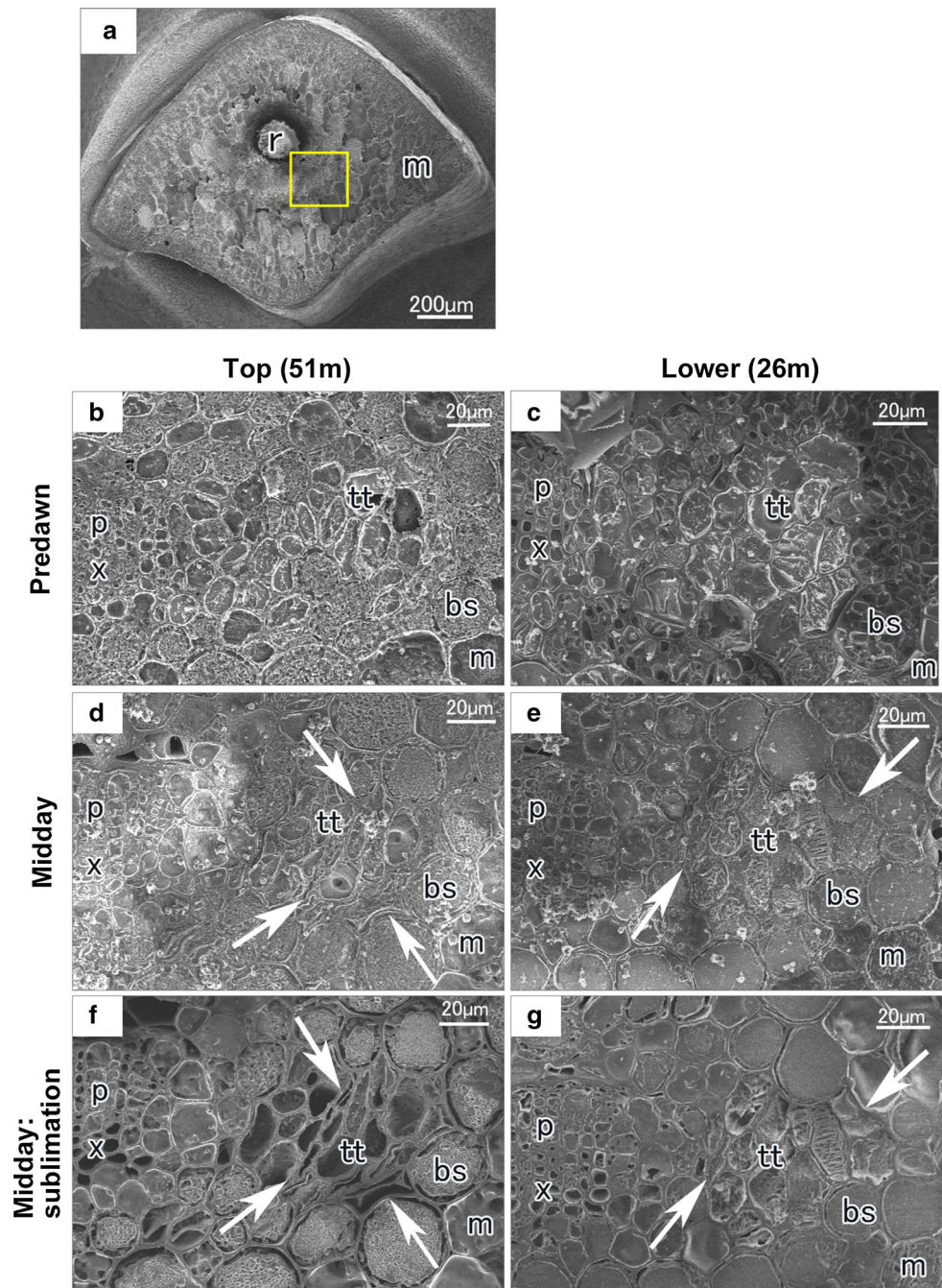
In the tallest *C. japonica* trees,  $\Psi_{tip}$  remained constant with height and canopy openness, indicating that drought tolerance of shoots was constant within the crown. On the other hand,  $C_L$  and  $RWC_{tip}$  increased with height and canopy openness, which suggested increasing drought avoidance with increasing height and irradiance. Typically, leaves with high  $C_L$  have elastic cell walls, which contribute to slow water potential decrease and maintenance of leaf turgor in response to decreasing water content (Salleo 1983). Leaves with elastic cell walls, however, have less

ability for water retention than those with hard cell walls (Salleo 1983). To compensate for this, the treetop leaves of *C. japonica* were succulent, having high capacity for water storage relative to evaporative surface area. Such drought avoidance of leaves is important for maintaining productivity at the treetop, where high light and high temperatures cause high vapor pressure deficit. In *Eucalyptus pauciflora*, which were watered after experimentally being exposed to severe water stress, recovery of stomatal conductance occurred much later than stem hydraulic conductance suggesting that in this species, leaf gas exchange is tightly regulated to avoid drought stress (Martorell et al. 2014). Although in *Callitris rhomboidea* (Cupressaceae), transpiration rates recovered rapidly to pre-drought levels after rewatering (Brodrigg and Cochard 2009), such regulation may result in reduced photosynthetic rates under constant water stress. While various mechanisms have been suggested as causes for hydraulically induced stomatal limitation in tall trees, in tall *C. japonica*, foliar water storage may be an adaptation, which allows the tallest trees to maintain physiological function in treetop leaves. A similar homeostatic response of  $\Psi_{tip}$  and foliar water storage in treetop leaves was also found in *S. sempervirens* (Ishii et al. 2014) and may be a common adaptation to hydraulic constraints in tall Cupressaceae.

In succulent leaves of olive (*Olea europaea* L.) growing in arid regions, osmotic adjustment and development of thick leaves are major adaptations against water stress (Xiloyannis et al. 1999; Bacelar et al. 2004). In the *C. japonica* in this study, osmotic adjustment was not observed but LMA increased with height and light availability. In tall trees, the increase in LMA is driven by height (i.e., water status) more than light availability (Coble et al. 2014) and leaves with greater LMA have less evaporative surface area per dry mass, which prevents decreases in leaf-specific hydraulic conductivity under water-stressed conditions (Niinemets 2002; Burgess et al. 2006). In addition, we found SPAR decreased with height and light availability. In conifer shoots, low SPAR is associated with increasing boundary layer resistance, which decreases evapo-transpirational demand under high vapor pressure deficit (Martin et al. 1999). Low SPAR of treetop leaves also contributes to avoiding the negative effects of excess irradiance on photosynthesis, allows more sunlight to penetrate into the lower crown, and compensates for decreasing photosynthetic rate per leaf dry mass (Niinemets 2002; Ishii et al. 2007, 2012).

Leaf anatomy also responds to water stress. For example, in the epiphytic plant, *Sarmienta repens*, plants in sun conditions develop thicker, water-storing leaf parenchyma than those in shade (Godoy and Gianoli 2013). In *C. japonica*, mesophyll anatomy and cuticle thickness did not change with height and light availability (data not shown),

**Fig. 4** **a** Cryo-SEM images of transverse surface of a second-year leaf of *C. japonica*. **b–g** Magnified images of transfusion tissue area, which correspond to the *rectangular* region in **a**. Treetop (**b, d, f**; 51 m) and lower-crown (**c, e, g**; 26 m) leaves were flash frozen in situ at predawn (**b, c**) and at midday (**d**;  $\Psi_L = -18.6$  MPa, **e**;  $\Psi_L = -15.5$  MPa) on a clear day. **f** and **g** were taken after deep sublimation of **d** and **e**, respectively. *Arrows* denote examples of deformed transfusion cells. *r* resin duct, *m* mesophyll, *p* phloem, *x* xylem, *tt* transfusion tissue, *bs* bundle sheath

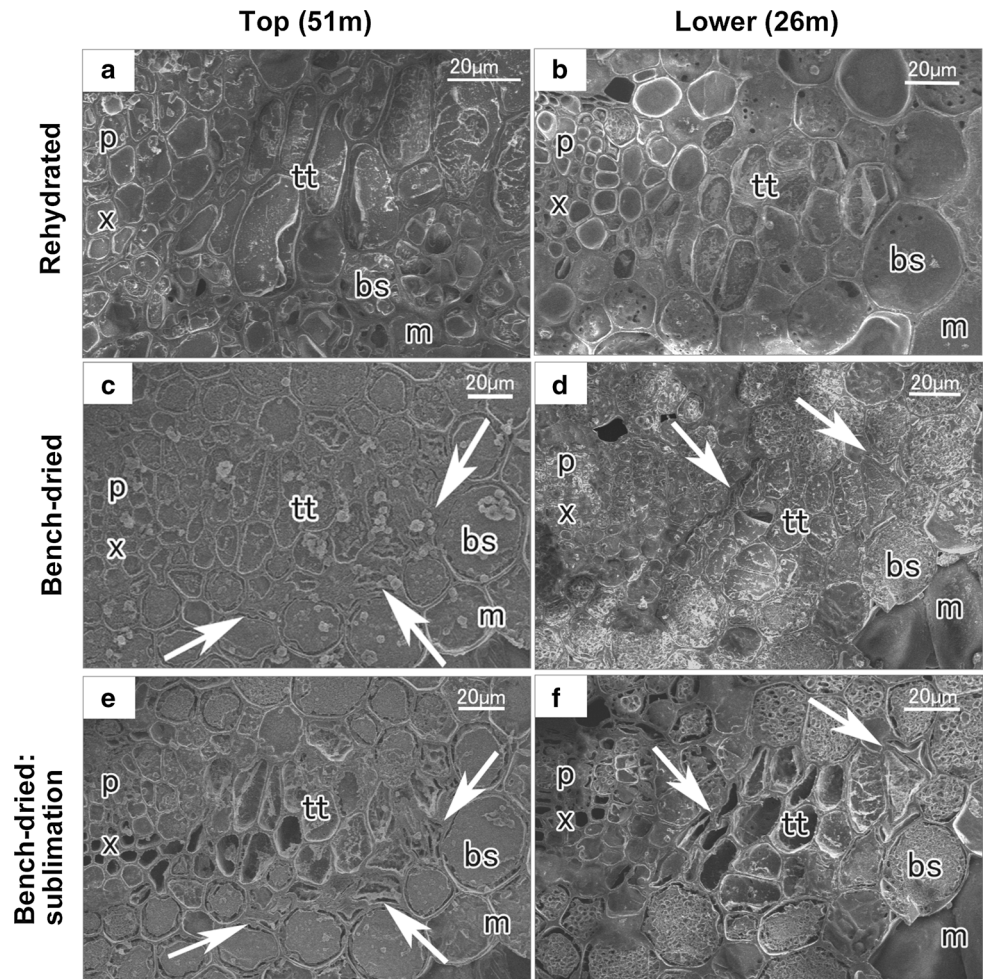


while  $A_{TT}$  increased. Our cryo-SEM images showed that transfusion cells were flattened when leaf water potential decreased in situ and by experimental dehydration. Deformation of transfusion cells surrounded by bundle sheath may be related to their anatomical characteristics, such as non-uniform shape, tracheary-element-like hollow structure, and cell walls with numerous bordered pits and less lignin deposition compared with normal tracheids (Esau 1997; Bouche et al. 2014). While collapse of xylem cells induces significant reduction in hydraulic conductance

(Cochard et al. 2004), in experimentally dehydrated leaves of some conifer species, transfusion cells dehydrate or shrink before embolism occurs in adjacent tracheids in the vascular bundle and contributes to preventing xylem dysfunction (Brodrigg and Holbrook 2005; Johnson et al. 2009; Zhang et al. 2014). We found that transfusion cells of *C. japonica* were flattened but not fully dehydrated, while no xylem cells were empty or deformed both in situ at midday and by experimental dehydration. In addition, recovery of the shape of transfusion cells at predawn as well as after



**Fig. 5** Cryo-SEM images of transverse surface of second-year leaves of *C. japonica*. Treetop (51 m; **a, c, d**) and lower-crown (26 m; **b, d, f**) leaves were flash frozen after rehydration (**a, b**) and subsequent bench drying until  $\Psi_L$  reached values similar to those at midday in the field (**c**;  $\Psi_L = -18.6$  MPa, **d**;  $\Psi_L = -15.5$  MPa). **e** and **f** were taken after deep sublimation of **c** and **d**, respectively. Arrows denote examples of deformed transfusion cells. *m* mesophyll, *p* phloem, *x* xylem, *tt* transfusion tissue, *bs* bundle sheath



**Table 1** Circularity ( $C_{TC}$ ) and cross-sectional area ( $A_{TC}$ ) of transfusion cells of treetop and lower-crown leaves of *C. japonica* under various conditions and water statuses

Condition	Water status	$C_{TC}$		$A_{TC}$ ( $\mu\text{m}^2$ )	
		Top	Lower	Top	Lower
In situ	Predawn	$0.79 \pm 0.11$	$0.86 \pm 0.08^{*+}$	$241 \pm 127$	$214 \pm 95^*$
	Midday	$0.57 \pm 0.10$	$0.66 \pm 0.12^{*+}$	$94 \pm 62$	$105 \pm 75^*$
In the laboratory	Rehydration	$0.80 \pm 0.11$	$0.83 \pm 0.12^*$	$184 \pm 121$	$188 \pm 79^*$
	Bench dried	$0.60 \pm 0.12$	$0.67 \pm 0.13^*$	$101 \pm 55$	$106 \pm 58^*$

Values are the mean ( $\pm$  one s.d.) of ten transfusion cells randomly chosen from each cryo-SEM image  
\* and + denote significant differences ( $P < 0.001$ ) between water statuses and crown position, respectively

experimental rehydration suggested that deformation of transfusion cells is a reversible process. Our observations suggest that, in addition to increasing leaf hydraulic conductance, like leaf veins of broadleaved species (Brodrribb et al. 2007), transfusion tissue has functions of both water storage and supply, much like a sponge, absorbing and releasing water according to leaf water status.

The decrease in cross-sectional area of transfusion cells at midday represents the amount of water withdrawn from these cells in response to increasing water demand caused

by daytime transpiration. If transfusion tissue volume translates directly to volume of water stored, the slope of the allometric relationship between  $A_{TT}$  and  $S_L$  would equal 0.5. Our analysis showed that it was 0.28 ( $S_L = 521.26 A_{TT}^{0.28}$ ,  $R^2 = 0.55$ ,  $P = 0.002$ ), suggesting that in addition to increasing transfusion tissue, mesophyll tissue may contribute to foliar water storage. Because water in mesophyll is easily lost via stomata, dehydration of mesophyll induces decline in photosynthesis as a result of stomatal regulation. Therefore, when transpiration

demand increases rapidly, water must be quickly supplied to mesophyll which involves the risk of putting a large load on leaf xylem water potential and inducing xylem embolism. The water stored in the transfusion tissue can function as a “circuit breaker” (sensu Zhang et al. 2014) against xylem embolism, protecting the xylem from such excessive loads while maintaining photosynthesis. Thus, the plastic response of transfusion tissue may act as a hydraulic buffer preventing disruption of water flow due to such rapid decrease in leaf water potential. We observed more marked deformation of transfusion cells in treetop leaves than in lower-crown leaves, suggesting that a greater hydraulic buffer is required for the more water-stressed, treetop leaves.

As with *S. sempervirens* (Oldham et al. 2010; Ishii et al. 2014), increasing cross-sectional area of transfusion tissue with height in the tallest *C. japonica* trees reflects increasing capacity for foliar water storage, an anatomical response to increasing water stress. In *S. sempervirens*, cross-sectional area of xylem decreased with height, suggesting decreasing reliance on water transport from roots and that the source of stored water in treetop leaves may not be from roots but from fog or other moisture absorbed via leaf surfaces (Ishii et al. 2014). In cloud forests, fog and cloud immersion result in increased photosynthesis, leaf conductance, and xylem water potential in conifers (Simonin et al. 2009; Berry and Smith 2013). In the north coast of California, where *S. sempervirens* occurs, summer fog arising from the Pacific Ocean is an important source of moisture in rainless summer (Dawson 1998; Johnstone and Dawson 2010). In contrast to *S. sempervirens*, cross-sectional area of xylem remained constant with height in *C. japonica*; this may be because the northwest coast of Japan receives more summer rain (ca. 400 mm during June, July, and August) than northern California, maintaining soil water supply.

Although stem water storage plays an important role in the water and carbon economy of tall trees (Phillips et al. 2003), experiments using small trees have shown that crown water storage contributes more to whole-tree transpiration rate than the stem (Zweifel et al. 2000). Water storage (hydraulic capacitance) and vulnerability to cavitation of the bole and branch xylem decrease with height to maintain water transport from root to leaves (hydraulic safety, Domec and Gartner 2001; Burgess et al. 2006; Schulte 2012). Increasing foliar water storage with height could compensate for increasing water demand at the treetop and realize hydraulic homeostasis within the crown. Changes in anatomical structure of a leaf and associated hydraulic properties may be an adaptive mechanism acquired by tall Cupressaceae trees to overcome hydraulic constraints on physiological function with increasing height.

**Author contribution statement** WA and HRI conceived the study, conducted the field work, and made physiological measurements. WA and Keiko K observed leaf anatomy. WA and Katsushi K conducted CryoSEM observations. WA analyzed the data. WA and HRI wrote the manuscript.

**Acknowledgments** We thank the Noshiro Education Board and Tohoku Forest Management Office, Ministry of Agriculture, Forestry and Fisheries for permission to conduct the research. We thank members of the Laboratory of Forest Resources, Kobe University for field assistance and Drs. A. Makita, K. Takata, K. Hoshizaki, and M. Matsushita of Akita Pref. University for facilitating the study. Special thanks to M. Nakatani and A. Shiraki for extensive assistance both in the field and laboratory. This research was funded by JSPS Research Fellow (#02502390) and JSPS Kakenhi (#23380085).

**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

## References

- Aloni R, Foster A, Mattsson J (2013) Transfusion tracheids in the conifer leaves of *Thuja plicata* (Cupressaceae) are derived from parenchyma and their differentiation is induced by auxin. *Am J Bot* 100:1949–1956. doi:10.3732/ajb.1300149
- Ambrose AR, Sillett SC, Koch GW et al (2010) Effects of height on treetop transpiration and stomatal conductance in coast redwood (*Sequoia sempervirens*). *Tree Physiol* 30:1260–1272
- Bacelar EA, Correia CM, Moutinho-Pereira JM et al (2004) Sclerophylly and leaf anatomical traits of five field-grown olive cultivars growing under drought conditions. *Tree Physiol* 24:233–239
- Barclay HJ, Goodman D (2000) Conversion of total to projected leaf area index in conifers. *Can J Bot* 78:447–454
- Barnard HR, Ryan MG (2003) A test of the hydraulic limitation hypothesis in fast-growing *Eucalyptus saligna*. *Plant Cell Environ* 26:1235–1245
- Bartlett MK, Scoffoni C, Sack L (2012) The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: a global meta-analysis. *Ecol Lett* 15:393–405. doi:10.1111/j.1461-0248.2012.01751.x
- Berry ZC, Smith WK (2013) Ecophysiological importance of cloud immersion in a relic spruce-fir forest at elevational limits, southern Appalachian Mountains, USA. *Oecologia* 173:637–648
- Bouche PS, Larter M, Domec J-C et al (2014) A broad survey of hydraulic and mechanical safety in the xylem of conifers. *J Exp Bot* 65:4419–4431
- Brodribb TJ, Cochard H (2009) Hydraulic failure defines the recovery and point of death in water-stressed conifers. *Plant Physiol* 149:575–584. doi:10.1104/pp.108.129783
- Brodribb TJ, Holbrook NM (2005) Water stress deforms tracheids peripheral to the leaf vein of a tropical conifer. *Plant Physiol* 137:1139–1146
- Brodribb TJ, Feild TS, Jordan GJ (2007) Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiol* 144:1890–1898. doi:10.1104/pp.107.101352
- Brodribb TJ, Feild TS, Sack L (2010) Viewing leaf structure and evolution from a hydraulic perspective. *Funct Plant Biol* 37:488–498. doi:10.1071/FP10010
- Burgess SSO, Dawson TE (2004) The contribution of fog to the water relations of *Sequoia sempervirens* (D. Don): foliar uptake and prevention of dehydration. *Plant Cell Environ* 27:1023–1034

- Burgess SSO, Pittermann J, Dawson TE (2006) Hydraulic efficiency and safety of branch xylem increases with height in *Sequoia sempervirens* (D. Don) crowns. *Plant Cell Environ* 29:229–239
- Coble AP, Autio A, Cavaleri MA et al (2014) Converging patterns of vertical variability in leaf morphology and nitrogen across seven *Eucalyptus* plantations in Brazil and Hawaii, USA. *Trees* 28:1–15. doi:10.1007/s00468-013-0925-6
- Cochard H, Froux F, Mayr S, Coutand C (2004) Xylem wall collapse in water-stressed pine needles. *Plant Physiol* 134:401–408
- Cowan IR (1982) Regulation of water use in relation to carbon gain in higher plants. In: Lange OL, Nobel PS, Osmond CB et al (eds) *Physiological plant ecology II*. Springer, Berlin, Heidelberg, pp 589–613
- Dawson TE (1998) Fog in the California redwood forest: ecosystem inputs and use by plants. *Oecologia* 117:476–485
- Domec JC, Gartner BL (2001) Cavitation and water storage capacity in bole xylem segments of mature and young *Douglas-fir* trees. *Trees* 15:204–214. doi:10.1007/s004680100095
- Esau K (1997) *Anatomy of seed plants*, 2nd edn. Wiley, New York
- Godoy O, Gianoli E (2013) Functional variation of leaf succulence in a cold rainforest epiphyte. *Plant Ecol Evol* 146:167–172
- Hinckley TM, Duhme F, Hinckley AR, Richter H (1980) Water relations of drought hardy shrubs: osmotic potential and stomatal reactivity. *Plant Cell Environ* 3:131–140
- Hu YS, Yao BJ (1981) Transfusion tissue in gymnosperm leaves. *Bot J Linn Soc* 83:263–272
- Ishii H, Kitaoka S, Fujisaki T et al (2007) Plasticity of shoot and needle morphology and photosynthesis of two *Picea* species with different site preferences in northern Japan. *Tree Physiol* 27:1595–1605
- Ishii H, Jennings G, Sillett S, Koch G (2008) Hydrostatic constraints on morphological exploitation of light in tall *Sequoia sempervirens* trees. *Oecologia* 156:751–763
- Ishii H, Hamada Y, Utsugi H (2012) Variation in light-intercepting area and photosynthetic rate of sun and shade shoots of two *Picea* species in relation to the angle of incoming light. *Tree Physiol* 32:1227–1236
- Ishii HR, Azuma W, Kuroda K, Sillett SC (2014) Pushing the limits to tree height: could foliar water storage compensate for hydraulic constraints in *Sequoia sempervirens*? *Funct Ecol* 28:1087–1093
- Johnson DM, Meinzer FC, Woodruff DR, McCulloh KA (2009) Leaf xylem embolism, detected acoustically and by cryo-SEM, corresponds to decreases in leaf hydraulic conductance in four evergreen species. *Plant Cell Environ* 32:828–836. doi:10.1111/j.1365-3040.2009.01961.x
- Johnstone JA, Dawson TE (2010) Climatic context and ecological implications of summer fog decline in the coast redwood region. *Proc Natl Acad Sci* 107:4533–4538
- Koch GW, Sillett SC, Jennings GM, Davis SD (2004) The limits to tree height. *Nature* 428:851–854
- Larjavaara M (2014) The world's tallest trees grow in thermally similar climates. *New Phytol* 202:344–349. doi:10.1111/nph.12656
- Martin TA, Hinckley TM, Meinzer FC, Sprugel DG (1999) Boundary layer conductance, leaf temperature and transpiration of *Abies amabilis* branches. *Tree Physiol* 19:435–443
- Martorell S, Diaz-Espejo A, Medrano H et al (2014) Rapid hydraulic recovery in *Eucalyptus pauciflora* after drought: linkages between stem hydraulics and leaf gas exchange. *Plant Cell Environ* 37:617–626
- McDowell N, Barnard H, Bond BJ et al (2002) The relationship between tree height and leaf area: sapwood area ratio. *Oecologia* 132:12–20
- Meinzer F, McCulloh K, Lachenbruch B et al (2010) The blind men and the elephant: the impact of context and scale in evaluating conflicts between plant hydraulic safety and efficiency. *Oecologia* 164:287–296
- Midgley JJ (2003) Is bigger better in plants? The hydraulic costs of increasing size in trees. *Trends Ecol Evol* 18:5–6
- Nabeshima E, Hiura T (2008) Size-dependency in hydraulic and photosynthetic properties of three *Acer* species having different maximum sizes. *Ecol Res* 23:281–288
- Negret BS, Perez F, Markesteijn L et al (2013) Diverging drought-tolerance strategies explain tree species distribution along a fog-dependent moisture gradient in a temperate rain forest. *Oecologia* 173:625–635
- Niinemets U (2002) Stomatal conductance alone does not explain the decline in foliar photosynthetic rates with increasing tree age and size in *Picea abies* and *Pinus sylvestris*. *Tree Physiol* 22:515–535
- Oldham AR, Sillett SC, Tomescu AMF, Koch GW (2010) The hydrostatic gradient, not light availability, drives height-related variation in *Sequoia sempervirens* (Cupressaceae) leaf anatomy. *Am J Bot* 97:1087–1097
- Pallardy SG (2007) *Physiology of woody plants*, 3rd edn. Academic Press, San Diego
- Parker WC, Colombo SJ (1995) A critical re-examination of pressure-volume analysis of conifer shoots: comparison of three procedures for generating PV curves on shoots of *Pinus resinosa* Ait. seedlings. *J Exp Bot* 46:1701–1709
- Peel MC, Finlayson BL, McMahon TA (2007) Updated world map of the Köppen-Geiger climate classification. *Hydrol Earth Syst Sci* 11:1633–1644
- Phillips NG, Ryan MG, Bond BJ et al (2003) Reliance on stored water increases with tree size in three species in the Pacific Northwest. *Tree Physiol* 23:237–245
- Renninger HJ, Phillips N, Hodel DR (2009) Comparative hydraulic and anatomic properties in palm trees (*Washingtonia robusta*) of varying heights: implications for hydraulic limitation to increased height growth. *Trees* 23:911–921. doi:10.1007/s00468-009-0333-0
- Ritchie GA, Roden JR (1985) Comparison between two methods of generating pressure volume curves. *Plant Cell Environ* 8:49–53
- Rodriguez P, Mellisho CD, Conejero W et al (2012) Plant water relations of leaves of pomegranate trees under different irrigation conditions. *Environ Exp Bot* 77:19–24
- Ryan MG, Yoder BJ (1997) Hydraulic limits to tree height and tree growth. *Bioscience* 47:235–242
- Ryan MG, Phillips N, Bond BJ (2006) The hydraulic limitation hypothesis revisited. *Plant Cell Environ* 29:367–381
- Salleo S (1983) Water relations parameters of two sicilian species of *Senecio* (groundsel) measured by the pressure bomb technique. *New Phytol* 95:179–188
- Scholz F, Phillips N, Bucci S et al (2011) Hydraulic capacitance: biophysics and functional significance of internal water sources in relation to tree size. In: Meinzer FC, Lachenbruch B, Dawson TE (eds) *Size- and age-related changes in tree structure and function*. Springer, Netherlands, pp 341–361
- Schulte PJ (2012) Vertical and radial profiles in tracheid characteristics along the trunk of *Douglas-fir* trees with implications for water transport. *Trees* 26:421–433. doi:10.1007/s00468-011-0603-5
- Schulte PJ, Hinckley TM (1985) A comparison of pressure-volume curve data analysis techniques. *J Exp Bot* 36:1590–1602
- Sillett SC, Van Pelt R, Kramer RD et al (2015) Biomass and growth potential of *Eucalyptus regnans* up to 100 m tall. *For Ecol Manage* 348:78–91. doi:10.1016/j.foreco.2015.03.046
- Simonin KA, Santiago LS, Dawson TE (2009) Fog interception by *Sequoia sempervirens* (D. Don) crowns decouples physiology from soil water deficit. *Plant Cell Environ* 32:882–892

- Stephenson NL, Das AJ, Condit R et al (2014) Rate of tree carbon accumulation increases continuously with tree size. *Nature* 507:90–93
- Takeda H (1931) A theory of “transfusion-tissue”. *Ann Bot os* 27:359–363
- Tyree MT, Hammel HT (1972) The measurement of the turgor pressure and the water relations of plants by the pressure-bomb technique. *J Exp Bot* 23:267–282
- Woodruff DR, Bond BJ, Meinzer FC (2004) Does turgor limit growth in tall trees? *Plant Cell Environ* 27:229–236
- Xiloyannis C, Dichio B, Nuzzo V, Celano G (1999) Defense strategies of olive against water stress. *Acta Hort* 474:423–426
- Zhang Y-J, Rockwell FE, Wheeler JK, Holbrook NM (2014) Reversible deformation of transfusion tracheids in *Taxus baccata* is associated with a reversible decrease in leaf hydraulic conductance. *Plant Physiol* 165:1557–1565. doi:[10.1104/pp.114.243105](https://doi.org/10.1104/pp.114.243105)
- Zimmermann MH (1983) Xylem structure and the ascent of sap. Springer, Berlin
- Zweifel R, Item H, Häsler R (2000) Stem radius changes and their relation to stored water in stems of young *Norway spruce* trees. *Trees* 15:50–57. doi:[10.1007/s004680000072](https://doi.org/10.1007/s004680000072)