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Vein density is independent of epidermal cell size in *Arabidopsis* mutants

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Abstract. Densities of leaf minor veins and stomata are co-ordinated within and across vascular plants. This maximises the benefit-to-cost ratio of leaf construction by ensuring stomata receive the minimum amount of water required to maintain optimal aperture. A 'passive dilution' mechanism in which densities of veins and stomata are co-regulated by epidermal cell size is thought to facilitate this co-ordination. However, unlike stomata, veins are spatially isolated from the epidermis and thus may not be directly regulated by epidermal cell expansion. Here, we use mutant genotypes of *Arabidopsis thaliana* (L.) Heynh. with altered stomatal and epidermal cell development to test this mechanism. To do this we compared observed relationships between vein density and epidermal cell size with modelled relationships that assume veins and stomata are passively diluted by epidermal cell expansion. Data from wild-type plants were consistent with the 'passive dilution' mechanism, but in mutant genotypes vein density was independent of epidermal cell size. Hence, vein density is not causally linked to epidermal cell expansion. This suggests that adaptation favours synchronised changes to the cell size of different leaf tissues to coordinate veins and stomata, and thus balance water supply with transpirational demand.

Additional keywords: pavement cells, xylem, vascular tissue.

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Introduction

The correlation between leaf conductances to liquid- and vapour-phase water present across a diverse range of plants (Brodribb *et al*. 2005; Lo Gullo *et al*. 2010) suggests that a balance between water supply and transpirational demand is established during leaf construction. The efficiency with which water is transported through the leaf mesophyll to supply evaporative surfaces near the stomata is determined by leaf vein density (total vein length per unit area) (Sack and Frole 2006; Brodribb *et al*. 2007) whereas transpirational demand is determined by stomatal density (total number of stomata per unit area) and aperture. If there is an imbalance between the production of veins and stomata during leaf development the plant suffers diminishing photosynthetic returns on energetic investments in vein construction (Lambers and Poorter 1992) or stomatal maintenance. The maximum benefit-to-cost ratio is expected when investment in the leaf vein network is sufficient that stomata are supplied with the minimum amount of water required to allow them to open to an optimal aperture set by leaf photosynthetic biochemistry (Medlyn *et al*. 2011) under well watered conditions (Brodribb and Jordan 2011; Franks *et al*. 2012). This view is supported by evidence for developmental coordination of veins and stomata within individual plants, within species, across populations of the same species and across species in a range of woody and herbaceous angiosperms and ferns (Brodribb and Jordan 2011; Carins Murphy *et al*. 2012; Zhang *et al*. 2012; Brodribb *et al*. 2013; Martins *et al*. 2014; Yang *et al*. 2014; Zhang *et al*. 2014; Fiorin *et al*. 2016; Carins Murphy *et al*. 2016).

It has been proposed that vein and stomatal development is coordinated by a 'passive dilution' mechanism in which densities of veins and stomata are co-regulated by epidermal cell size (Carins Murphy *et al*. 2016). Under a passive dilution model it is predicted that no new xylem or guard cells are added during leaf expansion and both are passively diluted by epidermal cell expansion. Xylem cells are also expected to expand in unison with epidermal cells. This would result in positive relationships between vein density and $1/\sqrt{}$ epidermal cell size, stomatal density and 1/epidermal cell size and between vein density and $\sqrt{}$stomatal density. However, unlike stomata, veins are spatially isolated from the epidermis and it is not known whether they are directly regulated by differential epidermal cell expansion. Although there is evidence that, across a wide range of angiosperms, cell sizes of all major tissues types are correlated within leaves (Brodribb *et al*. 2013; John *et al*. 2013), and that these changes in cell size are associated with changes

in vein and stomatal density such that vein density is correlated with modelled demand for water (Brodribb *et al*. 2013).

Despite this overall link, the size and abundance of different cell types are likely under independent control. For example, the differentiation of procambial cells into vascular cells during leaf vein development is regulated by movement of the phytohormone auxin through developing leaf tissue (Jacobs 1952; Sachs 1981; Uggla *et al*. 1996; Mattsson *et al*. 1999; Sieburth 1999; Avsian-Kretchmer *et al*. 2002; Aloni *et al*. 2003). According to the 'auxin-flow canalisation hypothesis' cell polarity determines the direction of auxin transport, and sustained transport induces increased polarity creating feedback that leads to canalisation of auxin flow and cell differentiation into defined strands (Sachs 1981). Carrier proteins that are located at the basal end of cells regulate the polarity of auxin flow by mediating the transfer of auxin (Rubery and Sheldrake 1974; Raven 1975; Gälweiler *et al*. 1998). Thus, vein formation and connection is thought to be driven by PIN-mediated auxin transport (Verna *et al*. 2015). In comparison, stomatal development occurs in a series of transitions from undifferentiated protodermal cells to meristemoid mother cells, meristemoids, guard mother cells and finally to mature stomata (Pant and Kidwai 1967). This process is tightly regulated by a complex signalling network involving an independent suite of genetic controls (Lau and Bergmann 2012; Pillitteri and Torii 2012). The prominent role of auxin in vascular development and evidence that mutations affecting auxin transport impact stomatal differentiation (Mayer *et al*. 1993; Spitzer *et al*. 2009) led to the suggestion that auxin may link vein and stomatal development (Brodribb and Jordan 2011). However, recent research has indicated that auxin is a negative regulator of stomatal development (Balcerowicz and Hoecker 2014). The positive relationship observed between vein and stomatal density is therefore unlikely to involve auxin signalling because the high concentration of auxin required to produce vein infrastructure would likely inhibit stomatal differentiation. Conversely, auxin may be responsible for the absence of stomata from the epidermis directly above or below vascular tissue (Hill 1980; Smith *et al*. 1989).

Thus, here we aimed to investigate whether the developmental control of veins and epidermal tissues are linked in a way that is consistent with the 'passive dilution' mechanism, and if this affects the coordination of water supply and demand. Recent advances in the understanding of stomatal development have produced a suite of genotypes that specifically alter stomatal density, stomatal index, and epidermal cell size, which consequentially alter the gas-exchange capacity of the leaf (Dow and Bergmann 2014). Isolated control of cellular development in the epidermis allowed us to observe whether changes to external tissues remain coordinated with developmental programs in adjacent and physiologically linked internal tissues. We predicted that stomatal density would be regulated by genetic changes to stomatal index and, more generally, to epidermal cell expansion, while vein density would remain closely tied to genotype-specific differences in epidermal cell size. To test this hypothesis, we compared observed relationships in wild-type (Col-0) *Arabidopsis thaliana* (L.) Heynh) plants and mutant stomatal development genotypes with modelled relationships that assume veins and stomata are passively diluted by epidermal cell expansion.

Materials and methods

Plant material and growth conditions

All genotypes included in this study were in the Col-0 ecotype of *Arabidopsis thaliana* (L.) Heynh. and Col-0 was used as the control genotype. Seven previously described mutant and transgenic genotypes were used. Genotypes were chosen because of their overall wild-type appearance and because manipulated genes were restricted to the stomata and epidermal cells. The four mutant genotypes were: *tmm-1* (Nadeau and Sack 2002), *basl-2* (Dong *et al*. 2009), *epf1-1* (Hara *et al*. 2007), and *epf1-1*;*epf2-1* (Hunt and Gray 2009), and the three transgenic genotypes were $SPCH_{pro}$::SPCH-YFP, $SPCH_{pro}$::SPCH 2–4A-YFP and SPCH SILENCE (Lampard *et al*. 2008; Dow *et al*. 2014*a*, 2014*b*). Seeds were surface-sterilised and stratified at 4°C for 3–5 days in 0.15% agarose solution and then sown directly into pots of size 8.26 cm × 8.26 cm × 7.62 cm filled with Pro-Mix HP soil (Premier Horticulture, Quakerstown) and supplemented with Scott's Osmocote Classic 14-14-14 fertiliser (Scotts-Sierra, Marysville). At 10–14 days, seedlings were thinned so that only one seedling per pot remained. Plants of all genotypes were grown to maturity in growth chambers where the conditions were as follows: day/night cycle, 16 h/8 h; day/night temperature, 22/20°C; light intensity, ~100 µmol m^{-2} s^{-1}. Additional Col-0 plants were grown under 50, 160 and 200 µmol m^{-2} s^{-1} of incident light.

Leaf size and anatomical traits

All leaves were scanned at 300 pixels per inch (dpi) using a Canon CanoScan CS8800F flatbed scanner (Canon, Sydney) and leaf size measured (cm^2) using ImageJ (National Institutes of Health). Vein density (mm mm^{-2}), stomatal density (mm^{-2}), stomatal size (mm^2), stomatal index, epidermal cell density (total epidermal cells per unit area; mm^{-2}), epidermal cell size (mm^2) and presence of stomatal clusters or pairs was then quantified from one rosette leaf from each of six plants per mutant or transgenic genotype (except *epf1*;*epf2* where four plant were sampled, and *epf1* and SPCH SILENCE where five plants were sampled) and one rosette leaf from each of eight, six, five and three Col-0 plants grown under 50, 100, 160 and 200 µmol m^{-2} s^{-1} of incident light respectively. Leaves were cleared with a 7 : 1 ethanol : acetic acid solution overnight or longer, softened for 30 min in 1 M potassium hydroxide and rinsed with water. Leaves from the mutant and transgenic genotypes and Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light were then stained with 1% crystal violet and mounted on microscope slides in phenol glycerine jelly. Leaves from Col-0 plants grown under 50, 160 and 200 µmol m^{-2} s^{-1} of incident light were left unstained and mounted on microscope slides in Hoyer's solution.

Ten fields of view were photographed from all leaves to determine vein density. Care was taken to ensure representative sampling across the middle of the leaf and leaf margin (the midrib was avoided). Photomicrographs of leaves stained with crystal violet were taken at 4 × magnification (field of view area 3.47 mm^2) using a Nikon Digital Sight DS-L1 camera (Nikon,

Melville) mounted on a Leica DM 1000 microscope (Leica, Nussloch). Unstained leaves were visualised by differential interference contrast microscopy and photomicrographs taken at 5 × magnification through a 0.7 × tube (field of view area 4.72 mm^2) using a Leica DFC450 C digital microscope camera mounted on a Leica DM 2000 LED microscope. A further 10 fields of view were photographed from both the abaxial and adaxial surfaces of all leaves to determine stomatal density, stomatal size, stomatal index, epidermal cell density, epidermal cell size and whether stomata were present in clusters or pairs. As mentioned above sampling comprised a representative sample of the leaf surface except for the midrib. Leaves stained with crystal violet were photographed at 20 × magnification (field of view area 0.141 mm^2) and unstained leaves were visualised by differential interference contrast microscopy and photographed at 20 × magnification through a 0.7 × tube (field of view area 0.301 mm^2) using the same camera and microscope setups described above.

All leaf anatomical traits were quantified using ImageJ. Stomatal size was measured from five stomata (comprising a pair of guard cells) per field of view. Epidermal cell size (S_{EC}) was calculated as:

$$S_{EC} = (1 - (D_S \times S_S))/D_{EC}, \quad (1)$$

where D_S is stomatal density, S_S is stomatal size and D_{EC} is epidermal cell density. Stomatal index (SI) was calculated as:

$$SI = (D_S/(D_S + D_{EC})) \times 100, \quad (2)$$

according to Salisbury (1927). Partial stomata and epidermal cells were included in density counts if visible along the top and right-hand border of photomicrographs and discarded if visible along the bottom and left-hand border. The presence of stomatal clusters or pairs was assessed visually from photomicrographs.

Statistical analysis

Values of stomatal density, epidermal cell size and stomatal index from the abaxial and adaxial leaf surfaces, leaf size and ratio of vein density to $1/\sqrt{}$abaxial epidermal cell size from the mutant and transgenic genotypes were compared with values from Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light using one-way ANOVA and Tukey's HSD in R (R Foundation for Statistical Computing). Log and square root transformations were applied when necessary to normalise the data. Mean abaxial and adaxial stomatal index of Col-0 plants grown under 50, 160 and 200 µmol m^{-2} s^{-1} of incident light were also compared with mean values from Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light using the same method. The correlation coefficient (r^2) and statistical significance of co-variation between parameters ($P < 0.05$) was then determined for the relationships between vein density and $1/\sqrt{}$abaxial epidermal cell size, stomatal density and 1/epidermal cell size (on both leaf surfaces) and between vein density and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density across all Col-0 plants using R. Data were log and square root transformed where necessary. Analyses were performed at the plant level because there were highly significant pooled within treatment correlations between vein density and $1/\sqrt{}$abaxial epidermal cell size, abaxial stomatal density and 1/abaxial epidermal cell size, adaxial stomatal density and 1/adaxial epidermal cell size and vein density and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density ($r^2 = 0.45$; $P < 0.01$, $r^2 = 0.81$; $P < 0.001$, $r^2 = 0.71$; $P < 0.001$ and $r^2 = 0.6$; $P < 0.001$, respectively with 17 degrees of freedom; i.e. $n - 5$ to allow for the loss of degrees of freedom from fitting means for four treatments). Note that the relationships within treatments were highly similar to those among treatments. The relative contribution of stomatal index and 1/epidermal cell size to the r^2 of the multiple regression in which they are predictors of stomatal density was also quantified for the abaxial and adaxial leaf surfaces in Col-0 plants as a relative importance metric (lmg) using the 'relimp' package in R.

'Passive dilution' models

Observed relationships between vein density, stomatal density and epidermal cell size in Col-0 plants were also compared with modelled relationships using ANCOVA in R. Modelled relationships were calculated according to the method outlined by Carins Murphy *et al.* (2016). The modelled relationships between vein density, stomatal density and epidermal cell size were based on the 'passive dilution' hypothesis whereby vein and stomatal density are coordinated by the expansion of epidermal cells. This assumes that vein and stomatal densities are uniquely related to epidermal cell size and that the epidermis comprises only epidermal and stomatal cells with a constant ratio between them (reflected by the stomatal index). Thus, epidermal cell size was calculated for a range of stomatal densities using Eqn 1 where epidermal cell density was calculated as:

$$D_{EC} = (D_S/SI) - D_S, \quad (3)$$

using the mean stomatal index of the Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light and the mean stomatal size across all plants (stomatal size was independent of changes to stomatal density (Fig. 1). This relationship was modelled separately for the abaxial and adaxial leaf surfaces.

The relationship between vein density and abaxial epidermal cell size was modelled using the simplified assumption that vein length is associated with a fixed proportion of the perimeter of an epidermal cell. According to this assumption one side of a theoretical square epidermal cell would always be in contact with vein tissue as it expanded. Thus, a geometric model of vein density as a function of abaxial epidermal cell size was determined for a fixed stomatal index (mean abaxial stomatal index of the Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light) incorporating the mean abaxial stomatal size across all plants. Epidermal cell size was calculated as above. Assuming vein density (D_V) was a function of abaxial epidermal cell size we fitted the function:

$$D_V = a \times \text{abaxial } S_{EC}{}^{-0.5}, \quad (4)$$

where a is proportional to the epidermal cell perimeter associated with vein length. This returned a value of 0.1197 for a. This value was then used to predict the impact of abaxial epidermal cell size on vein density (using the equation vein density = 0.1197 × abaxial epidermal cell $size^{-0.5}$). Finally the expected relationship between vein density and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density was modelled by combining the relationships above.

Results

All mutant and transgenic genotypes had a different stomatal phenotype from Col-0 (Table 1). In some cases the effect of mutation or transgenes on phenotype was more severe on the abaxial leaf surface than on the adaxial surface. However, the leaves of all mutant and transgenic genotypes except SPCH SILENCE were the same size as Col-0 leaves.

Relationships between vein density, stomatal density and epidermal cell size in wild-type plants

Co-variation of vein and stomatal density with epidermal cell size contributed to the coordination of veins and stomata in Col-0. Thus, light-induced changes to stomatal density and 1/epidermal cell size were coordinated on both leaf surfaces across the Col-0 plants (Fig. 2*a*, *b*; abaxial: $r^2 = 0.71$; $F_{1,20} = 49.59$; $P < 0.001$, adaxial: $r^2 = 0.68$; $F_{1,20} = 42.1$; $P < 0.001$). However, the slope of the observed relationship was 78.7% greater than the slope of the modelled relationship assuming stomatal density responds passively to epidermal cell expansion on the abaxial leaf surface and 67% greater on the adaxial leaf surface. Thus, the results of ANCOVA indicated that there were significant interaction effects between the covariate 1/epidermal cell size and relationship type (observed or modelled) for both leaf surfaces (abaxial: $F_{1,34} = 16.6$; $P < 0.001$, adaxial: $F_{1,34} = 14.04$; $P < 0.001$). Although the main effects of the covariate 1/epidermal cell size on stomatal density were large and significant (abaxial: $F_{1,34} = 1794.95$; $P < 0.001$, adaxial: $F_{1,34} = 1545.84$; $P < 0.001$) and the main effects of relationship type were non-significant (abaxial: $F_{1,34} = 0.22$; $P > 0.05$, adaxial: $F_{1,34} = 0.0021$; $P > 0.05$). In addition, stomatal index increased with light intensity on both leaf surfaces (Table 2). Despite this, 1/epidermal cell size was a more important determinant of stomatal density than stomatal index (explaining 55.5% of variation in stomatal density on the abaxial leaf surface versus the 44.5% explained by stomatal index and 51.2% versus 48.8% on the adaxial leaf surface). The relationship between vein density and $1/\sqrt{}$abaxial epidermal cell size was also coordinated across

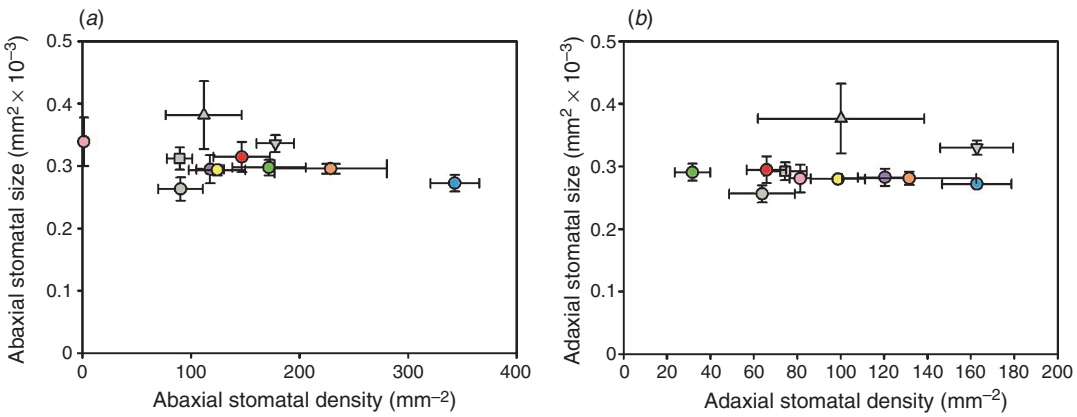

Fig. 1. Mean stomatal density and size (± s.d.) on the (*a*) abaxial and (*b*) adaxial leaf surfaces of Col-0 grown under four light treatments comprising 50, 100, 160 and 200 µmol m^{-2} s^{-1} of incident light (grey circles; $n = 8$, squares; $n = 6$, up-facing triangles; $n = 5$, and down-facing triangles; $n = 3$, respectively) and seven mutant and transgenic genotypes grown under standardised light conditions (100 µmol m^{-2} s^{-1} of incident light) (*basl*: purple circle, *epf1*: yellow circle, *epf1;epf2*: blue circle, SPCH 2-4A: orange circle, SPCH SILENCE: pink circle, SPCH-YFP: red circle and *tmm*: green circle) ($n = 6$ in all cases except *epf1* and SPCH SILENCE where $n = 5$ and *epf1;epf2* where $n = 4$).

Table 1. Phenotype of mutant and transgenic *Arabidopsis thaliana* genotypes compared with Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light

Significant differences from Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light are indicated: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$; values are means ± s.e.

Genotype	Stomatal density (mm^{-2})		Epidermal cell size ($mm^2 \times 10^{-3}$)		Stomatal patterning		Leaf size (cm^2)
	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	
Col-0	89.6 ± 4.8	74.5 ± 4	3.5 ± 0.2	5.2 ± 0.3	normal	normal	6.1 ± 0.4
basl	117.7 ± 5.1n.s.	120.4 ± 5.1***	2.8 ± 0.2n.s.	4.5 ± 0.2n.s.	clusters	clusters	5.2 ± 0.5n.s.
epf1	124.3 ± 11.7n.s.	98.8 ± 5.6n.s.	3.4 ± 0.2n.s.	5.4 ± 0.2n.s.	normal	normal	5.8 ± 0.4n.s.
epf1;epf2	343.1 ± 11.2***	162.8 ± 8***	1.3 ± 0.1***	3.9 ± 0.3*	normal	normal	5.2 ± 0.5n.s.
SPCH 2–4A	228.7 ± 21.1***	131.5 ± 12.6***	0.9 ± 0.1***	3.3 ± 0.3***	normal	normal	5.3 ± 0.3n.s.
SPCH SILENCE	1.4 ± 0.9***	81.4 ± 2.2n.s.	4.6 ± 0.2**	3.3 ± 0.2***	normal	normal	3.4 ± 0.4**
SPCH-YFP	146.8 ± 11.7*	65.9 ± 4.1n.s.	2.0 ± 0.2***	5.3 ± 0.3n.s.	normal	normal	5.6 ± 0.3n.s.
tmm	171.9 ± 13.8***	31.8 ± 3.3***	3.9 ± 0.2n.s.	6.3 ± 0.2*	clusters	pairs	6.3 ± 0.5n.s.

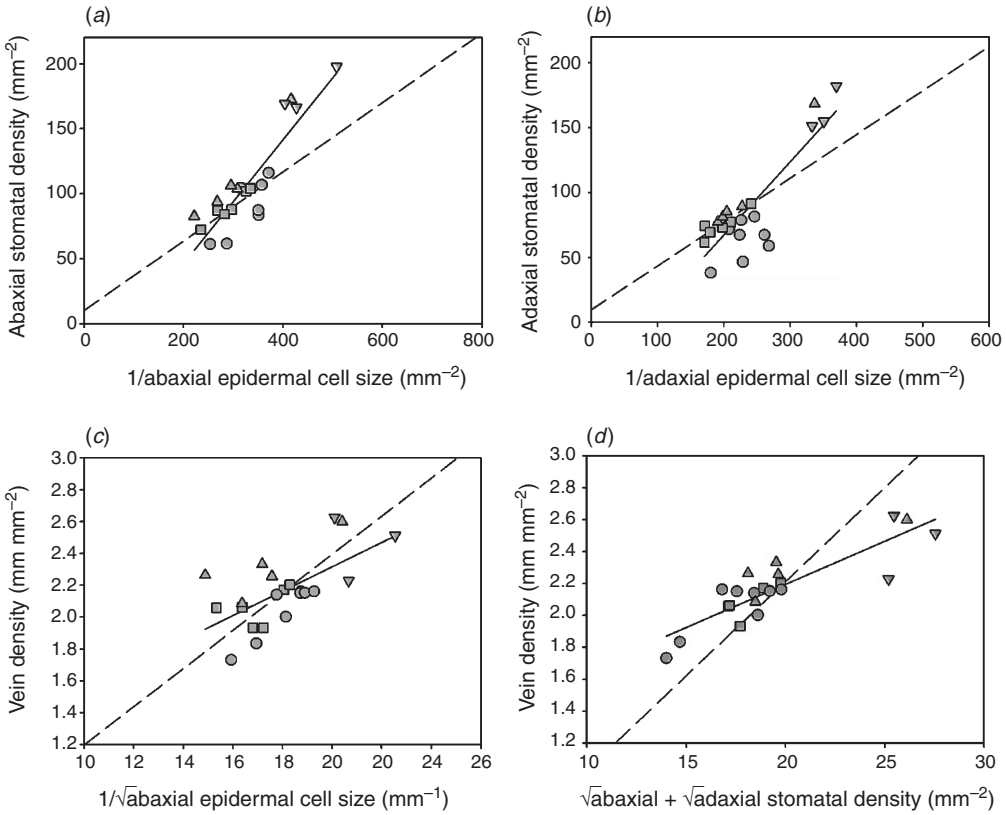

Fig. 2. Observed relationships between stomatal density and 1/epidermal cell size (each symbol represents the mean vein and stomatal density per leaf from 10 counts) on the (*a*) abaxial and (*b*) adaxial leaf surfaces, (*c*) vein density and $1/\sqrt{}$abaxial epidermal cell size and (*d*) vein density and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density across *Arabidopsis thaliana* Col-0 plants grown under a range of light conditions (grey symbols and black solid lines) compared with modelled relationships (black dashed lines). Light treatments comprised 50, 100, 160 and 200 μmol m^{-2} s^{-1} of incident light (circles, squares, up-facing triangles and down-facing triangles respectively). Modelled relationships assume veins and stomata are passively diluted by epidermal cell expansion and that stomatal index is constant (see 'materials and methods' for details). Black solid lines are regressions between stomatal density and 1/epidermal cell size (abaxial $D_S = 1.307 \times 1/\text{abaxial } S_{EC} - 1.268$, $r^2 = 0.71$, $F_{1,20} = 49.59$, $P < 0.001$ (log-transformed data); adaxial $D_S = 0.847 \times 1/\text{adaxial } S_{EC} - 3.747$, $r^2 = 0.68$, $F_{1,20} = 42.1$, $P < 0.001$ (square root transformed data)), vein density and $1/\sqrt{}$abaxial epidermal cell size ($D_V = 0.077 \times 1/\sqrt{}\text{abaxial } S_{EC} + 0.779$, $r^2 = 0.41$, $F_{1,20} = 13.93$, $P < 0.01$) and vein density and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density ($D_V = 0.054 \times \sqrt{}\text{abaxial} + \sqrt{}\text{adaxial } D_S + 1.113$, $r^2 = 0.74$, $F_{1,20} = 58.39$, $P < 0.001$). All observed relationships were significantly different from modelled relationships.

Table 2. Stomatal index of *Arabidopsis thaliana* Col-0 plants grown under 50, 160 and 200 μmol m^{-2} s^{-1} of incident light compared with plants grown under 100 μmol m^{-2} s^{-1} of incident light

Significant differences from Col-0 plants grown under 100 μmol m^{-2} s^{-1} of incident light are indicated: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$; values are means ± s.e.

Light treatment (μmol m^{-2} s^{-1} of incident light)	Stomatal index	
	Abaxial	Adaxial
100	23.4 ± 0.2	27.8 ± 0.7
50	21.5 ± 1n.s.	21.6 ± 1.4***
160	27.3 ± 0.8*	30.6 ± 1n.s.
200	29.1 ± 0.3**	33.1 ± 0.6n.s.

Col-0 plants (Fig. 2*c*; $r^2 = 0.41$; $F_{1,20} = 13.93$; $P < 0.01$), although the slope of the observed relationship was 35.8% less than the slope of the modelled relationship assuming vein density is determined by epidermal cell expansion alone (i.e. there was not a constant ratio between vein density and $1/\sqrt{}$abaxial epidermal cell size). This meant that changes to vein density and stomatal density (abaxial and adaxial leaf surfaces combined) were also coordinated (Fig. 2*d*) ($r^2 = 0.74$; $F_{1,20} = 58.39$; $P < 0.001$) although the slope of the observed relationship was 54.2% less than modelled. Thus, the results of ANCOVA indicated that there were significant interaction effects between both covariates ($1/\sqrt{}$epidermal cell size and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density) and relationship type ($F_{1,34} = 6.9$; $P < 0.05$ and $F_{1,34} = 90.54$; $P < 0.001$, respectively). However, the main effects of the covariates $1/\sqrt{}$epidermal cell size and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density on vein density were large and significant ($F_{1,34} = 1225.58$; $P < 0.001$ and $F_{1,34} = 2683.26$; $P < 0.001$ respectively) and the main effects of relationship type were non-significant ($F_{1,34} = 0.043$; $P > 0.05$ and $F_{1,34} = 1.39$; $P > 0.05$ respectively).

Relationships between vein density, stomatal density and epidermal cell size among modified genotypes

As predicted, relationships between stomatal density and 1/epidermal cell size on the abaxial and adaxial leaf surfaces in the mutant and transgenic genotypes differed from modelled relationships (Fig. 3*a*, *b*). Genotypes that deviated the most from modelled relationships had higher or lower stomatal index than Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light (Table 3), indicating that genetic controls determining stomatal index had a strong influence on stomatal density. However, contrary to the second part of our hypothesis, relationships between vein density and $1/\sqrt{}$abaxial epidermal cell size in the mutant and transgenic genotypes dramatically deviated from the modelled relationship (Fig. 3*c*). Thus, the ratio of vein density to $1/\sqrt{}$abaxial epidermal cell size in these genotypes differed from that observed in Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light (Table 3), demonstrating that vein density was not affected by large changes to epidermal cell size. Consequently, the relationship between vein density and stomatal density (abaxial and adaxial leaf surfaces) also deviated from the modelled relationship in some mutant and transgenic genotypes (Fig. 3*d*).

Discussion

Vein density is not causally linked to epidermal cell expansion

Epidermal cell size is a major determinant of vein and stomatal density in a diverse range of woody and herbaceous angiosperms (Carins Murphy *et al*. 2016). Consequently, it has been proposed that developmental coordination of these traits is facilitated by a 'passive dilution' mechanism in which densities of veins and stomata are co-regulated by epidermal cell size. However, the dramatic divergence of relationships between vein density and epidermal cell size in mutant and transgenic *Arabidopsis* genotypes from a modelled relationship that assumes vein density is passively diluted by epidermal cell expansion (Fig. 3*c*) shows that variation in vein density is not causally linked to epidermal cell size. In contrast, epidermal cell size was a strong predictor of vein and stomatal density in Col-0 plants (despite some deviation from modelled relationships). This is supported by previous work that found stomatal and epidermal cell density both increase with increasing light intensity in *Arabidopsis* (Šantrůček *et al*. 2014). Thus, vein density was correlated with light-induced changes to epidermal cell size but was unresponsive to variation in epidermal cell size that was driven by direct developmental cues in the stomatal lineage.

Vein density may respond to cells that mirror epidermal cells

The independence of vein density from epidermal cell size in the mutant and transgenic genotypes and dependence on epidermal cell size in Col-0 plants grown under a range of light conditions suggests that vein density does not respond directly to differential epidermal cell expansion but rather to another factor that mirrors any light-induced changes to epidermal cell size. Cell sizes of independent leaf tissues are correlated across species suggesting that changes to cell size commonly occur in unison within the leaf (Brodribb *et al*. 2013; John *et al*. 2013). Furthermore, cell volumes in the abaxial and adaxial epidermis and in the mesophyll tissue in *Arabidopsis* increase approximately in proportion with one another during leaf expansion (Wuyts *et al*. 2010). However, evidence from this study shows that abaxial epidermal cell size in mutant and transgenic genotypes of *Arabidopsis* can change independently from other leaf tissues. Because veins are embedded in the leaf mesophyll one would expect that mesophyll cell size may have a more direct influence on vein density. Thus, veins may be passively diluted by mesophyll cell expansion while unified changes to mesophyll and epidermal cell size may drive the proportional relationships observed between vein density, stomatal density and epidermal cell size in wild-type plants.

Control of cell size

We propose that synchronised changes to cell size in adjacent but independent epidermal and mesophyll leaf tissues coordinate the spacing of veins and stomata. As such we found that leaves from *Arabidopsis* plants grown under low light had larger epidermal cells (and presumably larger mesophyll cells) than leaves from plants grown under higher light intensity (Fig. 2). In growing plant cells wall relaxation drives the water uptake required to produce irreversible cell enlargement, reviewed in Schopfer (2006). In fact, shoot elongation (largely due to cellular expansion) in response to low light is thought to be regulated by the modification of cell wall extensibility (Sasidharan *et al*. 2008). Cell expansion during leaf growth is also sensitive to leaf water status, as reviewed by Pantin *et al*. (2012), and is responsive to hydraulic demand. If shade leaves maintain a higher apoplast water potential than sun leaves because transpiration is reduced, the steeper water potential gradient towards growing cells in these leaves may induce increased cell expansion. In support of this view, shade leaves of several herbs and woody angiosperms underwent greater epidermal cell expansion and had higher water potentials than sun leaves (Carins Murphy *et al*. 2016) and diurnal leaf expansion rates were depressed in *Arabidopsis* plants experiencing hydraulic limitation (Pantin *et al*. 2011). Thus, the *Arabidopsis* plants grown under low light in this study may have produced larger cells than those plants grown under higher light intensity because they had greater cell wall extensibility, and/or because the water potential gradients towards growing cells were steeper in these plants.

Water supply and demand may be mismatched in some mutant and transgenic genotypes

There was considerable variation in stomatal density across the mutant and transgenic genotypes, but little variation in vein density. Presumably this resulted in a mismatch between water supply and demand in these genotypes as vein density is closely linked to leaf hydraulic conductance (Sack and Frole 2006; Brodribb *et al*. 2007) and stomatal density to stomatal conductance. Under the growth conditions of our experiment, this potential mismatch had little impact on leaf size (Table 1). However, under conditions that induce drought or a high vapour pressure difference (VPD), producing too few veins

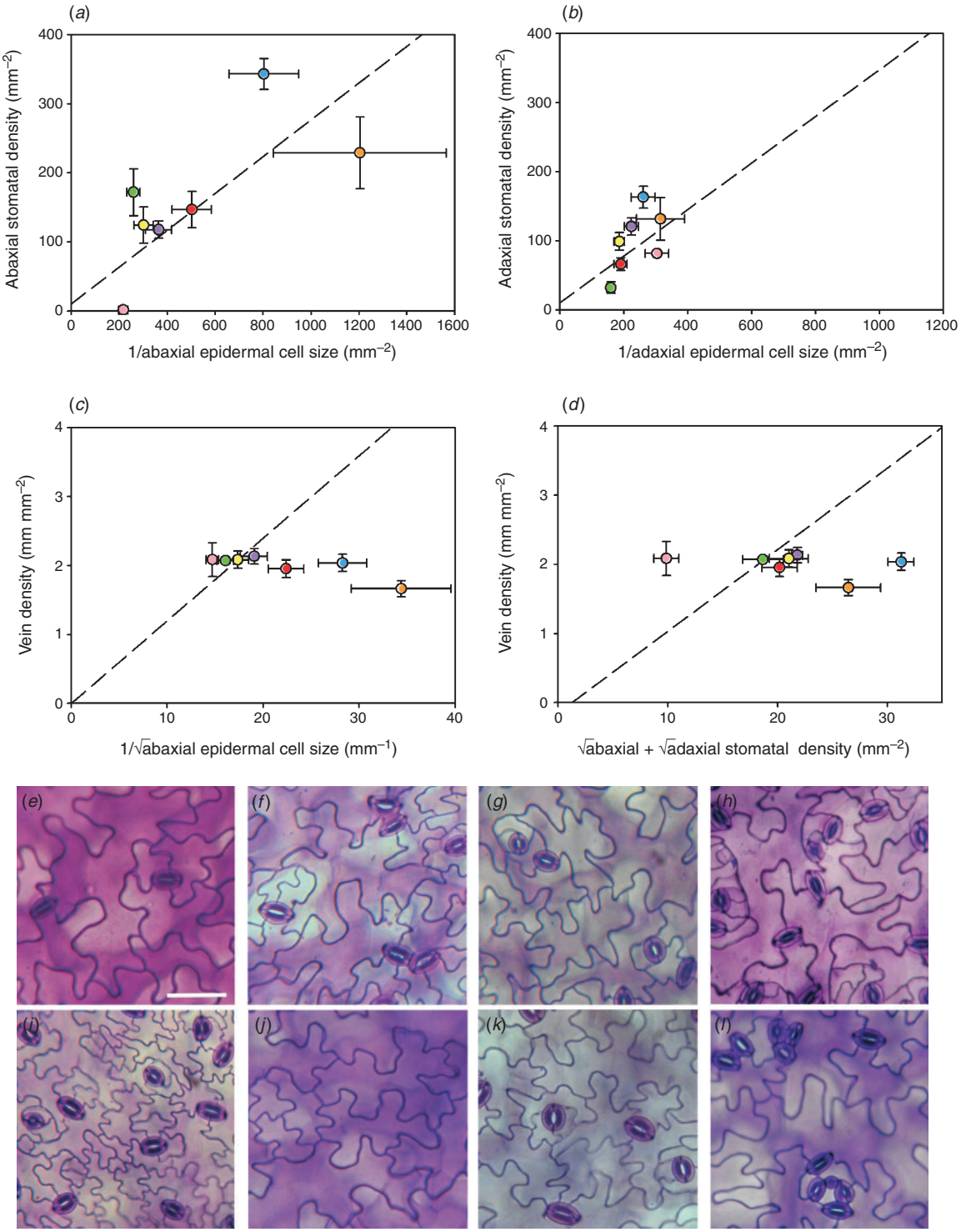

Fig. 3. Stomatal density and 1/epidermal cell size on the (*a*) abaxial and (*b*) adaxial leaf surfaces, (*c*) vein density and 1/$\sqrt{}$abaxial epidermal cell size and (*d*) vein density and $\sqrt{}$abaxial + $\sqrt{}$adaxial stomatal density of seven mutant and transgenic *Arabidopsis thaliana* genotypes grown under standardised light conditions (100 µmol m^{-2} s^{-1} of incident light) (*basl*: purple circle, *epf1*: yellow circle, *epf1*;*epf2*: blue circle, SPCH 2–4A: orange circle, SPCH SILENCE: pink circle, SPCH-YFP: red circle and *tmm*: green circle) compared with modelled relationships (black dashed lines). Symbols are means ± s.d. ($n = 6$ in all cases except *epf1* and SPCH SILENCE where $n = 5$ and *epf1*;*epf2* where $n = 4$). Modelled relationships assume veins and stomata are passively diluted by epidermal cell expansion and that stomatal index is constant (see materials and methods for details). Photomicrographs illustrate the range in epidermal cell size ((*e*) Col-0, (*f*) *basl*, (*g*) *epf1*, (*h*) *epf1*;*epf2*, (*i*) SPCH 2–4A, (*j*) SPCH SILENCE, (*k*) SPCH-YFP, (*l*) *tmm*). Scale bar = 50 µm.

Table 3. Stomatal index and ratio of vein density to $1/\sqrt{}$abaxial epidermal cell size of the mutant and transgenic *Arabidopsis thaliana* genotypes compared with Col-0 plants grown under 100 μmol m^{-2} s^{-1} of incident light

Significant differences from Col-0 plants grown under 100 μmol m^{-2} s^{-1} of incident light are indicated: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$; values are means ± s.e.

Genotype	Stomatal index		Vein density:$1/\sqrt{}$ abaxial
	Abaxial	Adaxial	epidermal cell size
Col-0	23.4 ± 0.2	27.8 ± 0.7	0.12 ± 0.0032
basl	24.6 ± 0.6n.s.	34.4 ± 0.5***	0.11 ± 0.0048n.s.
epf1	29.4 ± 0.9***	34.5 ± 0.5***	0.12 ± 0.0027n.s.
epf1;*epf2*	32 ± 1.1***	38.5 ± 0.8***	0.07 ± 0.0023***
SPCH 2–4A	16.6 ± 0.5***	29.9 ± 0.9n.s.	0.05 ± 0.0027***
SPCH SILENCE	0.5 ± 0.3***	20.9 ± 0.9***	0.14 ± 0.0062n.s.
SPCH-YFP	22.8 ± 0.6n.s.	25.6 ± 0.6n.s.	0.09 ± 0.0013***
tmm	39.8 ± 1.1***	15.9 ± 1.1***	0.13 ± 0.0037n.s.

relative to stomata may result in premature stomatal closure to prevent desiccation or reduced plant growth (Doheny-Adams *et al*. 2012; Carins Murphy *et al*. 2014). In contrast, an abundance of veins relative to stomata would produce an oversupply of water and an energetic overinvestment in vein development, potentially reducing other growth characteristics. In both cases, the carbon gain for energy investment in leaf infrastructure is reduced and a significant disadvantage during competition against more efficient species or ecotypes may arise in stressful growth environments. In support of this idea, the mutant genotypes used in this study that produce stomata in clusters have been found previously to have impaired stomatal function (Dow *et al*. 2014*b*). Thus, our data not only demonstrate that such changes often lead to mismatch between veins and stomata, but that these tissues are coordinated in wild-type plants, strongly suggesting that adaptation is driving the balance between water supply and demand.

Conclusions

Contrary to our hypothesis, vein density was independent of variation in epidermal cell size among mutant and transgenic *Arabidopsis* genotypes. Conversely, epidermal cell size was an important predictor of vein density in wild-type plants grown under a range of light conditions. Thus, we suggest that vein density responds to expansion of another cell type (most likely the mesophyll) that mirrors any light-induced changes to epidermal cell size. These results suggest that adaptation favours synchronised changes to cell size in different leaf tissues to coordinate vein and stomatal density, and thus, maintain a balance between water supply and transpirational demand. One would expect that there is selective pressure to achieve this kind of co-ordination as a mismatched supply of water would result in significant costs to plants in terms of vein and stomatal infrastructure or reduced CO_2 uptake due to stomatal closure. This is in line with recent research that found *Arabidopsis* genotypes with mutations that cause stomatal clustering have impaired stomatal function (Dow *et al*. 2014*b*).

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