The Developmental Basis of Stomatal Density and Flux[1][OPEN]

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Since the first published measurements of stomatal density by Johann Hedwig (1793) and Alexander von Humboldt (1798), the counting and measuring of stomata has been one of the most typical botanical activities, with an important role across fields of plant biology (Willmer and Fricker, 1996). Stomatal density (d) and size (s) are indicators of acclimation and adaptation to contrasting environments, and permit estimation of the theoretical anatomical maximum stomatal conductance (g_{max}; units: mol m^{-2} s^{-1}; Brown and Escombe, 1900; Lawson et al., 1998; Franks and Beerling, 2009; Franks et al., 2009), which represents a first quantitative estimate of the anatomical constraint on maximum stomatal gas exchange. While decades of theory have focused on d and g_{max}, their basis in traits with a transparent relationship to epidermal development has not been expressed. We derived exact mathematical equations for d and g_{max} as functions of stomatal differentiation rate, also known as stomatal index (i, no. of stomata per no. of epidermal cells plus stomata), s, and epidermal cell size (e). These equations unify the quantitative understanding of epidermal development and maximum flux, revealing the developmental bases for d and g_{max} across genotypes or species, and enabling targeting of specific epidermal development traits in plant breeding for productivity.

The genetic and developmental basis for high stomatal density and stomatal conductance is a research priority in plant physiology, agriculture, and paleobiology (Asl et al., 2011; Doheny-Adams et al., 2012; Dow et al., 2014; Franks et al., 2015; Roche, 2015; Wang et al., 2015b). Indeed, a higher g_{max} should benefit species under low CO_2, higher irradiance or nutrient supply, or under selection for high productivity or competition (Franks and Beerling, 2009; Taylor et al., 2012; Jones, 2014). Under the opposite conditions, a lower g_{max} would provide the potential benefits of reduced water loss and/or increased CO_2 gain relative to water loss (Franks and Beerling, 2009; Taylor et al., 2012; Jones, 2014; Franks et al., 2015). Decades of theory have focused on the basis of g_{max} in stomatal anatomy (Fig. 1, A–C). According to a classic formulation of g_{max} (Brown and Escombe, 1900) in a recently updated form,

$$g_{max} = D\left(\frac{d}{v}\right)\frac{a_{max}}{l + 0.5(\pi a_{max})^{0.5}}, \quad (1)$$

where D (m^2 s^{-1}) represents the diffusivity in air of water or CO_2 (which differ by a factor of 1.6); ν the molar volume of air (m^3 mol^{-1}); and d, a_{max}, and l, respectively, the stomatal density (pores m^{-2}), the mean maximum area of a single stomatal pore (m^2), and stomatal pore depth (m; Franks and Beerling, 2009; Franks et al., 2009). The most recent extensions of this equation incorporated basic assumptions about allometries among guard cell dimensions, which have become standard in the stomatal literature (e.g. Franks and Beerling, 2009; Franks et al., 2009; Taylor et al., 2012; Dow et al., 2014; McElwain et al., 2016) and enable the estimation of g_{max} as a function of d and s. In its simplest form:

$$g_{max} = \frac{bmds}{s^{0.5}} = bmds^{0.5}, \quad (2)$$

where $b = \frac{D}{v}$ and $m = \frac{\pi c^2}{j^{0.5}(4hj + \pi c)}$ such that b is a biophysical constant and m a morphological constant based on scaling factors representing the proportionality of stomatal length (L) and width (W), and pore length (p) and depth (l), with $c = p/L$, $j = W/L$, and $h = l/W$ all treated as constant for the estimation of g_{max} (c, h, and j = 0.5 for nongrasses with kidney bean-shaped guard cells, or c = 0.5, h = 0.5, and j = 0.125 for grasses with their dumbbell-shaped guard cells; Franks and Beerling, 2009; McElwain et al., 2016), though these ratios can be allowed to vary for individual species or genotypes when more detailed information is available on stomatal dimensions (Franks and Farquhar, 2007; Franks et al., 2014).

The g_{max} estimated this way strongly predicted the operating stomatal conductance measured with leaf gas

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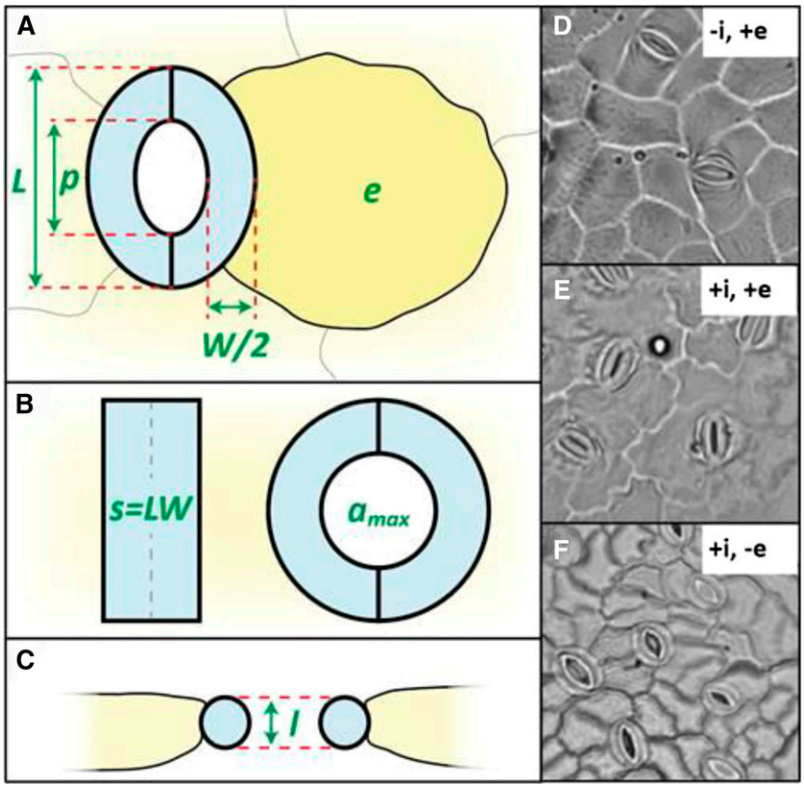

Figure 1. Anatomical variables determining maximum stomatal conductance (g_{max}). A to C, Stomatal dimensions (guard cell length, *L*; stomatal pore length, *p*; guard cell width, *W*; stomatal area, *s*; stomatal maximum pore area, a_{max}; stomatal depth, *l*) and epidermal development traits (epidermal cell area, *e*; stomatal index, *i*). D to F, The influence on stomatal density (*d*) and g_{max} of *e* and *i*: increasing *i* as from D to E would lead to higher *d* and g_{max}; reducing *e* as from E to F would lead to higher *d* and g_{max}. Larger *s* would also lead to lower *d* and g_{max}, though with a much smaller effect. Stomatal images after Beaulieu et al. (2008).

exchange systems (g_{op}) across Arabidopsis (*Arabidopsis thaliana*) genotypes under low CO_2, high humidity, and high red and blue light (Dow et al., 2014). However, across diverse species, the g_{max} values estimated by Equation 2 tend to be much higher than g_{op} (Feild et al., 2011; McElwain et al., 2016) for several reasons. First, for typical leaves transpiring even under the best conditions, the effective area of the stomatal pore (*a*′) is smaller than the anatomical maximum a_{max}, by an amount that varies across species, particularly as the actual pore geometry usually deviates from simplified cylindrical geometry (Franks and Farquhar, 2007). Second, as guard cells close under adverse conditions, *a*′ declines (Fanourakis et al., 2015). Third, there may be a substantial contribution of diffusion resistances in the intercellular airspaces, especially in the case of a partly cutinized substomatal chamber (Roth-Nebelsick, 2007; Feild et al., 2011). Fourth, leaf surface features such as hairs or papillae surrounding the stomata, or encryption of stomata, may affect the diffusion through stomata, and especially will influence the boundary layer, which in addition to stomatal conductance determines overall diffusional conductance and therefore gas exchange (Kenzo et al., 2008; Hassiotou et al., 2009; Maricle et al., 2009). Clearly, much more research is needed to establish models that include all the factors that determine the anatomical influence of stomata on gas exchange rates and to validate these against a wide diversity of plants, yet the anatomical maximum defined as in Equations 1 and 2 is a strong constraint: g_{max} correlates across diverse species with g_{op} and light-saturated photosynthetic rate (McElwain et al., 2016), and scales up, in combination with leaf area allocation, to the determination of ecosystem net primary productivity (Wang et al., 2015a). The anatomical g_{max} is therefore a theoretical value estimating the maximum stomatal diffusion capacity, and like other theoretical physiological variables, such as photosynthetic parameters including the maximum carboxylation rate (V_{cmax}), it cannot be reached in practice, but is useful for generating hypotheses regarding the capacity for stomatal diffusion in various domains, such as comparisons of genotypes or species, functional types, or trends in evolutionary time (Franks and Beerling, 2009; Doheny-Adams et al., 2012; Taylor et al., 2012; McElwain et al., 2016; de Boer et al., 2016).

Despite the well-recognized importance of both *d* and g_{max}, there has been limited understanding of their genetic and developmental basis and their relationships to other epidermal traits. Ever since the seminal work of E.J. Salisbury early last century, *d* has been known to be positively associated with stomatal initiation rate, also known as stomatal index (*i* = no. of stomata per no. of epidermal cells plus stomata; Salisbury, 1927; Wengier and Bergmann, 2012), and negatively with mean epidermal cell area (*e*), as increases in *e* would space stomata apart (Fig. 1, D–F). Studies of plants of different species (Beaulieu et al., 2008; Brodribb et al., 2013) or of given species grown in

different irradiance and vapor pressure deficit treatments (Carins Murphy et al., 2012, 2014) found that d related negatively to e. Further, a negative relationship of d with s within plant canopies or across species has been found numerous times and sometimes attributed to a "general association" or "trade-off" (e.g. Weiss, 1865; Grubb et al., 1975; Tichá, 1982; Hetherington and Woodward, 2003; Sack et al., 2003; Franks and Beerling, 2009; Brodribb et al., 2013; Wang et al., 2015a; de Boer et al., 2016). Yet, while numerous correlational studies within and across species have confirmed these relationships, their formal mathematical basis has remained unclear.

To directly link g_{max}, and thus stomatal flux, to underlying epidermal development traits, we derived new equations for d and g_{max} as functions of e, i, and s, where e and s are projected cell areas (units: m^2).

As defined by Salisbury (1927), i is the number of stomata (n_s) divided by the sum of n_s and the number of epidermal cells (n_e):

$$i = \frac{n_s}{n_s + n_e}. \quad (3)$$

Stomatal density (d) is related to n_s, n_e, s, and e as:

$$d = \frac{n_s}{area} = \frac{n_s}{n_s s + n_e e}, \quad (4)$$

where *area* is that of the whole leaf (units: m^2). Equation 4 can be rearranged as

$$d = \frac{1}{s + e(n_e/n_s)}. \quad (4a)$$

The ratio n_e/n_s can be expressed in terms of i by rearranging Equation 3:

$$\frac{n_e}{n_s} = \frac{1}{i} - 1. \quad (5)$$

Applying Equation 5 to Equation 4a gives

$$d = \frac{1}{s + (i^{-1} - 1)e} = \frac{i}{is + (1 - i)e}. \quad (6)$$

This equation gives d as a function of e, i, and s—traits with a transparent relationship to development, all being related to epidermal cell differentiation and expansion. Equation 6 can be applied to Equation 2 to give g_{max} as a function of e, i, and s:

$$g_{max} = \frac{bmis^{1/2}}{is + (1 - i)e}. \quad (7)$$

These expressions rely on mean values for e, s, and i, so their accuracy may be affected by variation of these variables within leaves, or by variation in sampling methods as there exists no standard measurement protocol (see "Materials and Methods"). We tested the correctness of the derivation of Equation 6 and its

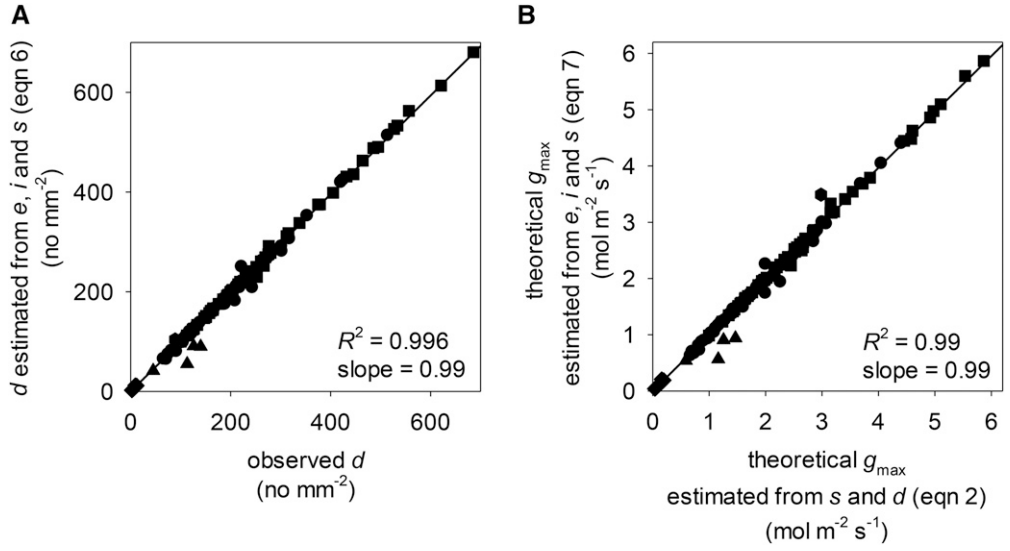

Figure 2. Developmental basis for maximum stomatal flux variables: the estimation of abaxial leaf stomatal density (d; A) and theoretical maximum stomatal conductance (g_{max}; B) as functions of epidermal cell area (e), stomatal index (i), and stomatal area (s). A, Values estimated using Equation 6 plotted against reported values of d; B, values estimated using Equation 7 plotted against values estimated using Equation 2 with inputs of s and d as standard in the current literature. Data were compiled from single or mean values for leaves from published papers for seedlings (circles) and adults (squares) of 54 European woody species; four species of annual herbs of genus *Gomphrena* (Amaranthaceae; triangles); 22 species of genus *Stanhopea*, Orchidaceae (diamonds); and one grass species (*Paspalum dilatatum*, Poaceae; hexagon). The lines are ordinary least squares regressions fitted to the data with fixed zero intercept. The high R^2 values indicate the correctness of the derivation, its applicability to real measurements, and the quality of the measurements.

applicability to real measurements of d, e, i, and s for abaxial leaf surfaces compiled from the published literature for 141 values from 81 species from 28 angiosperm families ("Supplemental Data"). We further checked for quantitative consistency between g_{max} as estimated from e, s, and i (Eq. 7) and the literature standard estimate of g_{max} from d and s (Eq. 2) using the same dataset. In both cases we found extremely tight correspondence (Fig. 2). Considering relationships within individual plant families for which ≥ 6 points were available showed similarly tight correspondence (R^2 = 0.96–1.0, $P < 0.001$, n = 6–30 for Betulaceae, Ericaceae, Fabaceae, Fagaceae, Orchidaceae, Rosaceae, and Sapindaceae; slopes and intercepts did not differ at $P < 0.05$ among families or from 1.0 and 0, respectively).

These equations clarify precise geometric linkages among stomatal flux, anatomy, and development. We propose five examples of potentially powerful applications of these relationships to inform fundamental research across plant development, physiology, paleobiology, and crop science.

(1) *An expansion of available data on stomatal differentiation.* Measurements of i can be technically challenging given the need to resolve all epidermal pavement cells in an image, but Equation 6 can be rearranged to allow estimation of i from measurements of d, e, and s, greatly expanding the data availability for this important developmental trait:

$$i = \frac{e}{\frac{1}{d} - s + e}. \quad (8)$$

(2) *Analysis of the developmental and genetic drivers of d and g_{max} across genotypes of a given species or across phylogenetically diverse species*; i.e. quantifying how much of the variation in d and g_{max} arises due to differences in i, e, or s. Thus, the developmental basis for observed shifts in g_{max} in response to climate, CO_2, and lifeform evolution can be inferred using Equations 6 and 7.

(3) *Clarifying the quantitative role of shifts in genome and cell sizes (i.e. e and s) on g_{max}.* The question of the role and impact of cell size is especially important given the strong developmental plasticity and evolutionary lability of cell size, and its relationship to other traits. For example, within some lineages, epidermal cell size correlates positively with genome size and leaf size and/or negatively with venation density (Beaulieu et al., 2008; Brodribb et al., 2013).

(4) *Resolving the coordinated shifts of stomatal traits in fossils and experimental plants, thereby improving inferences concerning shifts in response to global temperature and atmospheric CO_2.* Previous studies of adaptation and acclimation in response to CO_2 have tended to quantify d and/or i (e.g. Beerling et al., 1998; Royer, 2001) and/or more rarely s and e (e.g. Ogaya et al., 2011; Haworth et al., 2014), and assumed that a shift in any one of these traits was an important marker of adaptation. Equations 6 and 7 allow estimation of the quantitative dependency of shifts in d and g_{max} on other variables.

(5) *Prediction of how each trait should be adjusted, through breeding or genetic manipulation, to optimize productivity through changes in g_{max}.* Equations 6 and 7 clarify the separate roles of e, i, and s in determining higher g_{max}. Given the increasing resolution of the genetic basis for these traits in model species (e.g. Ferris et al., 2002; Delgado et al., 2011), these traits can be made specific targets for breeding for higher g_{max} and thereby for productivity. Other traits would also need to be targeted (e.g. hydraulic and photosynthetic traits) to enable higher productivity above and beyond the potential cost of constructing, maintaining, and operating additional stomatal apparatus (Assmann and Zeiger, 1987).

By linking leaf epidermal anatomy and development with physiological flux, these equations allow scaling from the differentiation and expansion of epidermal cells and stomata to plant productivity. Given ongoing improvement of models for the influence of the anatomy and dynamic behavior of stomata and of internal and external leaf tissues on gas exchange, consideration of these important traits in terms of their development will have potential applications across the widest range of fields in plant biology and earth system science.

MATERIALS AND METHODS

To test these equations, data were compiled for stomatal traits from the literature via searches using Google Scholar, Web of Science, and references from articles. Four papers were found containing data for e, i, s, and d for abaxial leaf surfaces for European woody species (greenhouse-grown seedlings and field-sampled adults; 54 species; Cornelissen et al., 2003); for annual herbs sampled in the field and in a garden (four species of genus *Gomphrena*, Amaranthaceae; Fank-de-Carvalho et al., 2010); for 22 species of genus *Stanhopea*, Orchidaceae (Ferry et al., 1997); and for one grass grown in a growth chamber (*Paspalum dilatatum*, Poaceae; Soares et al., 2008). Data compiled from the papers were either means for replicate leaves of given species and/or life stages, or represented individual leaves or plants (Supplemental Data S1). We focused on the abaxial leaf surface, given the much greater availability of data; in principle the equations would apply equally to the adaxial surface. One outlier was removed from the dataset, that for leaves of adult *Calluna vulgaris* (Cornelissen et al., 2003) for which calculations showed that only 67% of the leaf surface was accounted for by stomata and epidermal cells; including this outlier did not substantially affect the relationships. Notably, studies diverged in methods for visualization of leaf surfaces (e.g. acetate or nail polish impressions visualized with light microscopy or scanning electron microscopy of leaf surfaces) and measurement of stomatal traits (i.e. using a microscope graticule or image analysis software), and in replication of measurements within and across leaves for given species. The validation of the new equations despite such heterogeneity further demonstrates the robustness of the equations. Tests of relationships among variables across families were performed by comparing regression lines in slopes and intercepts (using SMATR; Warton et al., 2006).

Supplemental Data

The following supplemental materials are available.

Supplemental Data S1. Published measurements of stomatal density (*d*), epidermal cell area (*e*), stomatal index (*i*), and stomatal size (*s*); estimated values of *d* based on *e*, *i*, and *s* (Eq. 6); and maximum theoretical stomatal conductance (g_{max}) based on *s* and *d* (Eq. 2) and based on *e*, *i*, and *s* (Eq. 7).

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