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Original Article

Morphological and anatomical determinants of mesophyll conductance in wild relatives of tomato (*Solanum* sect. *Lycopersicon*, sect. *Lycopersicoides*; Solanaceae)

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ABSTRACT

Natural selection on photosynthetic performance is a primary factor determining leaf phenotypes. The complex CO_2 diffusion path from substomatal cavities to the chloroplasts – the mesophyll conductance (g_m) – limits photosynthetic rate in many species and hence shapes variation in leaf morphology and anatomy. Among sclerophyllous and succulent taxa, structural investment in leaves, measured as the leaf dry mass per area (LMA), has been implicated in decreased g_m. However, in herbaceous taxa with high g_m, it is less certain how LMA impacts CO_2 diffusion and whether it significantly affects photosynthetic performance. We addressed these questions in the context of understanding the ecophysiological significance of leaf trait variation in wild tomatoes, a closely related group of herbaceous perennials. Although g_m was high in wild tomatoes, variation in g_m significantly affected photosynthesis. Even in these tender-leaved herbaceous species, greater LMA led to reduced g_m. This relationship between g_m and LMA is partially mediated by cell packing and leaf thickness, although amphistomy (equal distribution of stomata on both sides of the leaf) mitigates the effect of leaf thickness. Understanding the costs of increased LMA will inform future work on the adaptive significance of leaf trait variation across ecological gradients in wild tomatoes and other systems.

Key-words: adaptation; LMA; photosynthesis.

INTRODUCTION

Leaves are the primary photosynthetic organs in most plants and many disparate features of leaf morphology and anatomy, including cell shape, stomatal patterning and chloroplast distribution, can affect photosynthetic efficiency. Photosynthetic performance also affects components of plant fitness (Arntz & Delph 2001), so that, unsurprisingly, many features of leaves are shaped by natural selection (Givnish 1987; Smith *et al*. 1997; Nicotra *et al*. 2011). The effect of leaf structure on fundamental processes such as CO_2 diffusion and hence photosynthetic performance has potentially important consequences for ecologically important differences between closely related species and for phenotypic plasticity within species. To determine what aspects of trait variation are most critical at the ecological and microevolutionary scales where natural selection acts, studies must examine variation in these traits and their relationships among diverse yet recently diverged species.

Different aspects of physiological response and photosynthetic performance are more or less determined by leaf structural variation. In particular, unlike stomatal conductance (g_s), which responds physiologically within minutes to changes in the external environment, the mesophyll conductance (g_m: the conductance of CO_2 from the substomatal cavity to the sites of carboxylation in the chloroplast) reflects, to a large degree, structural parameters such as leaf thickness, cell packing, shape and wall thickness (Terashima *et al*. 2011; Tosens *et al*. 2012b; Tomás *et al*. 2013), although rapid changes in g_m have been documented (Flexas *et al*. 2008; Warren 2008; Evans *et al*. 2009; Tazoe *et al*. 2011; Griffiths & Helliker 2013). These structures cannot be altered until new leaves develop (plastic responses) or until natural selection alters leaf structure following a changing environment (evolutionary responses). In addition, g_m is increasingly recognized as a significant limitation on photosynthetic rate in many species (Flexas *et al*. 2008, 2012) that affects carbon-, water- and nitrogen-use efficiencies (Barbour *et al*. 2010; Buckley & Warren 2013; Flexas *et al*. 2013). Understanding the determinants of mesophyll conductance is an active area of theoretical and empirical investigation (Tholen & Zhu 2011; Sharkey 2012; Tholen *et al*. 2012), but little is known about their potentially important evolutionary role in shaping leaf phenotypes in response to selection across environmental gradients.

Despite the variety of leaf morphology and anatomy observed among species and environments, some aspects of leaf structure are constrained by the fundamental physics of gas diffusion (Parkhurst 1994). As CO_2 diffuses into a leaf, it must pass though the boundary layer, the stomatal pore, the air-filled pore space within the leaf, the cell wall, plasma membrane and cytoplasm as well as the chloroplast membrane. Variation in leaf structures influences the conductance

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to CO_2 at each step of this diffusion pathway (e.g. Schuepp 1993; Flexas *et al*. 2006; Terashima *et al*. 2006), and therefore can impact photosynthetic performance. Leaf dry mass per area (LMA) is one of the many aspects of leaf structure that affect g_m, and is therefore one such phenotype that could be shaped by selection on photosynthetic performance. LMA is a composite of many underlying traits, such as mesophyll thickness, cell wall thickness, cell shape, cell packing and so forth. At a basic level, LMA can be decomposed into bulk leaf thickness and density (Witkowski & Lamont 1991; Niinemets 1999). A thicker leaf allows greater photosynthetic rate under high irradiance, explaining the strong plasticity of this trait in response to light (Poorter *et al*. 2010). Increased leaf thickness is also associated with succulence, which can prevent cellular dehydration by buffering the transpirational stream (Ogburn & Edwards 2010). Similarly, dense, sclerophyllous leaves are associated with cellular drought tolerance (Niinemets 2001, but see Bartlett *et al*. 2012) and/or mechanical defences against herbivores (Turner 1994; Onoda *et al*. 2011). Variation in LMA is therefore ecologically and evolutionary significant (Reich *et al*. 2003; Wright *et al*. 2005; Poorter *et al*. 2009). Generally, higher LMA is associated with greater (a)biotic stress tolerance but slower growth. Low LMA, indicating relatively little investment per leaf area, benefits plants by capturing light more efficiently and allowing faster whole plant growth and competitive ability (Poorter & Bongers 2006), often at the expense of stress tolerance. LMA is known to be highly variable between species (Reich *et al*. 2003; Wright *et al*. 2005; Poorter *et al*. 2009), and the optimal LMA for a given ecological strategy and/or environmental context depends on balancing these costs and benefits.

Studies across broad functional groups and within species indicate that high LMA limits photosynthetic efficiency by reducing the mesophyll conductance (Flexas *et al*. 2008; Niinemets *et al*. 2009a; Galmés *et al*. 2011). This is particularly evident among sclerophylls (Niinemets *et al*. 2009b; Hassiotou *et al*. 2010) and succulents (Maxwell, von Caemmerer & Evans, 1997), where extreme differences in anatomy among distantly related species explain variation in mesophyll conductance. In comparison, little is known about how LMA affects mesophyll conductance among species on the low LMA end of the spectrum (e.g. between herbaceous species) or whether LMA-mediated changes in mesophyll conductance are important over short evolutionary timescales (within species or between recently diverged species). A meta-analysis of LMA and g_m showed that while LMA sets up an upper limit on g_m, wide variation in g_m is possible on the low LMA (herbaceous) end of the spectrum (Flexas *et al*. 2008), suggesting that the relationship between LMA and photosynthetic performance in herbaceous species might be highly variable from one taxonomic group to another (Hanba *et al*. 1999).

Predicting the relationship between LMA and g_m in herbaceous leaves is difficult because the two basic components of LMA (leaf thickness and density) affect g_m in different ways. Firstly, in terms of leaf thickness, thicker mesophyll in tender, herbaceous leaves might actually increase g_m by allowing more chlorophyll surface area per leaf area (S_c), which provides more parallel paths for CO_2 diffusion from the intercellular airspace (IAS) into the chloroplasts (Terashima *et al*. 2006; Galmés *et al*. 2013). However, greater airspace resistance in thicker leaves eventually limits diffusion (Parkhurst 1994), especially in hypostomatous species (Syvertsen *et al*. 1995). Consequently, the relationship between leaf thickness and g_m could be positive, negative or even quadratic depending on the domain of leaf thickness over which g_m is measured. Furthermore, the ratio of stomatal density on the adaxial ('upper') to abaxial ('lower') surface, referred to as the stomatal ratio (SR), alters the effective leaf thickness. In terms of CO_2 diffusion through the IAS, amphistomatous leaves are essentially half as thick as comparable hypostomatous leaves, further complicating the relationship between thickness *per se* and g_m.

The relationship between LMA and g_m can also be affected traits that affect leaf density rather than thickness since increased cell packing increases airspace resistance by reducing the porosity of the mesophyll (Nobel 2009), usually measured as IAS fraction of leaf volume (f_{IAS}). However, in herbaceous species, leaves are generally not densely packed, so these factors might be relatively less important. Leaf density is also affected by mesophyll cell walls and chloroplast envelope thickness, which are the major components of liquid phase CO_2 diffusion. Because CO_2 diffusion through water and lipids is many orders of magnitude slower than through air, the liquid phase resistance explains much of variation in total g_m across species (Evans *et al*. 2009). Because of these potentially complex contributions of anatomical leaf traits to CO_2 diffusion, the relationship between variation in LMA and g_m in herbaceous species is currently difficult to predict in any particular group.

Wild tomato species provide an excellent opportunity to examine questions of the functional significance of physiological and anatomical traits to photosynthetic performance, over recent evolutionary history. The clade is young [<2.7 million years old (Kamenetzky *et al*. 2010)] but ecological niches span deserts, tropical rain forests and highlands (>4000 m) and leaf morphology is correspondingly diverse (Moyle 2008; Peralta *et al*. 2008). Ecological niche modelling indicates that climate variables thought to affect leaf morphology (temperature and precipitation) are the strongest contributors to niche divergence (Nakazato *et al*. 2010). However, few studies have examined the functional and/or ecological consequences of leaf trait variation in these species (Nakazato *et al*. 2008, 2012; Chitwood *et al*. 2012). Examining how variation in leaf structure influences photosynthetic performance within and between tomato species therefore could provide insight into the selective pressures governing the evolution leaf morphology and anatomy as well as identify those leaf traits most likely to contribute to plasticity and local adaptation. For example, to contribute significantly to ongoing evolutionary responses, there must be genetic variation in a trait within or among species. Therefore, even if one or more components of the CO_2 diffusion pathway accounts for a large fraction of overall photosynthetic limitations, if this trait does not vary, it will be less

evolutionarily significant than a trait that remains functionally variable within and between species.

To ascertain the evolutionary significance of leaf morphological and anatomical variation on g_m and photosynthetic performance, we measured anatomical and physiological variation in eight phenotypically diverse species of closely related wild tomatoes, in order to address four questions:

1 Do physiological traits, including g_m, vary significantly within and/or between wild tomato species?
2 How much does g_m limit photosynthetic rate?
3 How does LMA affect g_m?
4 What are the anatomical determinants of the LMA–g_m relationship in these species? Specifically, how do leaf density, thickness and/or S_c determine g_m?

MATERIALS AND METHODS

Plant material and cultivation

We obtained seeds of eight wild species (Supporting Information Table S1) from the Tomato Genetics Resource Center (TGRC) at UC Davis (http://tgrc.ucdavis.edu). On 19–20 August 2010, seeds were soaked in 50% household bleach for 30 min, rinsed thoroughly and placed on moist paper in plastic boxes and germinated in a growth chamber. After 1 week, seedlings were transplanted to cell-pack flats containing Metro-Mix 360 (Sun Gro Horticulture, Vancouver, British Columbia, Canada). Two weeks later, seedlings were transplanted again to 3.78 L pots containing a 1:1 mixture of compost soil and Metro-Mix 360. Plants were positioned randomly in the Indiana University greenhouse and grown under supplemental lighting to maintain a constant 16:8 h light:dark cycle. Plants were irrigated to field capacity daily to prevent drought stress and fertilized weekly with a nitrogen, phosphorus and potassium solution. We periodically trimmed plants and removed any developing fruit to maintain rapid vegetative growth throughout the experiment. Gas exchange measurements and tissue for anatomical and morphological measurements were taken from individual plants in a haphazard order between November 2010 and February 2011. All traits measured are listed in Table 1.

Gas exchange

We used an open-path infrared gas exchange analyser with a 2 cm^2 leaf chamber fluorometer (LI-6400-40, Li-Cor Inc., Lincoln, NE, USA) to simultaneously measure leaf gas exchange and chlorophyll *a* fluorescence. To minimize leaf position and age effects, all measurements were made on young, fully expanded leaves ($\bar{n} = 5.25$, range = [4, 7]). The ambient CO_2 concentration in the chamber (C_a) was 370 μmol CO_2 mol^{-1} air, leaf temperature was 25 °C, photosynthetic photon flux density (PPFD) was 1000 μmol m^{-2} s^{-1} with 90:10 red:blue light, and relative humidity was between 50 and 70%. Once a leaf reached steady-state photosynthesis (A) and stomatal conductance (g_s), usually after ~30 min, we measured the photosynthetic light response to obtain an estimate of a leaf's maximum photosynthetic rate (A_{max}). The photosynthetic light response was measured between PPFD of 1500 and 25 μmol m^{-2} s^{-1} (90:10 red:blue). Using non-linear least squares regression, we fit the data to a non-rectangular hyperbola:

$$A = \frac{\phi I + A_{max} - \sqrt{(\phi I + A_{max})^2 - 4\Theta\phi I A_{max}}}{2\Theta} \quad (1)$$

The response to changing substomatal CO_2 concentrations (C_i) was measured by adjusting the ambient CO_2 concentration in the leaf chamber (C_A) to concentrations ranging between 50 and 1300 μmol CO_2 mol^{-1} air. The flow rate was 300 μmol s^{-1}. Diffusional leaks for both CO_2 and H_2O were corrected by using the methods of Rodeghiero *et al.* (2007). We observed no differences between diffusion coefficients calculated for leaves of different species, dried leaves, lyophilized leaves, or an empty chamber (data not shown).

From fluorescence measurements and $A - C_i$ curves, we determined the photosynthetic rate, stomatal conductance (g_s), mesophyll conductance to CO_2 (g_m) and the maximum rate of carboxylation ($V_{c,max}$). Photosynthetic rate and stomatal conductance are estimated directly from gas exchange measurements. We estimated mesophyll conductance using the variable J method (Harley *et al.* 1992):

$$g_m = \frac{A}{C_i - \frac{\Gamma^*[J + 8(A + R_d)]}{J - 4(A + R_d)}} \quad (2)$$

For each leaf, g_s and g_m were not constant, but decreased at higher C_i, as seen in many studies (Tazoe *et al.* 2011 and references therein). We report g_s and g_m at ambient C_a

Table 1. Partitioning phenotypic variance within and between species. Most of the variation in physiological traits (upper half) is within species. In contrast, interspecific differences account for a large fraction of the total variation in anatomical traits (lower half). Variance components were estimated using restricted maximum likelihood (REML)

Trait	Variance component		% Variance explained by species
	Species	Residual	
Physiological			
$\log(g_s)$	6.03×10^{-3}	6.90×10^{-2}	8.03%
$\log(g_m)$	0	0.262	0%
$V_{c,max}$	0	812	0%
A_{max}	0	18.2	0%
Anatomical			
LMA	31.8	26.3	54.8%
$\log(T)$	6.46×10^{-2}	3.47×10^{-2}	65.1%
$\log(D)$	8.32×10^{-3}	6.15×10^{-2}	11.9%
$\log(S_c)$	3.83×10^{-2}	3.93×10^{-2}	49.4%
SR	4.48×10^{-2}	3.09×10^{-2}	59.2%

g_s, stomatal conductance; g_m, mesophyll conductance; $V_{c,max}$, maximum velocity of carboxylation; A_{max}, light-saturated maximum photosynthetic rate at ambient CO_2 concentration; LMA, leaf mass per area; T, leaf thickness; D, bulk leaf density; S_c, area of chloroplasts exposed to internal air space per leaf area; SR, stomatal ratio.

(370 ± 5 μmol CO_2 mol^{-1} air) because these values are most ecologically relevant and allowed us to directly analyse how g_s and g_m limited A_{max}, which was also measured at ambient C_a (see above). g_m is sometimes averaged over a larger range of C_i, but we found that average g_m was highly correlated with point measurements of g_m at ambient C_a, and hence the choice of method did not qualitatively change the relationship between g_m and leaf anatomy. For high g_m leaves such as tomatoes, gas exchange measurements can be sensitive to error. Points measurements of ambient g_m met Harley *et al.*'s (1992) criterion ($10 > dC_c/dA > 50$) in all cases, indicating that these measurements were reliable.

We estimated non-photorespiratory CO_2 evolution in the light (R_d) and the chloroplastic CO_2 compensation point (Γ^*) using the Laisk method (Laisk & Oja 1998). Briefly, for each plant, we measured the photosynthetic response to C_i over the linear portion of the response curve (generally, $30 < C_i < 120$ μmol CO_2 mol^{-1} air) at two irradiances (75 and 500 μmol m^{-2} s^{-1}). We fit linear regressions to the responses for each irradiance and calculated their intersection to obtain estimates of R_d and C_i^*, the intercellular CO_2 compensation point, for individual leaves. Since

$$C_i^* = \Gamma^* - R_d/g_m \quad (3)$$

the *y*-intercept of the line fit between R_d and C_i^* is an estimate of Γ^* (Evans & von Caemmerer 1991). As there is not a causal relationship between R_d and C_i^*, we calculated Γ^* using type II regression, which estimates the regression slope and intercept by minimizing error in both *x*- and *y*-axes, using the R package smatr version 3.2.6 (Warton *et al.* 2012).

The electron transport rate of photosystem II (J_f) was calculated from fluorescence measurements as $J_f = \Phi_{PSII} I \alpha\beta$, where Φ_{PSII} is the quantum yield (moles of CO_2 fixed per mole of quanta absorbed) of photosystem II, I is irradiance, α is the leaf light absorptance and β is the photosystem partitioning factor. Φ_{PSII} was estimated from the chlorophyll fluorescence data. We estimated the product $\alpha\beta$ using the relationship $\Phi_{PSII} = \frac{\alpha\beta\Phi CO_2}{4}$ under non-photorespiratory conditions (Genty *et al.* 1989). To vary the quantum yield, we measured photosynthetic light response curves under 2% O_2. The relationship became non-linear at low irradiance as stomatal conductance decreased. Since non-linearities can bias estimates $\alpha\beta$ and therefore g_m (Gilbert *et al.* 2012), we fit the curve using linear regression with only measurements over the range PPFD < 1500 μmol m^{-2} s^{-1}. Error in our estimates of Γ^* and/or $\alpha\beta$ could have potentially large effects on our inference about g_m. To examine the sensitivity of our results to measurement error, we reran our analyses using the 0.025 and 0.975 quantiles of Γ^* and $\alpha\beta$.

Using the calculated values of g_m, we determined $A - C_c$ curves to estimate the maximum velocity of carboxylation [$V_{c,max}$ (μmol m^{-2} s^{-1})], which is proportional to the concentration of activated ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco). We estimated $V_{c,max}$ following Long & Bernacchi (2003) from the linear, Rubisco limited portion of the $A - C_c$ curve, empirically determined to be $C_i < 200$ mol CO_2 mol^{-1} air. We used constants from Sharkey *et al.* (2007) to calculate an effective Michaelis–Menten constant of $K_m = 81.15$ Pa.

Leaf morphology and stomatal density

Morphological measurements were made on the same leaflets as gas exchange measurements. To calculate LMA, we used a 1 cm^2 cork borer to remove a constant area of laminar tissue from every leaf. The leaf samples were dried at 60 °C and dry mass determined with a Sartorius CP225D fine balance (Sartorius Corp., Edgewood, NY, USA). LMA was calculated as g dry mass divided by the area of fresh tissue. Since LMA is the product of thickness and density, we estimated bulk leaf density (D) by dividing LMA by leaf thickness (T) measured from leaf cross sections (estimated as we explain below). A limitation of this method is that any measurement error in LMA or T will be attributed to variation in D. This causes some spurious autocorrelation between D and T. However, for this to be a serious problem, the amount of measurement error would have to be comparable to the true biological variation in T and LMA, which is unlikely given the precision with which T, dry mass and leaf area were measured. Leaf surface impressions were made by applying a thin layer of nail polish. The nail polish was removed using transparent adhesive tape and mounted on a microscope slide. Stomata were counted from three fields each of the abaxial ('lower') and adaxial ('upper') leaf surfaces. The SR was calculated as ratio of the stomatal densities (SD) on the adaxial ('upper') and abaxial ('lower') side:

$$\text{Stomatal ratio} = \frac{SD_{adaxial}}{SD_{abaxial}} \quad (4)$$

Leaf anatomy

Following gas exchange measurements, leaf pieces from within the gas exchange chamber were fixed (2% glutaraldehyde, 1% formaldehyde; 0.05 M PIPES buffer), embedded in firm Spurr's resin, and cross sections (100–160 nm) were made using a microtome (Sorvall, Porter-Blum MT2, Norwalk, CT, USA). Images of cross sections were captured with a Nikon E800 microscope (Nikon USA, Melville, NY, USA) using bright field illumination. We calculated leaf thickness and S_c following Evans *et al.* (1994). Briefly, we used camera lucida drawings to measure the area and width of the sections. Leaf laminar thickness (T) was calculated as leaf cross-sectional area divided by the width of the section.

The number of chloroplasts facing intercellular air space in both palisade (N_p) and spongy mesophyll cells (N_s) was separately determined for each section. The length of chloroplast touching the plasma membrane appressed to airspace was measured on five representative chloroplasts in both the palisade (L_p) and spongy mesophyll (L_{sp}) cells. Length measurements were made in ImageJ version 1.45 s (Schneider *et al.* 2012) using the segmented line tool. S_c was then calculated as

$$S_c = \frac{N_p L_p + N_{sp} L_{sp}}{L_{sec}} \quad (5)$$

where L_{sec} is the length of the section examined. Finally, we measured f_{IAS}, the fraction of mesophyll occupied by IAS in micrographs.

Statistical analysis

Because of shared evolutionary history, species are not necessarily statistically independent units (Felsenstein 1985). To determine whether we should incorporate phylogenetic distance into our statistical analyses, we compared the fit of a Brownian motion (BM) and white noise (WN) model of trait evolution, for each measured trait. A better fit of the BM compared with the WN model indicates that phylogenetic relatedness is positively associated with trait values and should be incorporated into the analysis. We estimated model parameters using maximum likelihood methods implemented in the R package *geiger* version 1.3-1 (Harmon *et al*. 2008) on a phylogenetic tree from Rodriguez *et al*. (2009). We compared model fits using a parametric bootstrapping method (Boettiger *et al*. 2012) implemented in the R package *pmc* version 0.0–8 (Boettiger 2013). For all traits, the WN model fit the data as well or better than the BM model, indicating that phylogenetic relationships did not contribute significantly to trait variance (i.e. there was no significant phylogenetic signal in the measured traits). Therefore, species were treated as independent units in the statistical analysis.

Replicates within species likewise might not be statistically independent. Since the number of replicates per species was low ($\bar{n} = 5.25$) and unbalanced, we used linear mixed models (LMM) treating species as a random effect. Physiological (g_s, g_m, $V_{c,max}$, A_{max}) and anatomical (LMA, T, D, S_c, f_{IAS}, SR) traits were treated as fixed effects when used as predictor variables. For all analyses, we log-transformed response variables as necessary to normalize residuals, linearize relationships and/or decrease the leverage of a few data points. Elsewhere in this study, we generally do not log-transform the same variables when they are predictor variables and there is no assumption of normality unless it was desirable for linearization or leverage. Following the recommendations of Bolker *et al*. (2009), we fit LMMs using restricted maximum likelihood (REML) implemented in the lmer function, part of R package lme4 version 0.999375-42 (Bates *et al*. 2011). We used two approaches to assess statistical significance of fixed effects. Firstly, we used *F*-tests, calculating the residual degrees of freedom using the Kenward–Roger approximation (Kenward & Roger 1997) as implemented in the R package pbrktest version 0.3-1 (Halekoh & Højsgaard 2012). A significant *F*-test indicates that a statistical model that includes the term of interest explains more variance than expected by chance. In addition to *F*-tests, we determined 95% highest posterior density (HPD) intervals calculated from 10^4 Markov chain Monte Carlo simulations of fitted models. An HPD interval that does not overlap 0 indicates that a parameter is significantly different from 0. Note that it is possible for a term to be significant by one criterion but not the other.

We applied these statistical methods to our four questions as follows: (1) to assess the amount of interspecific variation for our physiological and anatomical traits, we compared the variance explained by species, as estimated by REML, with the total trait variation for each trait. (2) We used quantitative limitation analysis (Jones 1985, Grassi & Magnani 2005; Tomás *et al*. 2013) to partition controls on photosynthesis into stomatal (l_s), mesophyll (l_m) and biochemical (l_b) limitations:

$$l_s = \frac{g_{tot}/g_s \times \partial A/\partial C_c}{g_{tot} + \partial A/\partial C_c} \quad (6)$$

$$l_m = \frac{g_{tot}/g_m \times \partial A/\partial C_c}{g_{tot} + \partial A/\partial C_c} \quad (7)$$

$$l_b = \frac{g_{tot}}{g_{tot} + \partial A/\partial C_c} \quad (8)$$

Following Tomás *et al*. (2013), we estimated $\partial A/\partial C_c$ using the slope of the $A - C_c$ response curve between $C_c = 40$ and $C_c = 110$ μmol CO_2 mol^{-1} air. We complemented limitation analysis using partial regression to assess whether g_m *per se* influenced A_{max} independently of an indirect association between photosynthetic potential (measured as $V_{c,max}$), g_m and A_{max}. (3) To determine the effect of variation in leaf mass per area on mesophyll conductance, we tested whether LMA was correlated, positively or negatively, with g_m. (4) To determine the influence of focal anatomical traits (leaf density, leaf thickness and chloroplast surface area) on mesophyll conductance, we estimated the effect of D, T and S_c on g_m simultaneously in a single LMM. We also allowed a quadratic term for T to test our *a priori* prediction that thickness might increase g_m in thin leaves but decrease it as leaves become very thick. To test our prediction that SR mitigates the effect of leaf thickness on airspace resistance, we tested whether there was a positive correlation between SR and T.

RESULTS

Determination of biochemical parameters

Biochemical parameters were used to estimate g_m via the variable J method (see Eqn 2). We estimated the chloroplastic CO_2 compensation point (Γ^*) to be 42.7 (95% CI: 40.1–45.3) μmol CO_2 mol^{-1} air by regressing respiration (R_d) against the internal CO_2 compensation point (C_i^*) for all individual replicates (plants; Fig. 1). Note that while it is possible to estimate an average mesophyll conductance (g_m) from the slope of the regression (see Eqn 3), we did not use it for this purpose since we were interested in differences between plants rather than the average across plants. This method assumes that Γ^* is constant across species, an assumption that is likely justified since Γ^* is a property of Rubisco, which varies little across C_3 species. Furthermore, sequences of the gene encoding the large subunit of Rubisco

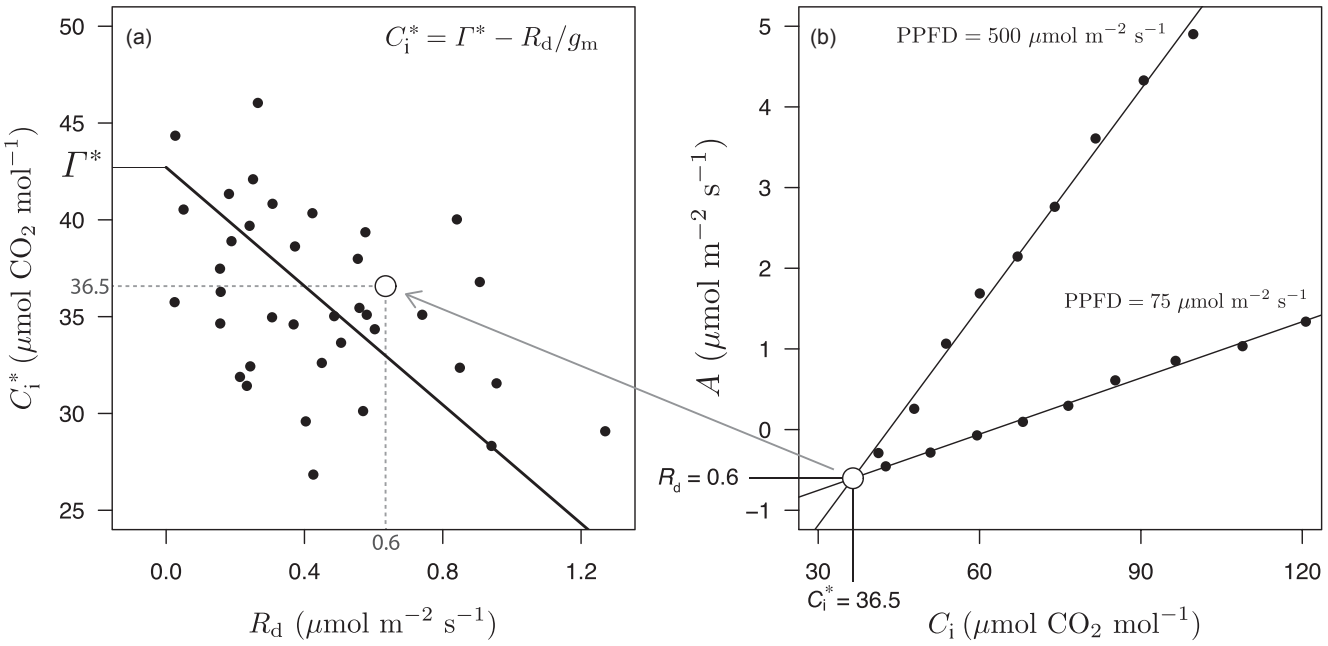

Figure 1. Estimating biochemical parameters for curve fitting. We calculated the chloroplastic CO_2 compensation point (Γ^*) as the intercept of the regression between mitochondrial respiration (R_d) and the intercellular CO_2 compensation point (C_i^*). (a) We fit the equation (top right corner) using type II regression. Each point represents an individual plant in our experiment for which we calculated R_d and C_i^* using the Laisk method (see Materials and Methods for details). The larger open circle is the data point for the representative example calculation illustrated in b. R_d and C_i^* were measured individually for each plant in the experiment as the intersection of CO_2 response curves conducted at two light intensities [photosynthetic photon flux density (PPFD) = 75 and 500 μmol m^{-2} s^{-1}].

(*rbcL*) indicate minimal functional divergence across all wild tomatoes used in this study (J. Galmés, personal communication). In addition to Γ^*, we calculated product of leaf absorptance and the photosystem partitioning factor ($\alpha\beta$). Using data from three species (*Solanum arcanum*, *S. chilense* and *S. habrochaites*), we estimated $\alpha\beta = 0.472$ (95% CI: 0.452–0.495), with little variance between species suggesting that this value was nearly constant in our experiment. Therefore, we used a single value for every plant. Assuming $\beta = 0.5$, $\alpha = 0.94$, which was slightly higher than the measured absorptance 0.88 ± 0.03 in these species (data not shown).

Intraspecific and interspecific trait variation

Our first objective was to determine whether there was significant variation in physiological and anatomical traits, and how trait variation was partitioned within and among species. Physiological parameters were measured using $A - C_i$ and light response curves (Fig. 2). Although there was substantial variation in physiological traits, most of the variation was within rather than between species (i.e. among plants; Table 1). For three of four traits, including g_m, the REML estimate of species' contribution to trait variance was 0. In comparison, we detected substantial interspecific variation in anatomical traits (Table 1). Interestingly, several of these traits were correlated with g_m (see below); we consider the meaning of this apparent contradiction in the Discussion section. Standard analyses of variance (ANOVAS) treating species as a fixed rather than as a random effect gave the same qualitative results as LMMs (data not shown).

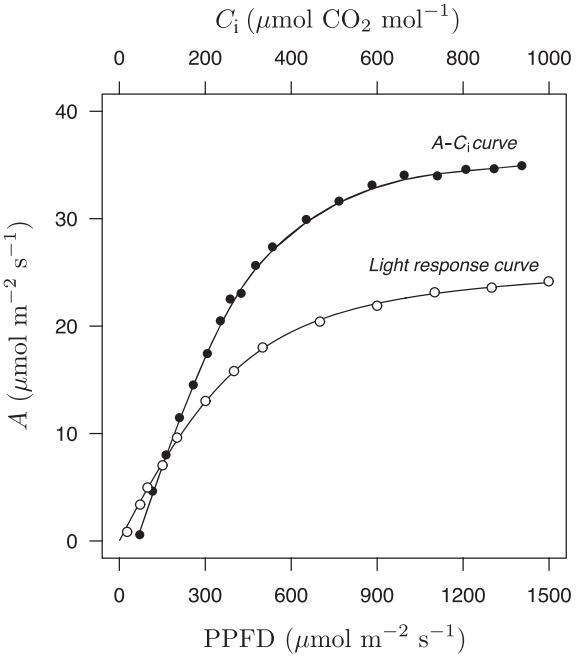

Figure 2. Representative $A - C_i$ (●; upper x-axis) and photosynthetic light response (○; lower x-axis) curves for a single plant. The $A - C_i$ curve captures the response of photosynthesis (A; y-axis) to intercellular CO_2 concentration (C_i; upper x-axis). The line was fit using the LOWESS algorithm (Cleveland 1979) and is intended solely for illustration rather than analysis. The light response curve captures the response of photosynthesis (A; y-axis) to photosynthetic photon flux density (PPFD; lower x-axis). The line was fit to a non-rectangular hyperbola (Eqn 1) using non-linear least squares.

Table 2. Quantitative limitation analysis comparing stomatal (l_s), mesophyll (l_m) and biochemical (l_b) limitations to photosynthetic rate. Note that, by definition, $l_s + l_m + l_b = 1$. In wild tomatoes, $l_s < l_m < l_b$ in non-stressed conditions

Limitation	Stomatal (l_s)	Mesophyll (l_m)	Biochemical (l_b)
Mean ± SEM (n = 44)	0.24 ± 0.007	0.33 ± 0.015	0.43 ± 0.014

How much does mesophyll conductance limit photosynthetic rate?

In general, biochemistry limited photosynthetic rate more than g_m, and g_m limited photosynthetic rate more than g_s (Table 2). Across plants, biochemical (l_b) were on average greater than stomatal (l_s) limitations (paired t-test, $t_{42} = 11.97$, $P = 4.00 \times 10^{-15}$) and mesophyll (l_m) limitations (paired t-test, $t_{42} = 3.58$, $P = 0.0009$). Likewise, l_m was on average greater than l_s (paired t-test, $t_{42} = 5.16$, $P = 6.42 \times 10^{-6}$). However, the relative limitation imposed by mesophyll versus stomatal conductance was sensitive to $\alpha\beta$ (Supporting Information Fig. S3), indicating that this result might not be robust to measurement error. We also used partial regression to examine whether variation in g_m *per se* affected photosynthetic performance, which we measured as the maximum photosynthetic rate (A_{max}) under saturating irradiance (Eqn 1). An alternative hypothesis is that g_m is correlated with photosynthetic potential, leading to an ostensibly strong but indirect relationship between g_m and A_{max}. For fitting purposes, g_m was log-transformed prior to analysis to produce a linear relationship with $A_{max.}$ g_m and $V_{c,max}$, an indicator of photosynthetic capacity, were both associated with greater A_{max} (Fig. 3a,b), but were uncorrelated with each other (Fig. 3c). The partial correlation between g_m and A_{max}, conditioned on $V_{c,max}$ ($\rho = 0.84$, $t_{41} = 10.08$, $P = 1.17 \times 10^{-12}$), confirms that the relationship between g_m and A_{max} is not mediated by $V_{c,max}$.

How does LMA affect mesophyll conductance?

LMA was negatively associated with mesophyll conductance (Fig. 4a, $F_{1,27.81} = 7.16$, $P = 0.012$), indicating that even in herbaceous tomato leaves, increased investment in leaf tissue (higher LMA) significantly inhibits g_m. The 95% HPD interval for the coefficient relating LMA to g_m does not overlap 0 (–1.41 – –0.23). For these results, we log-transformed both g_m and LMA prior to analysis because both traits were log-normally distributed, meaning that residuals are highly heteroscedastic. For comparison, the relationship is significant even if LMA is not log-transformed ($F_{1,25.38} = 8.28$, $P = 0.008$) and marginally significant if neither variable is transformed ($F_{1,19.56} = 3.98$, $P = 0.060$). The CO_2 drawdown from substomatal cavities to the chloroplast ($C_i - C_c$) was also significantly positively correlated with LMA (Fig. 4b, $F_{1,28.60} = 9.15$, $P = 0.005$). The 95% HPD interval for the coefficient relating LMA to CO_2 drawdown does not overlap 0 (19.41–89.91). The effect of LMA of g_m and CO_2 drawdown was not sensitive to measurement error in Γ^* or $\alpha\beta$ (Supporting Information Fig. S4). This provides further evidence that greater LMA hinders internal CO_2 diffusion. We also reanalysed a previously published global dataset of LMA, g_m and CO_2 drawdown (Niinemets *et al.* 2009a) to put our results in context. The slope of the log(g_m)–log(LMA) relationship in the global dataset (–0.66, 95% CI: –0.96 – –0.37) fell within 95% HPD interval determined from tomatoes (see above), indicating a general pattern of declining in g_m with increasing LMA (Supporting Information Fig. S1a). The slope of the global ($C_i - C_c$)–log(LMA) likewise fell within the interval determined from tomatoes (26.31, 95% CI: 11.97–40.69), although there was less overlap between datasets in this comparison than that with g_m (Supporting Information Fig. S1b).

What is the mechanistic basis of the LMA–g_m relationship?

LMA is equal to the product of leaf thickness (T) and density (D). Therefore, the relationship between LMA and g_m must be mediated by these traits. We hypothesized that leaf thickness could be associated with increased g_m in herbaceous leaves if thicker leaves have increased chloroplast surface area (S_c), a trait often positively correlated with g_m. Although

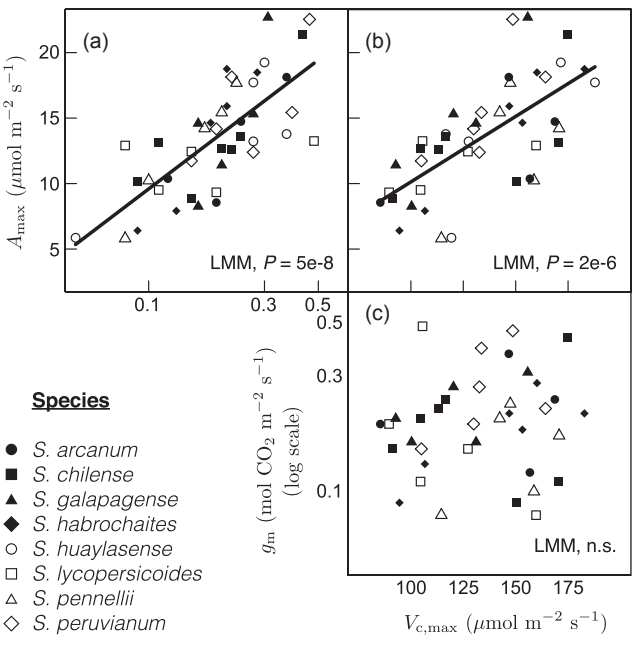

Figure 3. Pairs plot of physiological traits. Each data point is an individual plant from the species indicated by the legend. Maximum photosynthetic rate (A_{max}) is correlated with (a) mesophyll conductance (g_m) and (b) the maximum rate of carboxylation ($V_{c,max}$). (c) g_m is not correlated with $V_{c,max}$. The linear mixed model (LMM) regression lines were fit by restricted maximum likelihood (REML), treating species as a random effect.

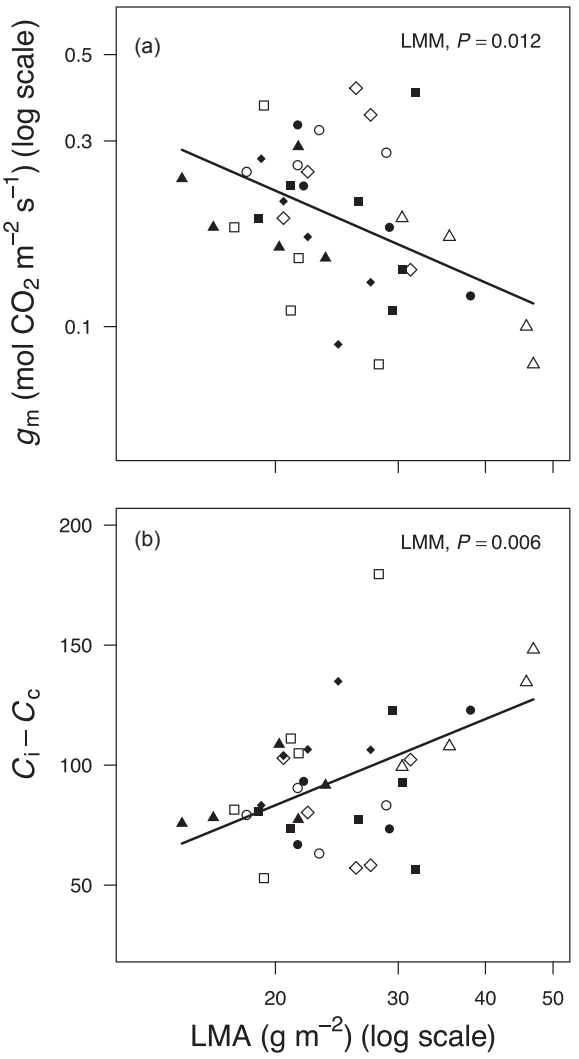

Figure 4. Leaf dry mass per area (LMA) decreases mesophyll conductance (a) and CO_2 drawdown from substomatal cavities to chloroplasts (b). (a) Mesophyll conductance (g_m; *y*-axis) is negatively correlated with LMA (*x*-axis). (b) Decreased g_m in higher LMA leaves lead to a greater CO_2 drawdown ($C_i - C_c$; *y*-axis). Each data point is an individual plant. Following Fig. 3, the different symbols indicate a different species. The linear mixed model (LMM) regression line was fit by restricted maximum likelihood (REML), treating species as a random effect.

T and S_c were positively correlated with each other (Supporting Information Fig. S2b, $F_{1,\ 29.09} = 9.24$, $P = 0.005$), neither was significantly positively correlated with g_m (Table 3, but see below). In fact, we detected no effect of S_c but a significant *negative* effect of *T* according to both *F*-tests and 95% HPD intervals (Table 3). We log-transformed *T* before analysis to reduce the leverage of a few *S. pennellii* individuals with exceptionally thick leaves and low g_m. Using untransformed *T* makes the result more significant ($F_{1,\ 14.68} = 6.16$, $P = 0.026$). Although the primary effect of *T* on g_m was negative, there was some evidence of a positive association between *T* and g_m at very low *T* (approx. $T < 125\ \mu m$). Specifically, a statistical model including quadratic and linear terms for *T* (Supporting Information Table S3) fit better than a model including only the linear term (Supporting Information Table S2) according to Akaike information criteria (AIC). However, the HPD intervals for the quadratic term overlapped 0 (Supporting Information Table S3) and this term was not significant according to *F*-tests ($F_{1,\ 18.23} = 1.49$, $P = 0.237$). Leaf density (*D*) had a stronger effect on g_m than *T* (Table 3). The effect of *D* might be mediated by changes in leaf porosity, as *D* was negatively correlated with the airspace fraction, f_{IAS} ($F_{1,\ 25.49} = 10.20$, $P = 0.004$). However, f_{IAS} itself was not significantly correlated with g_m ($F_{1,\ 29.24} = 1.24$, $P = 0.27$), indicating the changes in the liquid phase resistance were probably also relevant. Finally, the fact that leaf thickness did not affect mesophyll conductance as strongly as leaf density might be in part because SR was positively correlated with thickness (Fig. 5), reducing the airspace resistance in the thickest leaves by increasing the proportion of stomata on the upper surface of the leaf.

DISCUSSION

Efficient fixation of CO_2 is essential for plant growth and fitness. The physics of CO_2 diffusion is therefore a significant factor shaping the evolution of leaf morphology and anatomy. When comparisons are made between very broad functional groups, mesophyll conductance (g_m) to CO_2 appears to be an ecologically important limitation on photosynthetic performance that is determined largely by leaf structure. Greater investment in leaf tissue per area (LMA) limits photosynthetic performance by decreasing g_m, especially in succulent and sclerophyllous leaves (Maxwell, von Caemmerer & Evans, 1997; Hassiotou *et al*. 2010; Tosens *et al*.

log(g_m) ~	REML estimate	95% HPD interval		
Random effects				
Species	0.021	0–0.450		
Residual	0.183	0.108–0.325		
Fixed effects			$F_{df1, df2}$	*P*
(intercept)	3.72	–0.879–7.98	–	–
D	–5.67	–9.57 – –1.56	$F_{1, 27.97} = 7.14$	0.012
log(T)	–0.972	–1.84 – –0.125	$F_{1, 19.72} = 4.58$	0.045
S_c	0.089	–0.050–0.213	$F_{1, 24.87} = 1.48$	0.235

Table 3. Linear mixed model (LMM) table of anatomical traits and mesophyll conductance. Bolded terms are statistically significant fixed effects according to two criteria: they increase model fit (significant *F*-test) and are significantly different from 0 [95% highest posterior density (HPD) interval does not overlap 0]

T, leaf thickness; *D*, bulk leaf density; S_c, area of chloroplasts exposed to internal air space per leaf area.

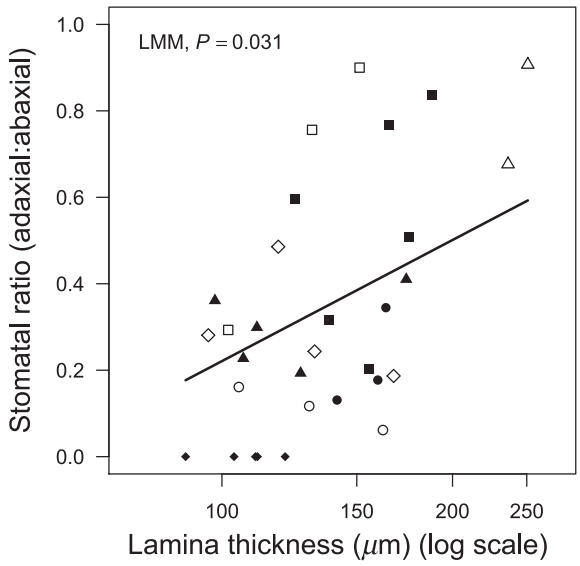

Figure 5. Thicker leaves have more stomata on the adaxial (upper) surface. Stomatal ratio (SR; *y*-axis) is positively correlated with leaf thickness (*T*; *x*-axis). SR is defined as the ratio of the adaxial to the abaxial stomatal density (see Eqn 5). Each data point is an individual plant. Following Fig. 3, the different symbols indicate a different species. The linear mixed model (LMM) regression line was fit by restricted maximum likelihood (REML), treating species as a random effect.

2012b). It is less clear how leaf structure affects CO_2 diffusion in herbaceous leaves with relatively high g_m. Here, one of our aims was to determine whether morphological and anatomical variation within and among closely related herbaceous species (wild tomatoes) affected photosynthetic performance by influencing g_m.

Under the non-stressed conditions of our experiment, we found no significant differentiation between species in physiological traits (A_{max}, $V_{c,max}$, g_s, g_m) even though there were significant interspecific differences in anatomical traits (Table 1) that impact g_m, especially LMA (Fig. 4). The discrepancy between physiological and anatomical traits with respect to interspecific differences probably reflects statistical limitations rather than biology. In contrast to easily measured traits like LMA, physiological traits like stomatal conductance can change rapidly in response to immediate environmental variation, leading to high trait variation among replicate plants. Given the additional variation in physiology caused by environmental fluctuations, the failure to detect strong physiological differentiation between species might reflect limited replication ($\bar{n} = 5.25$) and taxon sampling (eight species). Indeed, a broader analysis of physiological variation across 19 tomato species and close relatives found significant interspecific variation (Muir, unpublished data). In contrast, in this study, within-species variation in g_m and other physiological traits was more important than variation between species. Therefore, our conclusions are based primarily on within species (among plant) variation resulting from physiological plasticity and/or intraspecific genetic variation.

We found that variation in g_m strongly affected photosynthetic performance in wild tomatoes (Fig. 3a, Table 2). Quantitative limitation analysis showed that mesophyll limitations were generally greater than stomatal limitations, but less than biochemical limitations (Table 2). However, this conclusion is sensitive to measurement error in model parameters (Supporting Information Fig. S3). In many plants, high mesophyll conductance is coordinated with high photosynthetic capacity, suggesting that these traits might not be independent (Buckley & Warren 2013). However, we found that $V_{c,max}$ and g_m were uncorrelated (Fig. 3c) and partial correlation indicates that mesophyll limitations are independent of biochemical limitations.

We were also interested in examining the relationship between LMA, a highly variable leaf trait, and g_m. We found that LMA and g_m were negatively correlated (Fig. 4a). This negative association is consistent with other studies that have made comparisons across broad functional groups with a large range of LMA; in contrast, among species with low LMA leaves, g_m has been found to be highly variable (Flexas *et al.* 2008). Our study, however, found that a negative relationship held even among very closely related individuals and species within this group. Combined analysis of our dataset and a previously published, taxonomically broad dataset (Niinemets *et al.* 2009a) suggests that a single slope adequately describes the decline in g_m from low to high LMA ends of the leaf phenotypic spectrum (Supporting Information Fig. S1a). Furthermore, the CO_2 drawdown from substomatal cavities (C_i) to chloroplasts (C_c) increased with LMA (Fig. 4b), indicating that traits associated with greater LMA reduce diffusion through the mesophyll independent of how stomatal conductance affects C_i. Despite the fact that g_m is generally high in wild tomatoes, as in other herbaceous taxa (Flexas *et al.* 2008), it still strongly limited photosynthetic performance independent of both photosynthetic capacity and stomatal conductance.

Nonetheless, the negative relationship between LMA and g_m appears to be mediated by a relatively complex interaction between leaf tissue density, thickness, porosity, stomatal distribution and possibly other unmeasured traits. Unexpectedly, much of the association between LMA and g_m was apparently mediated by airspace rather than liquid phase resistance. Thicker leaves generally had lower g_m despite the fact that thicker leaves had higher S_c (Supporting Information Fig. S2) and higher SR (Fig. 5). The decline in g_m at higher T differs from the pattern seen across genotypes of cultivated tomato (Galmés *et al.* 2013), either because wild tomatoes vary in different ways compared to cultivars or because plants were raised under different experimental conditions. Airspace resistance might have also contributed to the negative effect of leaf density on g_m. Leaf density was partially explained by increased cell packing and therefore decreased leaf porosity (f_{IAS}). However, since f_{IAS} *per se* was not a significant predictor of g_m, other leaf properties, such as mesophyll cell wall thickness, must account for the negative effect of density on g_m. Direct estimates of parameters associated with liquid phase resistance are needed to partition the importance of air and liquid phase resistance (Tosens *et al.*

2012a,b; Tomás *et al*. 2013). We are currently measuring these parameters in a larger survey of photosynthetic and morphological variation in this group (Muir, unpublished data). Regardless, our conclusions are consistent with biophysical models indicating that airspace resistance can set an upper limit on photosynthesis in thick leaves (Parkhurst 1994; Terashima *et al*. 2006; Flexas *et al*. 2008, Niinemets *et al*. 2009a). However, our data also indicate that g_m is usually determined not by a single trait, but rather by complex trait covariation (Tosens *et al*. 2012b, Giuliani *et al*. 2013).

Two results from this study appear to be inconsistent with other theory and empirical data: S_c was not correlated with g_m and two lines of evidence suggested that airspace resistance was appreciable. In contrast, previous studies indicated that S_c is positively correlated with g_m and that liquid phase resistance is more important (e.g. Tosens *et al*. 2012b), especially in thin, amphistomatous leaves. Our results could be interpreted to mean that S_c and liquid phase resistance are not important in these species. For example, S_c does not strongly affect g_m when mesophyll cell walls are thick (Evans *et al*. 2009) and some studies similar to ours that compare g_m among closely related genotypes or species fail to show a significant affect of S_c under well-watered conditions (Galmés *et al*. 2013; Giuliani *et al*. 2013). Alternatively, it is possible that S_c and liquid phase resistance limit photosynthetic performance but, because they did not vary much among our experimental individuals, they do not contribute to explaining the A_{max} variation observed among our study individuals. That is, although these traits might be physiologically important because they are invariant (possibly because of selection to maximize S_c and/or minimize liquid phase resistance in all species), they cannot contribute to evolutionarily significant differences among wild tomatoes.

Although further work will be needed to understand the mechanistic basis for the negative effect of LMA on g_m in wild tomatoes, our data indicate that increased LMA inhibits CO_2 diffusion and photosynthetic performance in these species. Increased LMA is also known to reduce intrinsic biomass growth rate (Poorter *et al*. 2009), indicating that increased LMA has significant costs in terms of both photosynthesis and whole plant growth. How does this cost of LMA impact leaf phenotypic evolution across climatic gradients? One hypothesis is that increased LMA confers stress tolerance and that reduced growth and photosynthetic efficiency are the unavoidable, intrinsic costs associated with this stress tolerance strategy (Niinemets *et al*. 2009b). However, desert annuals, drought deciduous species and herbaceous perennials can respond dynamically to ephemerally optimal conditions, and actually have remarkably high photosynthetic performance under their optimal conditions (Gibson 1998), indicating adaptation to a stressful environment is not intrinsicially associated with slow growth rates. Indeed, in plants that employ a drought escape strategy (rapid growth during high water availability, followed by dieback during drought), tender leaves with efficient CO_2 diffusion might be beneficial, allowing them to take advantage of intermittent periods of water availability. For example, in *Populus balsamifera*, it has been hypothesized that high-latitude genotypes with shorter growing seasons have been selected to have greater photosynthetic performance via increased g_m specifically during their limited optimal growing season (Soolanayakanahally *et al*. 2009). This suggests that physiological plasticity in response to changing environmental conditions might be a critical factor in determining the nature of the relationship between LMA, photosynthetic performance and adaptation to resource limited environments, and therefore, the relationship between leaf phenotypes and climatic variation. The focus of this study was on functional relationships, and our limited taxon sampling did not permit us to investigate these ecological hypotheses here. A broader study of physiological and anatomical variation in this group, currently under way, will enable us to address these questions and better understand the mechanistic basis of adaptation to different environments.

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SUPPORTING INFORMATION

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Figure S1. (a) The decline of mesophyll conductance (g_m, *y*-axis) with increasing leaf mass per area (LMA, *x*-axis) in tomatoes (●) matches the global pattern (○). (b) Similarly, CO_2 drawdown from substomatal cavities to chloroplasts ($C_i - C_c$; *y*-axis) increases with greater LMA in both datasets. Data from wild tomatoes are from this study (same as Fig. 4); data from global analysis are from Niinemets *et al*. (2009a). Shaded polygons are 95% HPD intervals (wild tomatoes) and confidence intervals (global dataset) for the slope of the log(g_m)–log(LMA) relationship. Overlap between the intervals for the two datasets indicates a single, global slope is sufficient to explain the relationship between LMA and mesophyll conductance.

Figure S2. Pairs plot of anatomical traits. Each data point is an individual plant from the species indicated by the legend. (a) There is weak quadratic correlation between density (D) and the surface area of chloroplasts exposed to internal airspace (S_c), (b) a positive correlation between leaf thickness (T) and S_c, and (c) a positive correlation between T and D. The linear mixed model (LMM) regression lines were fit by REML, treating Species as a random effect.

Figure S3. Quantitative limitation analysis is sensitive to underlying parameters. For sensitivity analyses, we re-estimated photosynthetic limitation (l_s = stomatal limitations; l_m = mesophyll limitations; l_b = biochemical limitations) for all nine combinations of three values of Γ^* (40.1, 42.7, and 45.3 μmol CO_2 mol^{-1} air) and three values of $\alpha\beta$ (0.452, 0.472, and 0.495). For both parameters, these values represent the least-squares estimate as well as 2.5% and 97.5% quantiles of the confidence intervals. For each parameter combination, we compared l_s, l_m and l_b using paired *t*-tests. The values used in the main text are highlighted in the grey box. Key to statistical significance: n.s. ($P > 0.05$); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$);

Figure S4. The qualitative affect of LMA on (a) mesophyll conductance (g_m) and (b) CO_2 drawdown from substomatal cavities to chloroplasts ($C_i - C_c$) is not sensitive to estimate of the chloroplastic CO_2 compensation point (Γ^*) or the product of the photosystem partitioning factor and leaf absorptance ($\alpha\beta$). For sensitivity analyses, we re-estimated LMA-g_m and LMA-$C_i - C_c$ slopes ($\hat{\beta}_{LMA}$) for all nine combinations of three values of Γ^* (40.1, 42.7 and 45.3 μmol CO_2 mol^{-1} air) and three values of $\alpha\beta$ (0.452, 0.472 and 0.495). For both parameters, these values represent the least-squares estimate as well as 2.5% and 97.5% quantiles of the confidence intervals. For each parameter combination, we have plotted the REML estimate of the slope (○) and 95% HPD intervals (black lines) using the methods described in the main text. Regardless of parameter combinations, the affect of LMA on g_m and $C_i - C_c$ is significantly different than zero and similar to the results using the least squares derived parameter estimates (grey boxes).

Table S1. Species' means of physiological and morpho-anatomical trait used in this study.

Table S2. LMM results of the statistical model including leaf density (D) and thickness (T) as predictors of mesophyll conductance (g_m).

Table S3. LMM results of the statistical model including leaf density (D) as well as linear and quadratic terms of thickness (T) as predictors of mesophyll conductance (g_m).