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Effects of stomatal density and leaf water content on the ^{18}O enrichment of leaf water

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Key words: Arabidopsis, leaf water, leaf water content, oxygen isotope ratios, stomatal density.

Summary

- Leaf water isotopic composition is imprinted in several biomarkers of interest and it is imperative that we understand the isotopic enrichment of leaf water. Here, we test the effect of stomatal density and leaf water content on the oxygen isotopic composition of leaf water in transgenic Arabidopsis plants expressing different stomatal densities, and several other species showing a range of stomatal density.
- We grew Arabidopsis plants hydroponically and collected other species in the field. Stomatal density and leaf water content were determined for each plant. We measured transpiration and extracted leaf water for isotopic determination. Using these measurements and the current leaf water isotope model, we calculated several of the parameters related to leaf water isotopic enrichment.
- High stomatal density promoted leaf water isotope enrichment. No conclusion, however, can be drawn regarding the effect of leaf water content on leaf water isotope enrichment. Factors such as transpiration might mask the effect of stomatal density on leaf water isotopic enrichment.
- We propose a method by which stomatal density can be incorporated in the current Peclet model of leaf water isotope enrichment. These findings have important applications in the use of plant-based metabolic proxies in paleoclimate studies.

Introduction

Understanding the physiological and morphological processes that cause leaf water ^{18}O isotope enrichment is important in various fields, such as paleoclimatology and biogeochemistry. Paleoclimatologists, for example, often use the oxygen isotope ratios of tree ring cellulose as a proxy for climate (Jahren & Sternberg, 2003; Richter *et al.*, 2008). It is well known that the oxygen isotope ratio signal of tree ring cellulose is composed of *c.* 42% from the trunk water and 58% from the leaf water isotopic signal (Roden *et al.*, 2000; Cernusak *et al.*, 2005; Sternberg, 2009). Understanding paleo-signals from tree trunk cellulose oxygen isotope ratios requires knowing whether isotopic shifts occurred due to changes in source water (trunk water signal) or shifts in climate which affected leaf water isotopic composition. This, in turn, requires a full understanding of how leaf water becomes isotopically enriched and how this signal is passed on to phloem soluble sugars (Gessler *et al.*, 2013). Biogeochemists studying the atmospheric oxygen cycle are also interested in leaf water isotopic enrichment because the process of terrestrial photosynthesis generates oxygen having the isotopic signature of leaf water (Dole *et al.*, 1954; Guy *et al.*, 1993; Bender *et al.*, 1994). In addition, atmospheric CO_2 quickly equilibrates with leaf water, acquiring the oxygen isotope identity of leaf water (Farquhar *et al.*, 1993). Measurements of the oxygen isotope ratio of atmospheric carbon dioxide, because it reflects photosynthesis and leaf water isotopic identity, may provide important clues of biome productivity.

Our understanding of how leaf water becomes isotopically enriched relative to stem water is still evolving. Seminal experiments observed isotopic enrichment of artificial leaf membranes undergoing evaporation (Dongmann *et al.*, 1974). Dongmann *et al.* (1974) modeled such enrichment at steady state with the following slightly modified equation (Farquhar & Lloyd, 1993):

$$\Delta_e = \varepsilon^+ + \varepsilon_k + (\Delta_v - \varepsilon_k)\frac{e_a}{e_i}, \qquad \text{Eqn 1}$$

(Δ_e, oxygen isotopic enrichment of water undergoing evaporation relative to that of the source water (stem water); ε^+, equilibrium isotopic fractionation between water in the liquid and vapor phase; ε_k, kinetic fractionation which occurs during the diffusion of water vapor in the transpiration stream; Δ_v, oxygen isotopic enrichment of atmospheric vapor relative to the source water; e_a/e_i, ratio of atmospheric vapor pressure to that inside the leaf). This equation is still used today as a component of the more recent leaf water isotopic enrichment model. Very early in the investigations on how well bulk leaf water isotope ratios fitted the evaporative model in Eqn 1, it was observed that leaf water

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isotopic enrichment rarely reached the level of enrichment described by the above equation (Farris & Strain, 1978). The first explanations regarding this discrepancy between bulk leaf water isotope ratios and the above modeled evaporated water considered that there were separate pools of water, some of which did not undergo isotopic enrichment (Leaney *et al.*, 1985; Yakir *et al.*, 1989). However, these compartmentation models could not explain the observation that transpiration had an effect on the leaf water isotopic enrichment even at steady state (Flanagan *et al.*, 1991). The compartmentation models were eventually replaced by the Peclet model (Farquhar & Lloyd, 1993) which showed how transpiration rate could affect leaf water isotopic enrichment.

The Peclet model considers two opposing fluxes of leaf water: the rate of advection of isotopically unenriched water from the xylem to the sub-stomatal cavity and the diffusion rate of isotopically enriched water from the sub-stomatal cavity towards the leaf mesophyll. The ratio of these fluxes is expressed in terms of the unit-less Peclet number ($\wp$) (Farquhar & Lloyd, 1993):

$$\wp = \frac{EL}{DC}, \qquad \text{Eqn 2}$$

(E, transpiration rate per leaf surface area (mol m^{-2} s^{-1}); L, effective path length (m); D, diffusivity of $H_2{}^{18}O$ in water (m^2 s^{-1}); C, molar density of water (mol m^{-3})). The Peclet number affects the ratio of the oxygen isotope enrichment of lamina leaf water (Δ_L) to that of the evaporative pool by the following relationship:

$$\frac{\Delta_L}{\Delta_e} = \frac{1 - e^{-\wp}}{\wp}. \qquad \text{Eqn 3}$$

There are several new findings regarding the role of the Peclet ratio in determining leaf water isotopic enrichment (Ferrio *et al.*, 2012; Ellsworth *et al.*, 2013; Song *et al.*, 2013; Sternberg & Manganiello, 2014). A recent finding, for example, has shown that the rate of transpiration, a component of the Peclet ratio, can affect L, which is another component of the Peclet ratio (Song *et al.*, 2013). In addition, using an artificial leaf system, Sternberg & Manganiello (2014) showed that lower stomatal density also increases L and $\wp$, which probably explains the lower isotopic enrichment observed in Mangrove leaves compared to freshwater plants. The effect of stomatal density on leaf water isotopic enrichment has not been tested in real leaves. Further, mangrove leaves tend to have higher water content and it is not certain whether stomatal density or higher water content lowers the isotopic enrichment of leaf water. Clearly, leaf water isotopic enrichment is a complicated process with several factors, many of which are interacting with each other, contributing to the isotopic enrichment of leaf water. Here we test whether high leaf stomatal density and low water content will: (1) increase leaf water isotopic enrichment (Δ_L), (2) increase the ratio of leaf water isotopic enrichment relative to that of the evaporative pool (Δ_L/Δ_e), (3) decrease the Peclet ratio ($\wp$) and (4) decrease the effective path length (L). We first test for the effects on transgenic *Arabidopsis thaliana* plants with differential expression of the stomatal growth inducing the hormone stomagen and showing different stomatal densities, but similar water content (Sugano *et al.*, 2010; Tanaka *et al.*, 2013). Second, we test the effects on several species of plants showing a range of stomatal densities, but having variation in factors such as guard cell length and leaf water content that could alter the isotopic composition of leaf water. We realize that there might be other anatomical factors, such as specific leaf weight or leaf thickness, which could influence leaf water isotopic enrichment. A previous study (Rosado *et al.*, 2013), however, showed no relationship between these factors and leaf water isotopic enrichment. We therefore did not pursue these potential relationships.

Materials and Methods

Arabidopsis thaliana culture

The *Arabidopsis thaliana* (L.) Heynh transgenic lines were described previously (Sugano *et al.*, 2010; Tanaka *et al.*, 2013). We selected representatives of the lines of STOMAGEN overexpressing (ST-OX10) with high stomatal density and amiRNA mediated silencing (ST-RNAi10 and ST-RNAi12) (Sugano *et al.*, 2010) with low stomatal density. We used the line Columbia-0 (CS60000) as the wild-type (WT).

The transgenic lines were grown in a single hydroponic container under laboratory conditions, at room temperature (*c.* 22°C), with WT and transgenic lines randomly distributed. We maintained the experiment at 120 μmol s^{-1} m^{-2} of irradiance using four 20W bulbs at a distance of 8 cm from the plants. Seeds were placed in rockwool (Rock-Wool, Leeds, AL, USA) and watered from the top until seed germination. A prepared nutrient solution using a 3 : 2 : 1 mixture of FloraGro®, FloraMicro® and FloraBloom® solutions, from General Hydroponics® (GH Inc., Sebastopol, CA, USA) for promoting vegetative growth was introduced after seed germination, to prevent algal growth. The pH was adjusted to 6.5 with General Hydroponics® pH Control Kit®(GH Inc.). We added distilled water to the nutrient solution as needed to preserve its original volume and maintain contact with the rockwool. The solution was aerated using an aquarium pump. All plants were collected after the development of a flower stalk, on the same day.

Field collection of multiple species

Leaf samples were obtained from the Gifford Arboretum at the University of Miami. The average annual rainfall for this region is 1480 mm. The mean temperature varies from 30.4°C in the summer to 24.8°C in the winter months, and the annual mean RH is 83% (data from NOAA Climate Services, Miami, FL, USA, 25.65°N, 80.30°W). Three to five maximal sunlight exposed mature leaves were collected from an individual of each species, at 4–5 nodes under the apex of the shoot. We first collected leaf samples to determine stomatal density, size and water content. A second collection was made where transpiration was measured and leaves sampled for water extraction and isotopic composition. The following species were collected showing a range of stomatal densities: *Byrsonima crassifolia* (L.) Kunth,

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Conocarpus erectus L., *Chrysobalanus icaco* L., *Hamelia patens* Jacq., *Malpighia emarginata* DC., *Oncoba spinosa* Forssk., *Psidium cattleianum* Sabine, *Punica granatum* L., *Polyathia suberosa* (Roxb.) Thwaites and *Rhizophora mangle* L.

Stomatal density, size and leaf water content

Stomatal density and guard cell length were measured in five leaves for each line of *Arabidopsis*. For analysis, small square sections were cut with a razor blade from the middle of the leaf avoiding the central vein. The surfaces were wiped clean and the sample squares placed in slides with the abaxial surface of the leaf facing down. An image was prepared via high-resolution confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA), fluorescing the tissue with a helium neon laser at a wavelength of 458 nm, an optimal setting corresponding to the green chlorophyll of the plant. For the multiple species measurements, five replicate leaves were sampled for each species. A square *c.* 1 cm^2 was cut from each leaf avoiding the central vein and used for microscopy. Stomata were measured and counted for three areas within each sample square, using ImageJ software (NIH, Bethesda, MD, USA). In the case of *Conocarpus erectus* L. stomata were also present on the top surface of the leaf, but with similar averaged values as the abaxial surface. To determine leaf water content we first estimated leaf area for five replicate fresh leaves for each species including the three Arabidopsis lines using ImageJ software. Then, leaves were weighed and dried to constant mass at 60°C and weighed again, to estimate DW and water content.

Transpiration

Stomatal conductance (G_s, mol m^{-2} s^{-1}) of five leaves (one from each individual) for each Arabidopsis line was determined on the lower (abaxial) surface using a diffusion porometer (Leaf Porometer, model SC-1; Decagon Devices Inc., Pullman, WA, USA). Leaf temperature for each line was measured with an infrared thermometer (62Max+, Fluke, Everett, WA, USA), whereas the ambient relative humidity and temperature were measured with EXTECH humidity and temperature sensor (EXTECH instruments, Noshua, NH, USA). We calculated transpiration rate (E, mol m^{-2} s^{-1}) from the total conductance (G_t), which included stomatal and boundary layer conductance and the vapor mol fraction inside (w_l) and outside (w_a) the leaf using the following equation:

$$E = (w_l - w_a) G_t. \qquad \text{Eqn 4}$$

We assumed a boundary layer conductance of 1 mol m^{-2} s^{-1}. For field-collected species, instantaneous transpiration was measured with a steady-state porometer LI-1600 (Li-Cor Inc., Lincoln, NE, USA) on two consecutive sunny days (PAR > 1000 μmol m^{-2} s^{-1}). Ambient temperature, relative humidity and leaf temperature were also measured with the same instrument during transpiration measurements. Transpiration was measured between 10:00 h and 12:00 h for each leaf sampled for isotope analysis.

Isotope analyses

We pooled five Arabidopsis plants for one leaf water sample. This was necessary to distill sufficient water for isotope analysis. There were a total of five replicate leaf water samples per Arabidopsis line. For the field samples two stems were sampled for each species for water extraction and isotope analysis. We measured transpiration just before collecting leaves for water extraction and isotope analysis. If any leaf showed a large prominent central vein, we dissected it out before sampling. We pooled enough leaves (ranging from 1 to 5) in each collection tube to be able to obtain enough water for isotopic analysis. There were three replicates of leaf water samples per species. Water was extracted from the leaves and stems and analyzed isotopically by the method of Vendramini & Sternberg (2007). Oxygen isotope ratios are expressed in $\delta^{18}O$ units where:

$$\delta^{18}O\,‰ = \left(\frac{R_{sample}}{R_{std.}} - 1\right)1000, \qquad \text{Eqn 5}$$

and R_{sample} and $R_{std.}$ represent the $^{18}O/^{16}O$ ratio of the sample and standard, respectively. The standard is the $^{18}O/^{16}O$ of the Vienna Standard Mean Ocean water (vSMOW). The precision of analysis was ± 0.1‰.

We calculated Δ_L for the Arabidopsis samples by subtracting the average oxygen isotope ratio of the culture water from the beginning (0.0‰) to the end of the experiment (0.1‰) from the corresponding five pooled leaf samples. For the field-collected multispecies we subtracted the average oxygen isotope ratio of the two stem samples from the isotope ratio of each leaf sample for a particular species. We estimated Δ_e according to Eqn 1. We use the word 'estimated' here, because there are feedback processes between transpiration and the isotopic composition and vapor pressure of the water vapor surrounding the leaf. Nevertheless, because the boundary layer resistancev – particularly for the field-collected samples – is rather small, we assume that our measurements of external vapor are a close approximation. Using the leaf temperature in degrees Kelvin (T), ε^+ was calculated according the following equation (Majoube, 1971):

$$\varepsilon^+ = 1.137\frac{10^6}{T^2} - 0.4156\frac{10^3}{T} - 2.0667. \qquad \text{Eqn 6}$$

The value of ε_k was assumed to be 28‰ (Barkan & Luz, 2007). For the *Arabidopsis* study we used the $\delta^{18}O$ value of atmospheric vapor in the laboratory measured with a cavity ringdown spectrometer (L2130-I; Picarro, CA, USA) having an average $\delta^{18}O$ value of −13.8‰. For field-collected samples we assumed that the atmospheric vapor was in isotopic equilibrium with source water; that is water having the average isotopic composition of stem water (Cernusak *et al.*, 2002). The value of $\Delta^{18}O_v$ was calculated by subtracting the $\delta^{18}O$ value of the stem water from that of the atmospheric vapor. Using the ratio Δ_L/Δ_e, and assuming that leaf water isotope ratios are predicted by the steady-state model, we were able to calculate $\wp$ in Eqn 3 by

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iteration using the Excel solver function. We decided to use the steady-state model (Farquhar & Lloyd, 1993), as previous comparisons between the prediction of the steady-state and non-steady-state models showed that both were in close agreement during the daytime under normal circumstances (Farquhar & Cernusak, 2005). We also assume, as suggested by Farquhar & Cernusak (2005), that changes in leaf water content were minimal and did not affect the isotopic ratios of the leaf water. The effective path length (L) was then calculated by using Eqn 2 with the respective values of transpiration and diffusivity for each species.

Statistical analyses

We tested for differences among species, using ANOVA. *Post-hoc* Tukey's tests were performed as pairwise comparison tests. We tested the significant correlations between parameters first with a correlation table. We used linear regressions to test the effect of leaf stomatal density and water content on the ratio of bulk leaf water isotopic enrichment relative to the evaporative pool (Δ_L/Δ_e) and the Peclet ratio ($\wp$). ANOVA, Tukey and regressions were performed with R (v2.15.2; R foundation for statistical computing, Vienna, Austria).

Results

Arabidopsis measurements

There were significant differences in stomatal density for the different lines of *Arabidopsis*, with the high stomagen expression line (ST-OX10) showing the greatest density, averaging 297 stomatal pores per mm^2, followed by the wild type (90) and finally the lowest density in the low stomatal density line (ST-RNAi) with a density of 43 stomatal pores per mm^2 (Fig. 1a). Although the relative proportion of stomatal density between the three *Arabidopsis* lines were as expected, their values were much lower than previously reported (Tanaka *et al.*, 2013). It's possible that our light conditions were different and affected stomatal density. There were no significant differences in guard cell length between the three lines averaging 12 microns in length (Fig. 1b). Nor were there differences in leaf water content between the different lines (Fig. 1c). Transpiration rates were significantly different for the three *Arabidopsis* lines with the high stomatal density line having the highest transpiration rate averaging 3.5 mmols m^{-2} s^{-1}, followed by the wild-type (3.0 mmol m^{-2} s^{-1}), with the low stomatal density line having the lowest transpiration rate averaging 2.3 mmol m^{-2} s^{-1} (Table 1). Although all three lines had

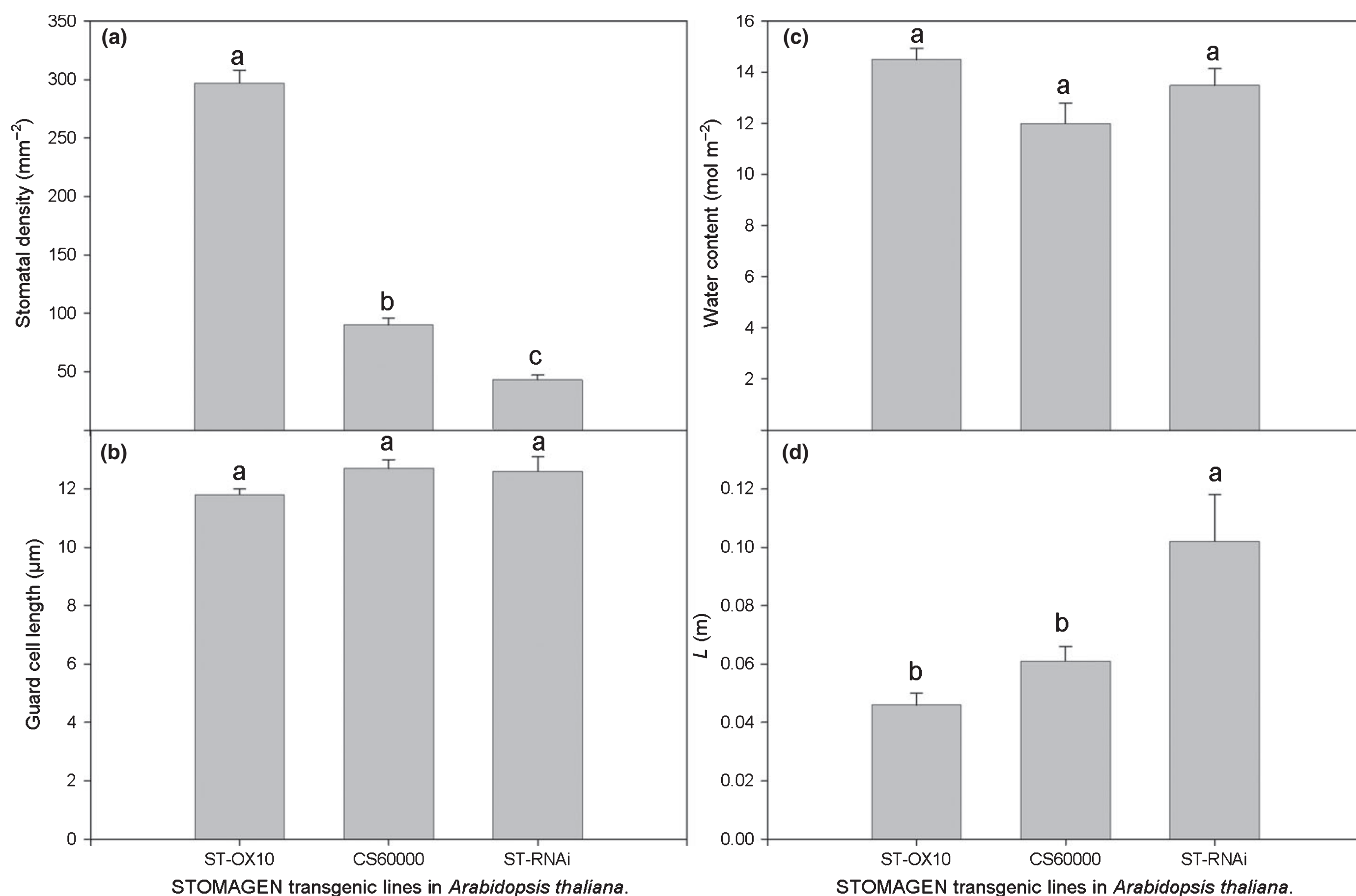

Fig. 1 (a) Stomatal density, (b) guard cell length, (c) water content and (d) effective path length that water travels from the xylem to the evaporative surface (L) of STOMAGEN-overexpressing (ST-OX), wild-type (CS60000) and -silencing (ST-RNAi) lines in *Arabidopsis thaliana*. Error bars are ± SEM ($n = 5$). Values marked with different letters are significantly different among transgenic lines at $P < 0.05$.

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different transpiration rates, there were no differences in the oxygen isotopic enrichment of leaf water, the ratio of the isotope enrichment of bulk leaf water to that of the evaporative water and the Peclet ratio (Table 1). The effective path length (L) between the different lines, however, was different with significant differences in L between the high stomatal and low stomatal density lines and the wild-type having an average intermediate value of L (Fig. 1d).

Multispecies measurements

There were significant differences in stomatal density between species (Figs 2, 3a), with the mangrove *Rhizophora mangle* having the lowest stomatal density, averaging 68 stomata per mm^2, followed by the mangrove-associated species *Conocarpus erectus*, with an average density of 155 stomata per mm^2 (Fig. 3a). The highest stomatal density was observed in *Punica granatum*, with an average density of 404 stomata per mm^2 (Fig. 3a). There were significant differences in guard cell length between the species (Fig. 3b) with the general trend of increasing guard cell length with a decrease in stomatal density (Fig. 4). Although, there were significant differences in leaf water content between the species (Fig. 3c), it was not correlated with stomatal density ($r = -0.56$, $P > 0.05$, Table 2). There were significant differences in transpiration rates between species with *R. mangle* and *P. granatum* having the highest transpiration rate and *P. cattleyanum* having the lowest transpiration rates (Table 3). Unlike Arabidopsis, transpiration rates were not correlated with stomatal density ($r = -0.24$, $P > 0.05$, Table 2). There were significant differences in the isotope enrichment of leaf water with *C. erectus*, *R. mangle* and *O. spinosa* having the lowest enrichment (Table 3). Likewise these same species had the lowest ratios of bulk leaf water isotopic enrichment to that of evaporative water and the highest Peclet ratios (Table 3). There were approximately three categories of effective path length: *B. crassifolia*, *M. emarginata* and *P. granatum* having significantly lower values of L compared to *C. erectus* and the rest of the species having an intermediate range of L values, but not significantly different from the above species (Table 3). A correlation analysis indicated that for those factors that are independently measured (stomatal density, water content and transpiration), only stomatal density and leaf water content contribute to factors related to the isotopic enrichment of the leaf water (Δ_L, Δ_L/Δ_e, $\wp$ and L; Table 2, Figs 5, 6). The high correlations between water content and Δ_L, Δ_L/Δ_e, $\wp$ and L (Table 2, Fig. 6), however, is driven by one outlier species (*C. erectus*) having high leaf water content. Elimination of this one species causes no correlation ($P > 0.10$) between water content and any of the above parameters, whereas the correlations between stomatal density and the above parameters are maintained or only lowered to $P < 0.10$. High correlation coefficients in the blue shaded cells (Table 2) are probably due to autocorrelation effects as one of the variables was used to calculate the corresponding variable. For example, P was calculated using Δ_L/Δ_e, or transpiration was used to calculate L. Therefore, high correlations for these cells will not be considered.

Table 1 Transpiration (mmol m^{-2} s^{-1}), leaf water isotopic enrichment relative to source water (Δ_L), ratio of leaf water isotopic enrichment over that of evaporative water (Δ_L/Δ_e) and Peclet ratio ($\wp$) of STOMAGEN-overexpressing (ST-OX), wild-type (CS60000) and -silencing (ST-RNAi) lines in *Arabidopsis thaliana* (SEM, $n = 5$)

STOMAGEN Transgenic lines	Transpiration	Δ_L	Δ_L/Δ_e	$\wp$
Low stomatal density (ST-RNAi)	2.32 ± 0.06c	6.2 ± 1.5a	0.45 ± 0.11a	1.97 ± 0.71a
Wild-type (CS60000)	3.00 ± 0.02b	6.7 ± 0.7a	0.51 ± 0.05a	1.55 ± 0.30a
High stomatal density (ST-OX)	3.50 ± 0.11a	6.5 ± 0.7a	0.55 ± 0.06a	1.38 ± 0.30a

Values marked with different letters are significantly different among genotypes/treatment at $P < 0.05$.

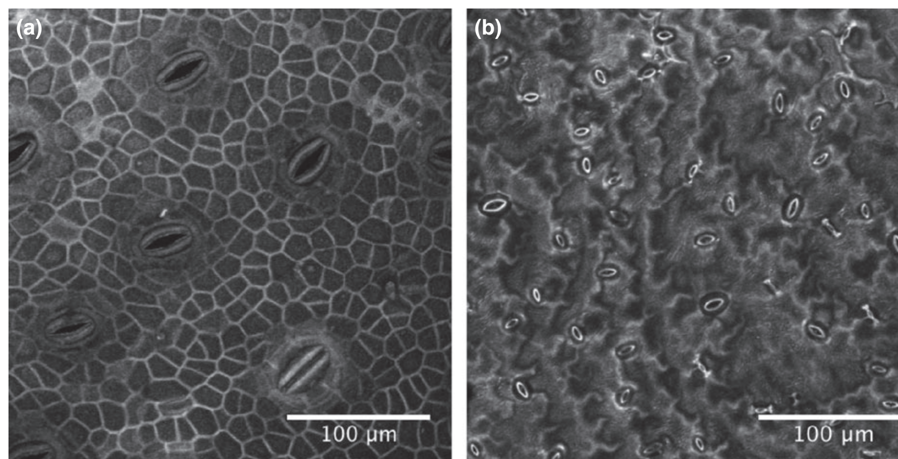

Fig. 2 Leaf abaxial surface of (a) *Rhizophora mangle* and (b) *Punica granatum* exhibiting differences in stomatal density and guard cell length. Bars, 100 μm.

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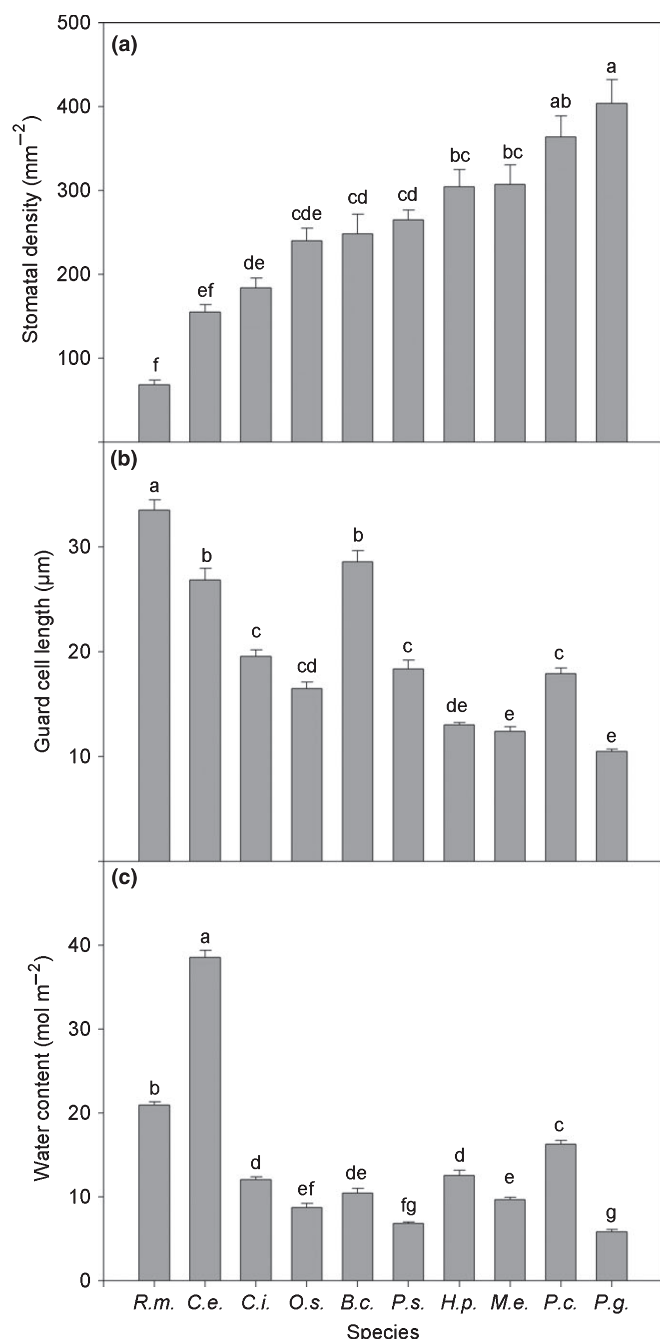

Fig. 3 (a) Stomatal density, (b) guard cell length and (c) water content of studied species. Bars not sharing a common letter have values that are significantly different from each other, ± SEM ($n = 5$). Abbreviations for species: *R.m.*, *Rhizophora mangle*; *C.e.*, *Conocarpus erectus*; *C.i.*, *Crhysobalanus icaco*; *O.s.*, *Oncoba spinosa*; *B.c.*, *Byrsonima crassifolia*; *P.s.*, *Polyalthia suberosa*; *H.p.*, *Hamelia patens*; *M.e.*, *Malpighia emarginata*; *P.c.*, *Psidium cattleianum*; *P.g.*, *Punica granatum*.

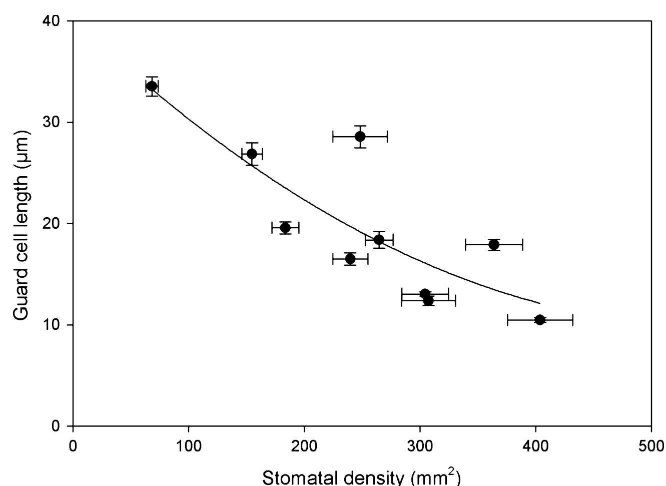

Fig. 4 Stomatal density vs guard cell length for *Rhizophora mangle*, *Conocarpus erectus*, *Crhysobalanus icaco*, *Oncoba spinosa*, *Byrsonima crassifolia*, *Polyalthia suberosa*, *Hamelia patens*, *Malpighia emarginata*, *Psidium cattleianum* and *Punica granatum*. Error bars are ± SEM ($n = 5$). Black line represents the best-fit exponential decay fit to the values of studied species (guard cell length: $y = 36.85e^{0.003x}$, $r = 0.76$, $P < 0.01$).

Table 2 Correlation coefficients for various parameters leading to leaf water isotopic enrichment

	Stom. Den.	Water Cont.	Trans.	D_L	D_L/D_e	Peclet	L
Stom. Den.	1.00						
WaterCont	−0.56	1.00					
Trans.	−0.24	−0.05	1.00				
D_L	0.56	***−0.66***	−0.16	1.00			
D_L/D_e	***0.66***	***−0.68***	−0.40	0.92	1.00		
Peclet	***−0.66***	***0.76***	0.34	−0.92	−0.99	1.00	
L	−0.20	***0.65***	−0.67	−0.59	−0.40	0.47	1.00

Light gray shaded cells reflect correlation between morphological and physiological characteristics that can lead do differences in leaf water isotopic enrichment. Dark gray shaded cells reflect possible auto-correlations between parameters as one factor is used to calculate the other. Bold italicized numbers reflect significant correlations ($P < 0.05$) with the sign reflecting the nature of the relationship, where negative signs imply an inverse correlation.
Stom. Den, stomatal density; WaterCont, water content; Trans., transpiration; Δ_L, leaf water isotopic enrichment; Δ_L/Δ_e, ratio of leaf water isotopic enrichment to the isotopic enrichment at the evaporative site; $\wp$, Peclet ratio; L, effective path length that water travels from the xylem to the evaporative surface.

Discussion

Arabidopsis measurements

Our hypothesis that stomatal density affects Δ_L, Δ_L/Δ_e and $\wp$ is not shown for the transgenic *Arabidopsis* (Table 1). However, there was a significant effect on L (Fig. 1d). We propose that the effect of stomatal density on leaf water isotopic enrichment would be seen if only other parameters related to leaf water isotopic enrichment remain constant. In the case of the transgenic *Arabidopsis*, transpiration rates were tightly coupled to stomatal density; that is, greater stomatal density was associated with greater transpiration (Table 1). Because increased transpiration rates increase the Peclet ratio and decrease leaf water isotopic enrichment, it would dampen any changes in these factors brought about by high stomatal density. Hence, potential differences in leaf water isotopic enrichment were masked by these two opposing factors: transpiration and stomatal density. One of the

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Table 3 Transpiration (mmol m^{-2} s^{-1}), leaf water isotopic enrichment relative to source water ($\Delta^{18}O$ leaf), ratio of leaf water isotopic enrichment over that of evaporative water (Δ_L/Δ_e), Peclet ratio and effective path length that water travels to the evaporative surface (*L*, m) of field collected species (SEM, $n = 3$)

Species	Transpiration	$\Delta^{18}O$ leaf	Δ_L/Δ_e	Peclet	*L*
Rhizophora mangle	7.5 ± 0.0a	9.0 ± 0.4c	0.45 ± 0.02bc	1.9 ± 0.1b	3.7E-02 ± 2.3E-03ab
Conocarpus erectus	5.5 ± 0.5abc	7.4 ± 0.1c	0.38 ± 0.01c	2.4 ± 0.1a	6.5E-02 ± 8.6E-03a
Chrysobalanus icaco	5.6 ± 0.8abc	13.0 ± 1.1a	0.54 ± 0.01ab	1.4 ± 0.1cde	3.7E-02 ± 6.9E-03ab
Oncoba spinosa	5.6 ± 0.9abc	8.2 ± 0 .4c	0.47 ± 0.02bc	1.8 ± 0.1bc	4.9E-02 ± 1.3E-02ab
Byrsonima crassifolia	5.8 ± 0.6ab	14.2 ± 0.5a	0.59 ± 0.02a	1.2 ± 0.1de	3.0E-02 ± 2.9E-03b
Polyalthia suberosa	3.9 ± 0.3bc	13.0 ± 0.3ab	0.57 ± 0.02a	1.3 ± 0.0de	4.8E-02 ± 5.4E-03ab
Hamelia patens	6.3 ± 0.7ab	11.8 ± 0.2b	0.51 ± 0.00b	1.6 ± 0.0bcd	3.7E-02 ± 4.0E-03ab
Malpighia emarginata	6.7 ± 0.8ab	13.6 ± 0.4ab	0.59 ± 0.02a	1.2 ± 0.1de	2.6E-02 ± 0.9E-03b
Psidium cattleianum	2.6 ± 0.4c	12.7 ± 0.3ab	0.62 ± 0.01a	1.0 ± 0.0e	5.7E-02 ± 8.3E-03ab
Punica granatum	7.4 ± 0.0a	12.4 ± 3.6ab	0.54 ± 0.01ab	1.4 ± 0.0cde	2.8E-02 ± 1.0E-03b

Values marked with different letters are significantly different among species at $P < 0.05$. Species are arranged from lowest stomatal density (*R. mangle*) to highest density (*P. granatum*).

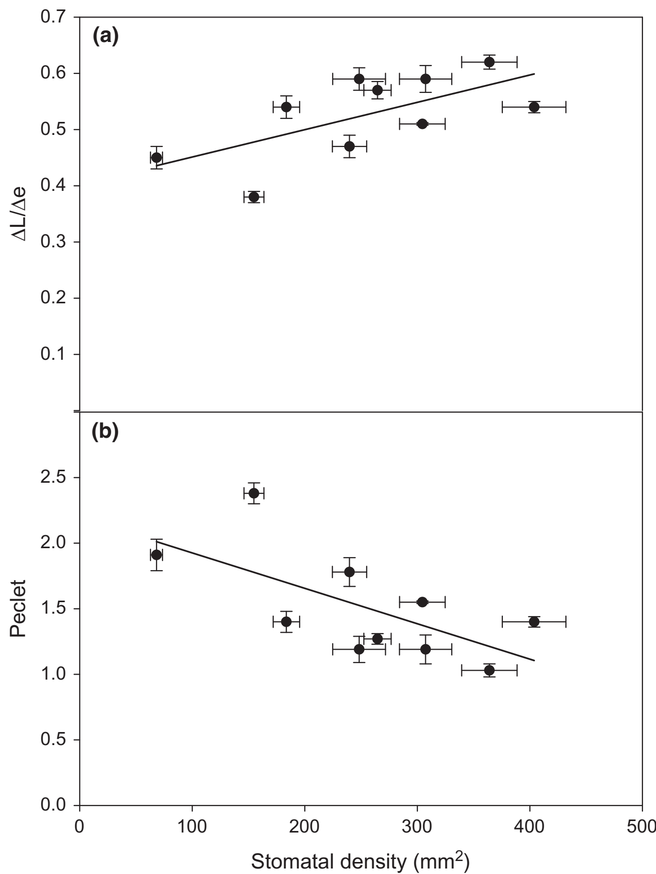

Fig. 5 (a) Ratio of leaf water isotopic enrichment over that of evaporative water (Δ_L/Δ_e) and (b) Peclet ratio as a function of stomatal density for *Rhizophora mangle*, *Conocarpus erectus*, *Crhysobalanus icaco*, *Oncoba spinosa*, *Byrsonima crassifolia*, *Polyalthia suberosa*, *Hamelia patens*, *Malpighia emarginata*, *Psidium cattleianum* and *Punica granatum*. Error bars are ± SEM ($n = 5$ for density count and 3 for isotope measurement). Black line represents the best-fit linear fit to the values of studied species.

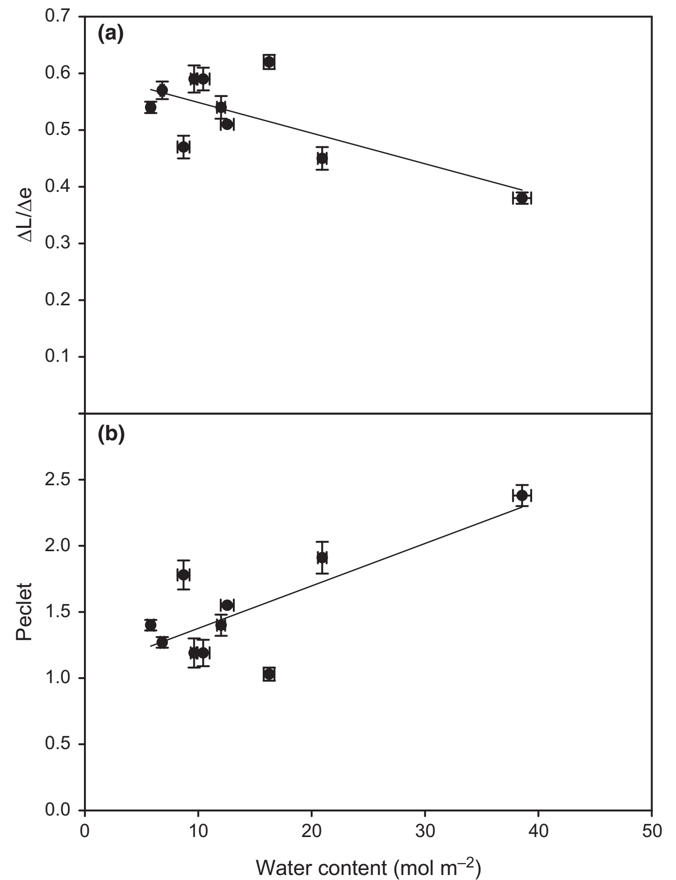

Fig. 6 (a) Ratio of leaf water isotopic enrichment over that of evaporative water (Δ_L/Δ_e) and (b) Peclet ratio as a function of water content for *Rhizophora mangle*, *Conocarpus erectus*, *Crhysobalanus icaco*, *Oncoba spinosa*, *Byrsonima crassifolia*, *Polyalthia suberosa*, *Hamelia patens*, *Malpighia emarginata*, *Psidium cattleianum* and *Punica granatum*. Error bars are ± SEM ($n = 5$ for water content and 3 for isotope measurement). Black line represents the best-fit linear fit to the values of studied species.

factors that contribute to leaf water isotopic enrichment is *L*, the effective path length water must travel from the xylem to the stomatal cavity. The term *L* is actually a scaling factor that takes into account differences in path lengths and cross-sectional areas of the path from the xylem to the stomatal cavity (Song *et al.*, 2013; Sternberg & Manganiello, 2014). This scaling is necessary as the water flux out of the leaf (*E*) used in the estimation of $\wp$ is expressed in terms of loss of water via transpiration per leaf

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surface area. In actuality, liquid water is traveling through a complex pathway in the leaf mesophyll that differs in length from the thickness of the leaf and the external leaf area of transpiration. A decrease in the path length or an increase in the cross-sectional area through which mesophyll water travels would cause a decrease in L, drive the Peclet ratio down and cause an overall greater enrichment of leaf water. This would occur, providing that transpiration does not increase to compensate for the decrease in L and maintain the $\wp$ constant (see Eqn 2). In the case of increased stomatal density one would expect a decrease in path length as the probability of a stomatal pore being close to vein increases. In addition, an increase in stomatal density would increase the total cross-sectional area through which mesophyll water flows (Sternberg & Manganiello, 2014). This was confirmed by our results showing a significant difference in L from the three transgenic lines, with the expected pattern of the *Arabidopsis* line with the highest stomatal density showing the lowest average value of 46.0 mm, followed by the wild-type line with intermediate stomatal density and finally the low stomatal density line showing an average value of L of 102.0 mm (Fig. 1d).

Field collected samples

When we examine the relationship between stomatal density vs Δ_L, Δ_L/Δ_e, $\wp$ and L for the field samples, there are other factors that could affect these parameters in addition to transpiration and stomatal density. Guard cell length differed significantly between the species and correlated with stomatal density: the lower the stomatal density the greater the guard cell length (Figs 2, 4). This inverse correlation between guard cell length and density is a general pattern and observed for several plant species (Franks & Beerling, 2009). The possible effect of this relationship is that any decrease in the cross-sectional area of water flow from the xylem to the evaporative site, due to lower stomatal density, is compensated by an increase in the surface area of the stomatal cavity volume, if it scales to stomatal pore size. However, we note that the surface area to volume ratio decreases with an increase in volume and the total evaporative surface area for larger and fewer stomatal cavities may still be smaller compared to that of leaves having smaller and more frequent stomata stomatal cavities (Sternberg & Manganiello, 2014). In contrast to the *Arabidopsis* lines, there were significant differences in leaf water content between the different species, but there was no relationship between water content and stomatal density (Tables 2, 3). Of the three independently measured leaf parameters of stomatal density, water content and/or transpiration that could have an effect on Δ_L, Δ_L/Δ_e, $\wp$ and L, only stomatal density and water content showed a significant correlation with Δ_L, Δ_L/Δ_e, $\wp$ and/or L. Transpiration showed a significant relationship with L, but it could simply be an autocorrelation effect, as L is calculated with the Peclet ratio and transpiration rate as variables (see Eqn 2). Both stomatal density and leaf water content contribute to Δ_L/Δ_e and $\wp$, whereas only the leaf water content was significantly correlated with Δ_L and L (Table 2). We note, however, that the correlation between leaf water content and the above parameters is tenuous, as it is driven by the value of one outlier species (*Conocarpus erectus*). Elimination of this one species causes no correlation between water content and the above parameters. Therefore, no conclusions, regarding the effect of leaf water content on the isotopic enrichment of leaf water, can be drawn from this study. We note that several other investigators observed no relationship between water content and leaf water isotopic enrichment (Kahmen *et al.*, 2008, 2009; Rosado *et al.*, 2013).

Our findings that leaf stomatal density is correlated with Δ_L/Δ_e and $\wp$, but not with Δ_L and L, can be explained in the context of the steady-state Peclet model. First, the lack of correlation between stomatal density and Δ_L but the presence of a correlation with Δ_L/Δ_e can be explained if Δ_e differed between species due to micro-environmental conditions, as well as differences in stem water isotopic composition. Differences in Δ_e would drive the variation in Δ_L in addition to stomatal density, but would be factored out in Δ_L/Δ_e and $\wp$. Although there was a significant correlation between stomatal density and $\wp$, there was no relationship between stomatal density and L. L is calculated from $\wp$ and the only variable that could affect L would be transpiration. This leads us to the conclusion that transpiration varied sufficiently so as to not always reflect the transpiration rate that led to the leaf water isotopic enrichment. Future studies should concentrate on a prolonged period of transpiration measurements before sampling of leaves for isotopic analysis.

Integrating stomatal density in the current Peclet model

Understanding how the findings reported here can be incorporated into the current Peclet-based leaf model requires a deeper understanding of the Peclet ratio. The Peclet ratio is originally defined as:

$$\wp = \frac{vl}{D}, \qquad \text{Eqn 7}$$

(v, velocity of the advective flow of water through the mesophyll; l, path length that water travels from the xylem to the evaporative surface of the substomatal cavity). We note that l is probably greater than the leaf thickness as water flow through the mesophyll might very well be a tortuous pathway. In the above expression of the Peclet number, diffusivity (D) is easily calculated as a function of temperature (Cuntz *et al.*, 2007). We cannot directly measure v or l, but we do know that the velocity of liquid water flow through the mesophyll will be related to the transpiration rate (E, mol m^{-2} s^{-1}). We, therefore, measure leaf transpiration rates and estimate vl indirectly. We first transform transpiration to velocity across the area of transpiration (A_E) by dividing transpiration by the molar volume of water (C, 5.5×10^4 mol m^{-3}):

$$v' = \frac{E}{C}, \qquad \text{Eqn 8}$$

(v' (m s^{-1}), velocity of liquid water moving through the mesophyll if it moved along the same cross-sectional area of the transpiring surface). But, in the mesophyll, liquid water does not

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necessarily move through the same cross-sectional area as that of the transpiring surface. This velocity v', must be corrected to the actual velocity liquid water is moving through in the mesophyll (v). This will depend in the actual cross-sectional area of water flow in the mesophyll. The greater the cross-sectional area that water moves through in the mesophyll relative to the transpiring surface, the lower the actual velocity v of advective flow in the mesophyll in relation to v'. To correct v' to v, we first define a term k which is the ratio of the cross-sectional area of water flow in the mesophyll to that of the transpiring surface and multiply it by v' (Barbour & Farquhar, 2003):

$$k = A_E/A_m \quad \text{Eqn 9}$$

$$v = v'k \quad \text{Eqn 10}$$

(A_m, average cross-sectional area of water moving through the mesophyll). In attempting to understand the morphological bases for the Peclet number, Barbour & Farquhar (2003) dissected the mesophyll pathway of water movement into their respective cross-sectional areas and added them up serially. We will discuss this approach when applying the variation in stomatal density to the current Peclet model. The Peclet ratio with the above corrections is then expressed as:

$$\wp = \frac{Ekl}{DC} = \frac{EL}{DC}, \quad \text{Eqn 11}$$

in which k and l are usually bundled into a single term L ($kl = L$) called the effective path length. L (m), however, cannot be measured directly and it is calculated by solving for the Peclet ratio based on lamina water isotopic enrichment and the isotope composition of water at the evaporative surface (Δ_L/Δ_e). Further, factoring out the transpiration, diffusivity and molar volume from the Peclet ratio yields an estimate of L. Here, we will not predict absolute values of L as a function of stomatal density; rather, we will predict the type of expected relationship between L and stomatal density.

Barbour & Farquhar's calculation for the overall ratio of transpiration surface to the cross-sectional areas where water flows through the mesophyll is given by the following equation:

$$k = \frac{\frac{A_E}{A_{m1}} l_1 + \frac{A_E}{A_{m2}} l_2 + \ldots \frac{A_E}{A_{mn}} l_n}{l_1 + l_2 + \ldots l_n}, \quad \text{Eqn 12}$$

in which the subscripted symbols A_{mi} and l_i represent the respective mesophyll cross-sectional area and length of water flow for each layer of water movement. Because $L = k(l_1 + l_2 + \ldots l_n)$ than the effective path length is given by:

$$L = \frac{A_E}{A_{m1}} l_1 + \frac{A_E}{A_{m2}} l_2 + \ldots \frac{A_E}{A_{mn}} l_n. \quad \text{Eqn 13}$$

Here we use this same formulation, but add another layer of water movement towards multiple sub-stomatal cavities (Fig. 7). We assume that at some point water flow must branch-off to each sub-stomatal cavity and the cross-sectional areas of each of these 'branches' to the sub-stomatal cavity are the same (and equal to A_S), but the path lengths may differ (Fig. 7). The above equation, which includes the path to the sub-stomatal cavities, is modified to:

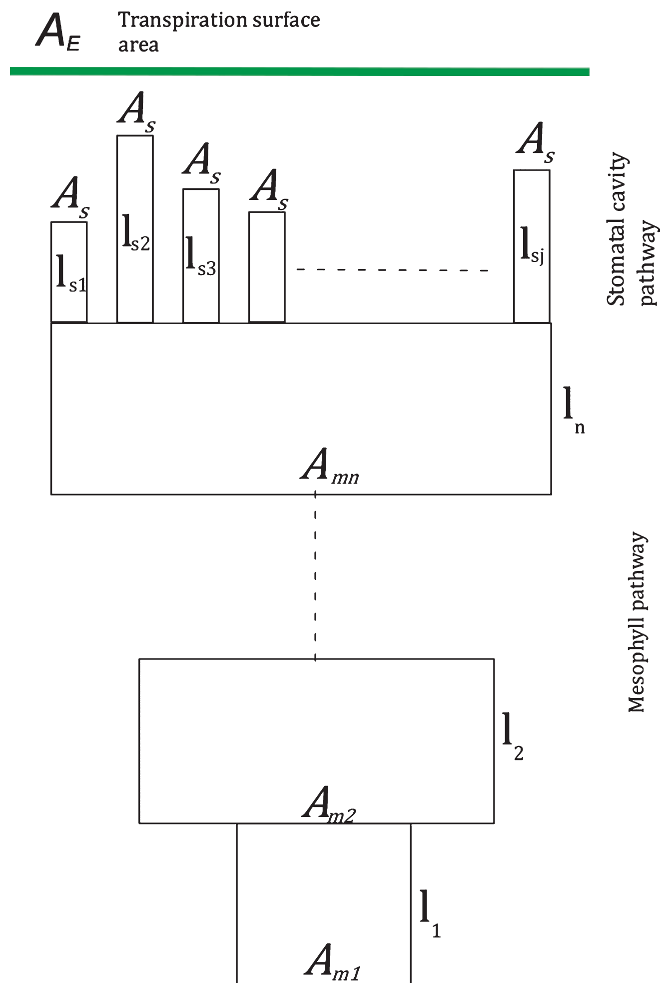

Fig. 7 Model of water flow in the mesophyll with water movement encountering cross-sectional areas (A_{m1}, $A_{m2} \ldots A_{mn}$) for the respective distances (l_1, $l_2 \ldots l_n$). Water flow then branches off to different sub-stomatal cavities each branch having the same cross-sectional area (A_S) but different lengths (l_{S1}, l_{S2}, l_{S3}, $\ldots l_{Sj}$). Water evaporates at the sub-stomatal cavity and exits as vapor across the transpiration surface (A_E).

$$L = \frac{A_E}{A_{m1}} l_1 + \frac{A_E}{A_{m2}} l_2 + \ldots \frac{A_E}{A_{mn}} l_n + \frac{A_E}{jA_S} \bar{l}_S, \quad \text{Eqn 14}$$

in which j is the stomatal density per unit area and $\bar{l}_S$ is the average length of all the branches of water flow to each sub-stomatal cavity. In the above equation and subsequent derivations we use mm units for A_E, A_{mi} (mm^2), j (stomatal density per mm^2) and path length l (mm). This equation can be simplified to:

$$L = \left(\sum_{i=1}^{n} \frac{A_E}{A_{mi}} l_i \right) + \frac{A_E \bar{l}_S}{A_S} j^{-1}. \quad \text{Eqn 15}$$

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There may be interactions between stomatal density and the average length and cross-sectional area of flow branches from the mesophyll to the sub-stomatal cavity. For example, as the stomatal frequency increases cross-sectional areas of branches may diminish, branch lengths may decrease, or there might be overlap between branches. For this reason the exponent of the variable j (the stomatal density) may not be exactly one and in general the equation will be of the form:

$$L = \left(\sum_{i=1}^{n} \frac{A_E}{A_{mi}} l_i\right) + \frac{A_E \bar{l}_S}{A_S} j^{-\varnothing}, \quad \text{Eqn 16}$$

($\varnothing$, exponent of stomatal density (j)). How do our *Arabidopsis* data, where the only anatomical variable is stomatal density, fit the above equation? A best-fit relationship with an r^2 value of 0.99 is described by the following equation:

$$L\,(\text{in mm units}) = 43 + (18\,789 \times j^{-1.538}). \quad \text{Eqn 17}$$

Therefore, the first term of Eqn 17, the sum of the ratios of 1 mm^2 transpiration surface to the internal cross-sectional areas before water flows towards the sub-stomatal cavity multiplied by their respective path lengths, is 43 mm. The ratio of 1 mm^2 of the transpiring surface to cross-sectional area of water flowing towards a single sub-stomatal cavity multiplied by the average path length is 18 789 mm.

Conclusions

Stomatal density affects leaf water isotopic enrichment as evidenced by the correlation with L for the transgenic *Arabidopsis* and with Δ_L/Δ_e and $\wp$ for the field-collected samples. High stomatal density causes an increase in Δ_L/Δ_e and a decrease in L and $\wp$. The relationship between water content and parameters related to leaf water isotopic enrichment, however, pivots on one outlier species. Therefore, no conclusions can be drawn regarding the effect of water content on leaf water isotopic enrichment. There are several factors which contribute to leaf water isotopic enrichment and these factors, such as transpiration and L, often interact with each other. No single factor is overwhelmingly responsible for leaf water isotopic enrichment and although one factor may promote the isotopic enrichment of leaf water, another factor may decrease it. A case in point is our observation that high stomatal density in transgenic *Arabidopsis* would normally promote leaf water isotopic enrichment by decreasing the effective path length. However, any increase in leaf water isotopic enrichment that would be promoted by high stomatal density, is counteracted with an increase in transpiration associated with higher stomatal density. The incorporation of leaf water content and stomatal density in the current steady-state leaf water isotopic enrichment model will improve our ability to predict leaf water isotopic enrichment. The findings reported here are important in the use of plant isotopic proxies in the interpretation of paleoclimate. Periods of warm weather, such as the Eocene, are associated with a higher CO_2 concentration (Doria *et al.*, 2011). It is well known that high CO_2 concentration lowers stomatal frequency (Woodward & Kelly, 1995). There are several lines of evidence that fossil leaves, indeed, show fluctuations in stomatal density (Beerling & Royer, 2002; Doria *et al.*, 2011). According to our observation, this fluctuation in stomatal density will affect the leaf water isotopic composition and lead to isotopic differences in metabolites produced by the leaf.

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