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Research

The determination of leaf size on the basis of developmental traits

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Summary

- Mature leaf area (LA) is a showcase of diversity – varying enormously within and across species, and associated with the productivity and distribution of plants and ecosystems. Yet, it remains unclear how developmental processes determine variation in LA.
- We introduce a mathematical framework pinpointing the origin of variation in LA by quantifying six epidermal 'developmental traits': initial mean cell size and number (approximating values within the leaf primordium), and the maximum relative rates and durations of cell proliferation and expansion until leaf maturity. We analyzed a novel database of developmental trajectories of LA and epidermal anatomy, representing 12 eudicotyledonous species and 52 Arabidopsis experiments.
- Within and across species, mean primordium cell number and maximum relative cell proliferation rate were the strongest developmental determinants of LA. Trade-offs between developmental traits, consistent with evolutionary and metabolic scaling theory, strongly constrain LA variation. These include trade-offs between primordium cell number vs cell proliferation, primordium mean cell size vs cell expansion, and the durations vs maximum relative rates of cell proliferation and expansion. Mutant and wild-type comparisons showed these trade-offs have a genetic basis in Arabidopsis.
- Analyses of developmental traits underlying LA and its diversification highlight mechanisms for leaf evolution, and opportunities for breeding trait shifts.

Introduction

The sizes of organisms and their organs span many orders of magnitude (Peters, 1983; Niklas, 1994; Wright *et al.*, 2017) and are often constrained by developmental, evolutionary and ecological trade-offs (Krist, 2011; Self *et al.*, 2018; Church *et al.*, 2019). The leaf, the plant's metabolic engine, varies in size across species over 150 000-fold, and the mature individual leaf area (LA) is associated globally with climate and ecology (Givnish, 1987; Wright *et al.*, 2017; Baird *et al.*, 2021), and with the productivity and distribution of species and ecosystems (Wright *et al.*, 2017; Li *et al.*, 2020; Smith *et al.*, 2023). The aim of this study was to clarify the proximate developmental basis of LA, which has remained incompletely resolved within and across diverse species (Avery, 1933; Tsukaya, 2005; Gonzalez *et al.*, 2012; Grubb, 2020).

A previous pioneering meta-analysis of leaf growth highlighted the multiple potential developmental drivers of variation in LA (Gazquez & Beemster, 2017). In eudicotyledons, leaf development begins with the emergence of the primordium from the shoot apical meristem, followed by overlapping periods of cell proliferation, expansion, differentiation and maturation, in distinct spatial zones (Fig. 1a; Granier & Tardieu, 2009; Gonzalez *et al.*, 2012; Kalve *et al.*, 2014; Hisanaga *et al.*, 2015), such that, overall, leaf expansion increases exponentially, then slows and ceases (Granier & Tardieu, 2009). In the last century, a rich literature based on correlation analyses has reported many associations of LA with developmental factors within and across species, from gene activities (Ferjani *et al.*, 2007; Tisne *et al.*, 2008; Gonzalez *et al.*, 2010) to the rates and durations of cell proliferation or of cell or leaf expansion (Moles & Westoby, 2000; Beemster *et al.*, 2005). Yet, these studies have often reported contrasting results (reviewed with references in Table 1). While most studies, but not all, reported that larger LA arose due to greater numbers of leaf epidermal cells rather than to larger cell sizes (reviewed in Table 1, row 1), LA variation within and among species was attributed to various developmental drivers, including leaf primordium cell numbers or sizes, or rates and/or durations of cell proliferation or expansion (Table 1, row 2). We consider such factors as 'developmental traits' that underlie mature LA, and which are in turn dependent on genes and influenced by environmental variation; in general, leaves develop a smaller LA under lower water supply and higher irradiance, and across species, smaller leaves are on average associated with

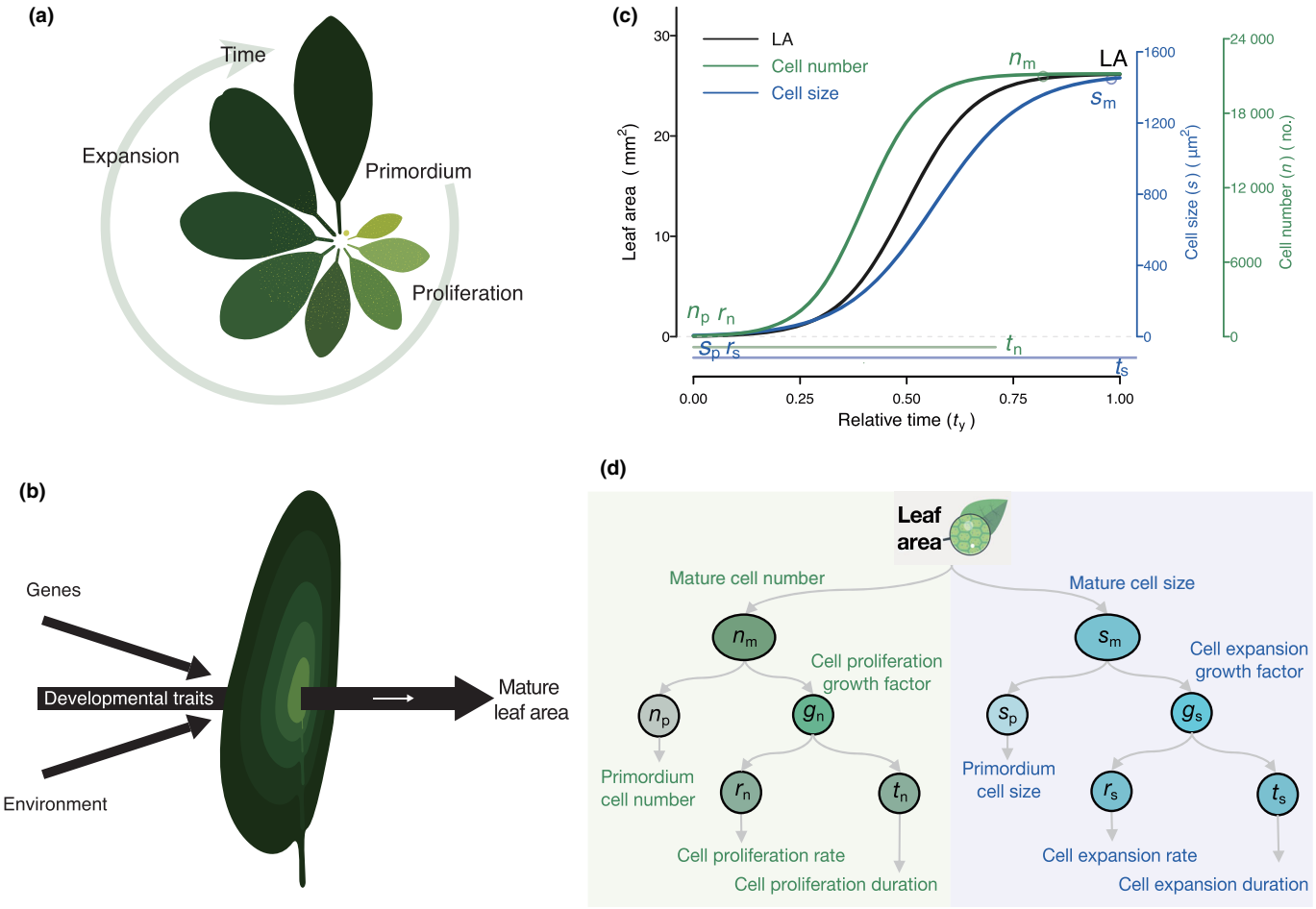

Fig. 1 Mature leaf area (LA) on the basis of 'developmental traits'. (a) In eudicots, the leaf originates from the primordium on the shoot apical meristem and undergoes overlapping phases of cell proliferation, expansion, differentiation, and maturation. (b) Mature LA depends on developmental traits based on genes and influenced by environmental variation, including mean primordium cell number and size (n_p and s_p, respectively), and maximum relative rates of cell proliferation and expansion (r_n and r_s) and their durations (t_n and t_s). (c) Developing LA is the product of leaf epidermal cell number and mean size; all increase as three-parameter sigmoidal functions of time, achieving maximum values of, respectively, LA, cell number (n_m), and mean cell size (s_m). Thus, LA is determined by three traits for the cell number function and three for the cell size function, and thus, by six developmental traits, the epidermal cell number and mean size in the primordium (n_p and s_p; y-intercepts), and the subsequent maximum relative rates of cell proliferation and expansion, which occur during initial growth (r_n and r_s) and their durations (t_n and t_s; denoted by horizontal lines). (d) Schematic of the hierarchical determination and causal analysis of mature LA based developmental traits: LA is the product of n_m and s_m, which are the products, respectively, of n_p and s_p and their proportional growth factors from primordium to mature leaf (g_n and g_s), which in turn are functions of r_n and t_n, and r_s and t_s, respectively (Box 1).

adaptation to cold, dry and sunny environments (Wright *et al.*, 2017) (Fig. 1b).

Additionally, previous studies have hypothesized contrasting trade-offs among developmental factors that would constrain LA within and across species. Thus, trade-offs have been proposed between primordium mean cell size or number and their growth rates, and between the rate and duration of cell proliferation, and between the rate and duration of cell expansion (Table 1, row 3). These putative trade-offs might be considered as versions at the leaf developmental scale of trade-offs previously recognized to limit the growth of whole organisms and populations, predictable from metabolic scaling and evolutionary theory (Peters, 1983; Niklas, 1994; West *et al.*, 1997). Thus, trade-offs between primordium cell size or number and their growth rates would be cases of *growth rate* vs *body size* trade-offs (Brown *et al.*, 2004; Savage *et al.*, 2007), analogous to the common finding that a larger organism or population grows more slowly. Trade-offs between the rates and duration of cell proliferation or those of cell expansion would be cases of *growth rate* vs *duration* trade-offs (Gillooly *et al.*, 2002; Wright *et al.*, 2004; Kempes *et al.*, 2012), analogous to the general finding that a faster-growing organism or population grows for a shorter time. By contrast, other studies have proposed that LA is constrained by *compensation* trade-offs between the determinants of final cell size vs cell number (Tsukaya, 2003; Ferjani *et al.*, 2007; Horiguchi & Tsukaya, 2011; Hisanaga *et al.*, 2015; Table 1, row 4). Yet, evaluation of these hypotheses yielded mixed results when based on testing for negative associations between developmental factors (Table 1, rows 3 and 4), potentially due to contrasting derivations of developmental traits and/or the often weak inference available from

Table 1 Alternative conclusions and incomplete evidence based on correlation analyses in the published literature for the developmental basis of mature leaf area (LA), and resolution by causal analyses of the novel database in this study.

Types of hypotheses for the determination of leaf size by developmental traits or for trade-offs between developmental traits	Mixed evidence based on correlational analyses	Resolution in this study
1: Determination of mature leaf size by leaf epidermal cell number vs mean cell size.	Mature leaf size variation has been attributed to differences in: (1a) cell numbers (Granier *et al*., 2000; Cookson *et al*., 2005; Gonzalez *et al*., 2010; Gazquez & Beemster, 2017). (1b) cell size (Tisne *et al*., 2008; Perez-Perez *et al*., 2011; Zhang *et al*., 2019).	On average, cell number strongly determines final leaf area (LA) within Arabidopsis and across species, and cell size is also important within Arabidopsis.
2: Determination of mature leaf size by developmental traits that determine leaf size via cell number and cell size.	Mature leaf size variation has been attributed to: (2a) shoot apical meristem or primordium size or cell numbers (Korner *et al*., 1989; Higuchi *et al*., 2004; Schnablová *et al*., 2017). (2b) cell proliferation rate (Eloy *et al*., 2011). (2c) duration of cell proliferation (Autran *et al*., 2002). (2d) duration of cell expansion (Gazquez & Beemster, 2017).	On average, LA depends most strongly on maximum relative cell proliferation rate across species, and on primordium cell number within Arabidopsis.
3: Trade-offs between developmental traits that determine leaf size via cell size, or via cell number, constraining final leaf size.	Leaf size development has been variously proposed to be constrained by trade-offs between: (3a) primordium cell number and cell proliferation (Cockcroft *et al*., 2000; Autran *et al*., 2002; Horiguchi *et al*., 2005). (3b) primordium cell size and cell size growth (Lee *et al*., 2009). (3c) rate and duration of cell proliferation (Fox *et al*., 2018). (3d) rate and duration of cell expansion (Cookson *et al*., 2005).	LA development is strongly constrained by multiple trade-offs that result in a constrained LA: between the primordium cell size and cell size growth, primordium cell number and cell proliferation, and between the maximum rates and durations of cell proliferation and expansion.
4: 'Cell size–number compensation' trade-offs between developmental traits that determine leaf size via cell number vs those that determine leaf size via cell size, constraining final leaf size.	Leaf size development has been proposed to be constrained by a 'compensation' trade-off between: (4a) mature cell size and mature cell number (Tsukaya, 2003; Horiguchi *et al*., 2005; Horiguchi & Tsukaya, 2011; Clauw *et al*., 2016). (4b) cell proliferation rate and cell expansion rate (Hepworth & Lenhard, 2014).	No support for any general cell size–number compensation trade-offs that would result in a constrained LA.
5: Genetic and environmental factors may differently influence the developmental traits underlying leaf size.	Leaf size development has been proposed to be influenced by (5a) genetic factors, mainly via cell proliferation (Li *et al*., 2008; Guo & Simmons, 2011). (5b) environmental factors, via both cell proliferation and cell expansion (Aguirrezabal *et al*., 2006; Cookson & Granier, 2006; Pantin *et al*., 2011).	Regulation of LA by genetic and environmental factors are both mainly determined by cell numbers: single gene mutations on average driving shifts in LA mainly by influencing primordium cell number, and environmental conditions mainly by influencing primordium cell number and size, and maximum relative cell proliferation rate.

correlation analyses when variables covary (John *et al*., 2017). Additionally, previous studies have proposed that genetic and environmental factors would differently influence the developmental traits underlying LA (Table 1, row 5), with genetic factors primarily affecting cell proliferation, and environmental factors affecting both cell proliferation and cell expansion.

Previous studies have considered the relative influences of developmental drivers of plant organ growth, including a pioneering meta-analysis that systematically applied correlational analyses (Gazquez & Beemster, 2017). We extended that approach while overcoming conceptual and analytical challenges to establishing causality, and using the most comprehensive database to date. We present a novel analytical schema, the 'number-size-rate-time' (NSRT) framework, defining 'developmental traits' and their relationships to disentangle how they constrain LA within and across eudicotyledonous species (Box 1; Fig. 1b–d; Table 2). This framework extends to leaf development an approach frequently applied in functional trait-based ecology (Garnier *et al*., 2015), namely, the analysis of higher level traits based on the contributions of underlying traits, such as the

Box 1. A developmental trait framework for the determination of mature leaf area (LA)

In the 'number-size-rate-time' (NSRT) framework presented herein, mature LA is determined exactly by 'developmental traits': LA is the product of mature leaf epidermal cell number (n_m) and size (s_m), themselves the products of, respectively, the initial cell number and mean cell size (approximating their values within leaf primordium) (n_p and s_p) and their proportional increases during growth (g_n and g_s), which in turn are functions of the maximum relative growth rates and the durations of growth in cell number (proliferation) and size (expansion) (respectively, r_n and t_n, and r_s and t_s; Fig. 1c; Table 2; see 'Derivation of the leaf developmental trait framework' in the Materials and Methods section). We focused on the role of epidermal cells, which are space-filling (Marcotrigiano, 2010), though the analysis is robust to alternative theories for determinants of leaf expansion, for example the leaf venation directing the development of the epidermis (Van Volkenburgh, 1999). Thus,

$$\mathrm{LA} = n_m s_m = (n_p g_n) \cdot (s_p g_s) \qquad \text{Eqn 1}$$

Assuming generalized sigmoidal growth trajectories for both cell number and mean cell size (Massonnet *et al*., 2010) (i.e. $y(t) = y_m/(1 + \exp(-r_y(t - t_{50y})))$, where y = size or number, y_m = mature value of y, r_y = maximum relative growth rate of y, and t_{50y} = the time at which $y = 50\%$ of y_m) and defining the duration of growth (t_y) as the time at which $y = 99\%$ of y_m, it follows (Eqns 4–8) that the proportional increase (growth) of y (g_y) between the primordium and mature leaf is

$$g_y \equiv \frac{y_m}{y_p} \approx \frac{1 + 0.01 \cdot \exp(r_y t_y)}{1.01} \qquad \text{Eqn 2}$$

Applying Eqn 2 (with $g_y = g_n$ and g_s, $r_y = r_n$ and r_s, and $t_y = t_n$ and t_s), gives

$$\mathrm{LA} \equiv L_m = \left[n_p \cdot \frac{1 + 0.01\exp(r_n t_n)}{1.01}\right] \cdot \left[s_p \cdot \frac{1 + 0.01\exp(r_s t_s)}{1.01}\right] = [n_p g_n] \cdot [s_p g_s] \qquad \text{Eqn 3}$$

This approach enables the extraction of developmental traits that determine LA in a three-tier hierarchy whereby LA is determined by n_m and s_m; n_m and s_m are determined, respectively, by n_p and g_n, and s_p and g_s; and g_n and g_s are determined, respectively, by r_n and t_n, and r_s and t_s (Fig. 1d). This approach also enables a causal analysis of LA, beyond simple tests of the correlation of LA with developmental factors. Causal analysis is critical, because when factors covary, an unimportant or even negative causal driver may be positively correlated with LA (John *et al*., 2017). Thus, in the NSRT framework, Eqn 3 enables differences in LA between any two leaves to be partitioned into the exact causal roles of the six traits n_p, s_p, r_n, t_n, r_s, and t_s (Fig. 1d; see 'Causal partitioning analysis of LA with respect to developmental traits' in the Materials and Methods section).

The 'intrinsic' influences of the six developmental traits on LA, all else being equal, are shown with a sensitivity analysis of Eqn 3, that is, quantifying how LA changes when a given trait value is increased by a small percentage. Thus, r_n and t_n have strongest intrinsic impacts, followed by r_s and t_s, and then n_p and s_p (Fig. 2c). However, in any comparison within or across species, the realized causal importance of developmental traits on LA depends not only on intrinsic influences, but on the relative variation of all the traits, and would be strongly affected by trade-offs among traits (John *et al*., 2017).

determination of leaf mass per area by anatomical variables (Niinemets, 1999; John *et al*., 2017), or of ecosystem productivity by physiological and structural traits (He *et al*., 2023).

The overall aim of our developmental trait analysis was to build a bridge for ecologists and physiologists to developmental biology (and vice versa) with approaches that synergize these fields, to clarify the evolution of leaf size diversity. We applied the NRST framework to identify important constraints on the development of leaf size in a unique database, including all published experiments, to our knowledge, on leaf anatomical developmental trajectories in eudicotyledonous plants, including 12 diverse species, with 52 experiments on mutant and wild-type (WT) genotypes of *Arabidopsis thaliana*, and further, including drought stress experiments on three species.

Materials and Methods

Compilation of leaf growth datasets

We compiled time-series data extracted from published studies that quantified the growth trajectories of leaves, and leaf epidermal cell numbers and sizes. We searched for studies using the keywords 'leaf development', 'cell proliferation', 'cell expansion', 'cell division', or 'development' combined with 'leaf size', 'leaf area', and 'leaf expansion', using the Web of Science and Google Scholar search engines, and searching papers that cited or were cited by these studies. The final database includes data from 36 studies for 12 diverse eudicotyledons species from 10 families (*Arabidopsis thaliana* (L.) Heynh., *Cucumis sativus* L., *Helianthus annuus* L., *Lonicera maackii* (Rupr.) Maxim., *Phaseolus vulgaris* L., *Pisum sativum* L., *Prunus yedoensis* Matsum., *Quercus ilex* L., *Solanum lycopersicum* L., *Syringa oblata* Lindl., *Trifolium repens* L., *Xanthium italicum* Moretti.), and 52 mutant and WT genotypes of Arabidopsis (*Arabidopsis thaliana*). The dataset for Arabidopsis was analyzed as three datasets: *WT* ($n = 30$), *mutants* ($n = 22$), and their combination, *all experiments* ($n = 52$), which allowed 24 paired comparisons of *A. thaliana* mutants with their background WT. We also compiled datasets to quantify the effects of environmental influences based on paired comparisons: five studies of droughted plants compared with well-watered plants of three species: one study of tomato (*S. lycopersicum*), and two of each for *Arabidopsis* and sunflower (*H. annuus*). Notably, relying on a compilation of data from disparate published studies can generate uncertainty, and we assumed that at worst, there would have been only minor errors in the published work. Indeed, such compiled data approaches have been powerful in analyzing general patterns in ecophysiology (e.g., Wright *et al*., 2004; Ochoa *et al*., 2024), and we here extend that approach to leaf development within and across diverse species. We believe that by comprehensively including in our analysis all the published time series for leaf size, cell size and cell number developmental trajectories, our broad conclusions from all these data are thus well supported.

For each time-series plot, we re-captured data for epidermal cell number, mean epidermal cell size, and LA (using Web Plot Digitizer; Rohatgi, 2019). We focused on the growth curves of the upper epidermis, with the exception of *P. sativum*, for which data were only available for the lower epidermis. When data were not provided for the epidermal cell number as a function of time,

Table 2 Leaf size and its underlying developmental traits.

	Symbol	Description	Units
Mature leaf	LA	Mature leaf area	mm^2
	n_m	Cell number in the epidermis of the mature leaf	no.
	s_m	Mean cell size in the epidermis of the mature leaf	μm^2
Determinants of mature leaf cell number	n_p	Epidermal cell number in the primordium, approximated as initial cell number at time = 0	no.
	r_n	Maximum relative growth rate of leaf epidermal cell number	d^{-1}
	t_n	Duration of cell proliferation	d
	t_{50n}	Time for cell number to increase to half of its mature value	d
	g_n	Cell proliferation factor, that is, the proportional increase in epidermal cell number from primordium to mature leaf	
Determinants of mature leaf mean cell size	s_p	Mean epidermal cell size in the leaf primordium, approximated as initial cell number at time = 0	μm^2
	r_s	Maximum relative growth rate of leaf epidermal cell size	d^{-1}
	t_s	Duration of cell expansion	d
	t_{50s}	Time for cell size to increase to half of its mature value	d
	g_s	Cell expansion factor, that is, the proportional increase in mean epidermal cell size from primordium to mature leaf	

we estimated these by dividing the LA by the average cell area at each time interval. When cell sizes were provided from multiple leaf locations, these were averaged for the time point. When growth curves were plotted without measured point data, we extracted the fitted lines in the paper by capturing points from the line (42/266 of the compiled datasets, i.e. 16% of studies), using the default settings of the software.

We addressed uncertainty arising in the published data for leaf size development due in particular to two issues. First, studies varied slightly in the designation of the initial leaf and its time of formation; ideally that designation would correspond to the primordium at the time it was first distinct from the rest of the shoot apical meristem. Studies applied typical designations: for Arabidopsis experiments, these were after seed stratification, after sowing, after germination, or when both cotyledons were visible; for the diverse eudicot species, these were after sowing, or after primordium emergence/initiation or after bud break (compiled in Supporting Information Datasets S1, S2). Notably, the first day of measurements was 5.4 ± 0.37 (mean 95% ± confidence interval) days later for Arabidopsis experiments and 5.2 ± 1.0 for the eudicot species (Datasets S1, S2). We conducted two analyses to test the impact of uncertainty in the true time zero on inferred trade-offs between developmental traits (see Methods S1). Second, studies varied in the time points at which leaves were measured during growth; in some cases, cell number and cell size were assessed at different specific time points. In those cases, we standardized the timepoints by using the mean of the time points, and interpolated Y-values for cell number and cell size at that mean timepoint. We also conducted an analysis to test for the uncertainty in the estimation of developmental traits due to timepoint selection (see Methods S2).

In quantifying epidermal cell numbers and sizes, we did not consider guard cells separately from epidermal pavement cells. The majority of compiled studies did not contain data for stomatal development or differentiation, and, given that stomata differentiate fully at a late stage of leaf development (Lau & Bergmann, 2012), we assumed they could reasonably be omitted in the calculation of LA developmental traits. Indeed, given that we considered the final leaf size as the sum of adaxial epidermal cell areas, only small errors would arise in the amphistomatous species due to the lack of distinction of the areas of stomatal guard cells from those of epidermal pavement cells. While guard cells do tend to differ from epidermal pavement cells in their area, by 4.2% on average (range: 0.3% to 26% across 101 eudicotyledonous species; Beaulieu *et al.*, 2008), even in amphistomatous species, the guard cells make up a small proportion of the cells on the adaxial surface, for example *c.* 13% on average for 111 Arabidopsis genotypes (Perez-Perez *et al.*, 2011) and *c.* 5% on average for 116 species of Proteaceae (Jordan *et al.*, 2020). We conducted an analysis to test for the uncertainty in the estimation of developmental traits associated with focusing on epidermal pavement cells not including guard cells (see Methods S3).

Derivation of the 'number, size, rate, time (NSRT)' leaf development framework

We derived a framework for considering leaf size as an exact function of developmental traits (Box 1; Fig. 2a,c). For each leaf development time series, that is, for cell number (n), or cell size (s) vs time (t), we fitted a sigmoid function previously used extensively in growth analyses (Cookson *et al.*, 2005; Aguirrezabal *et al.*, 2006):

$$y(t) = \frac{y_m}{1 + \exp\left(-r_y\left(t - t_{50y}\right)\right)} \quad \text{Eqn 4}$$

where $y(t)$ is the instantaneous value at time t of either epidermal cell number $n(t)$, or cell size $s(t)$; y_m is the mature value of y (n_m for cell number, or s_m for cell size); t_{50y} is the time at which y reaches 50% of its maximum value (t_{50n} for cell number, or t_{50s} for cell size); and r_y is the maximum relative growth rate ($RGR = [1/y] \cdot [dy/dt]$) for y = cell number (r_n), or cell size (r_s). We fitted Eqn 4 to the data using R (nlme, minpack.lm) (Elzhov *et al.*, 2010) and extracted the maximum final value (y_m), maximum relative growth rate (r_y), and the midpoint (t_{50y}) from the fitted models.

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An innovation of the NSRT framework is the ability to probe the causality of the fitted line parameters n_m and s_m, that is the final values of cell number and cell size and the final value of LA. Our derivation allows these parameters to be considered as functions of their underlying causal parameters: namely, primordial cell number (n_p) and size (s_p), and the maximum relative rates and durations of cell proliferation (r_n and t_n) and expansion (r_s and t_s). Note that n_p and s_p are, respectively, defined as cell number and mean cell size in the primordium, that is approximated as initial cell number at time = 0. The conceptual framework enables the derivation of Eqn 3 that defines mature leaf size as an explicit function of six

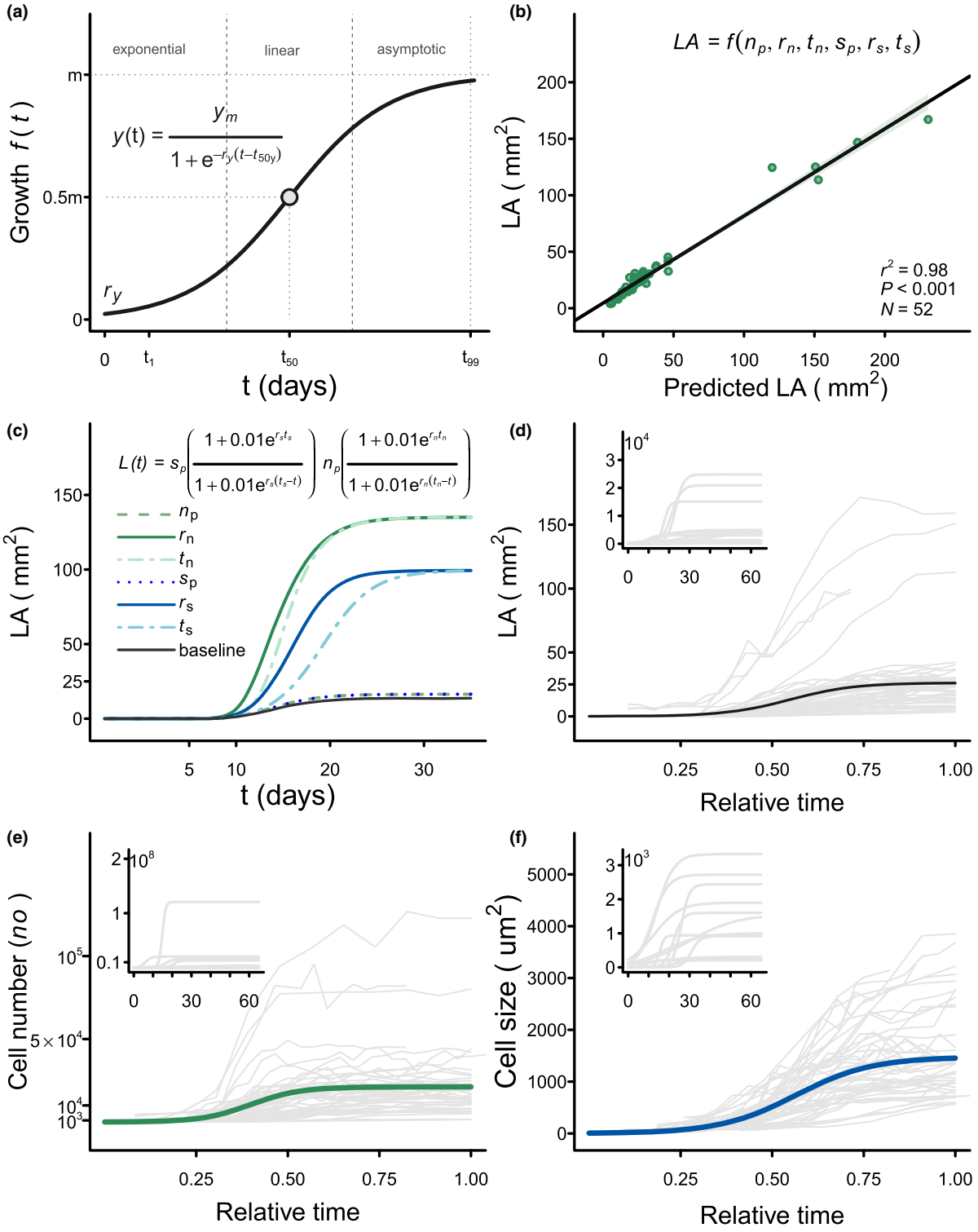

Fig. 2 Sigmoidal growth model applied to the development of leaf area (LA), and its intrinsic sensitivity to developmental traits. (a) Fitting the sigmoidal model to time-series data to extract key cellular traits within and across species. Cell number or size or whole LA was considered as a sigmoidal trajectory with exponential, linear, and asymptotic phases; y_m is the maximum value or asymptote; t_{50y} is the timepoint corresponding to half y_m; r_y is the maximum relative growth rate corresponding to initial growth; and t_y is the duration of growth, that is, the time to achieve 99% of y_m. (b) Observed LA was predicted well by applying Eqn 3 to the extracted six developmental traits of LA for data of 52 Arabidopsis experiments. (c) Intrinsic sensitivity of Eqn 3 describing LA as a function of developmental traits; as a baseline, we considered LA as a function of median values for developmental traits from 52 experiments of Arabidopsis (black solid line) and then tested the effect on the trajectory of LA of increasing by 20% the median value of each trait individually, with all others held at their median values. (d–f) LA, epidermal cell number, and epidermal cell size growth curves, in gray, for 52 experiments in Arabidopsis, with the fitted function calculated from median trait values as the solid line (and fitted functions for 12 diverse eudicotyledonous species, plotted against absolute development time, insets, for clarity, given diversity across species in leaf development time). n_p is the mean cell size in the epidermis of the mature leaf; r_n is the mean cell size in the epidermis of the mature leaf; t_n is the duration of cell proliferation; s_p is the mean epidermal cell size in the leaf primordium, approximated as initial cell number at time = 0; r_s is the maximum relative growth rate of leaf epidermal cell size; and t_s is the duration of cell expansion.

developmental traits, which can be extracted from the time series of the increases of cell numbers and sizes with time. Those traits were specifically defined to be, in principle, independent; that is, we used initial cell numbers and sizes, maximum cell size and number relative growth rates and growth times. In principle, each of those traits could be shifted independently – and we conducted an 'intrinsic' sensitivity analysis to show how this would influence LA (Fig. 2c). Our causal partitioning of the roles of the traits in determining leaf size differences across study sets (Arabidopsis genotypes or eudicot species) considers how the true variation in traits and final leaf sizes mathematically contributes to variation in leaf size. With these objectives, we derived LA as a function of these traits.

We determined the durations of cell proliferation and expansion (t_n and t_s, respectively) based on the fitted values of their respective halftimes (t_{50n} and t_{50s}), according to Eqn 4, defining duration as the time required for cell number or size to reach 99% of its mature value; that is, t_y is defined such that $y(t_y) \equiv 0.99 \times y_m$, so that

$$0.99y_m = \frac{y_m}{1+\exp\left(-r_y\left(t_y - t_{50y}\right)\right)} \rightarrow t_y = t_{50y} - \frac{\ln Z}{r_y} \qquad \text{Eqn 5}$$

where $Z \equiv 1/0.99 - 1 \approx 0.01$. We defined leaf primordium cell number (n_p) and size (s_p) as their respective values at $t = 0$ in Eqn 4, thus:

$$y_p = \frac{y_m}{1+\exp\left(r_y t_{50y}\right)} \qquad \text{Eqn 6}$$

The sigmoidal function can be re-expressed in terms of leaf primordium cell size and number values and total growth durations (y_p and t_y) rather than final mature leaf cell size and number values and halftimes (y_m and t_{50y}) by solving Eqn 5 for t_{50y} and applying the result to Eqns 4 and 6, to give

$$y(t) = y_p \cdot \frac{1+0.01\exp\left(r_y t_y\right)}{1+0.01\exp\left(-r_y\left(t - t_y\right)\right)} \qquad \text{Eqn 7}$$

Thus, the area of a growing leaf can be expressed in terms of NSRT traits:

$$L(t) = \left[n_p \cdot \frac{1+0.01\exp\left(r_n t_n\right)}{1+0.01\exp\left(-r_n(t-t_n)\right)}\right] \cdot \left[s_p \cdot \frac{1+0.01\exp\left(r_s t_s\right)}{1+0.01\exp\left(-r_s(t-t_s)\right)}\right] \qquad \text{Eqn 8}$$

In words, Eqn 8 says that LA at any given time t depends on primordium cell number (n_p) and size (s_p), and on the increase in number and size, which depend, respectively, on the maximum relative rates (r_n and r_s) and durations (t_n and t_s) of increase in cell number and size, respectively. Mature LA ($LA = L_m$) is found by replacing t with the total durations of cell proliferation (t_n) and expansion (t_s) in the denominators of the first and second sets of square brackets in Eqn 8, respectively, to give

$$LA \equiv \left[n_p \cdot \frac{1+0.01\exp\left(r_n t_n\right)}{1.01}\right] \cdot \left[s_p \cdot \frac{1+0.01\exp\left(r_s t_s\right)}{1.01}\right] = \left[n_p g_n\right] \cdot \left[s_p g_s\right] \qquad \text{Eqn 9}$$

where $g_n \equiv (1+0.01\exp(r_n t_n))/1.01$ and $g_s \equiv (1+0.01\exp(r_s t_s))/1.01$ are proportional *growth factors* for cell number and size, respectively. Importantly, Eqn 9 represents the causal determination of mature LA by primordium cell number and size and the maximum relative rates and durations of cell proliferation and expansion.

Estimation of developmental traits as model coefficients

We applied the sigmoid function (Eqn 4) to data for 266 measured time series for cell number and cell size. Then, we extracted three traits: the maximum relative growth rate (r_y), the midpoint time (t_{50y}), and mature value (y_m); and calculated leaf primordium values (y_p), duration (t_y), and growth factors (g_y) for each time series using Eqns 5, 6, and 9. For the comparisons of the 12 eudicotyledonous species, for which we compiled multiple curves and, in some cases, multiple studies for given species, we used the median value for the extracted developmental traits.

Analysis of the intrinsic sensitivity of final LA to developmental traits

The NSRT framework enables analysis of the intrinsic mathematical importance of developmental traits in determining LA. We conducted a sensitivity analysis of Eqn 9, quantifying the change in LA arising from shifts in each input variable in the NSRT equations by 20% of its median value, holding all other variables at their median values (Fig. 2c).

Causal partitioning analysis of LA with respect to developmental traits

The NSRT framework enables quantification of how given developmental traits cause differences in LA across genotypes and species. The causal influence of a trait on LA between two species (or genotypes) would depend on the relative variation in all traits, and on the intrinsic sensitivity of LA to given traits. We conducted a three-level hierarchical partitioning analysis of the causal importance of the developmental traits with traits at each level adding up to 100% contribution: the determination of LA by n_m and s_m; n_m and s_m by, respectively, n_p and g_n, and s_p and g_s; and g_n and g_s, respectively, by r_n and t_n, and r_s and t_s (Fig. 1d).

Our causal partitioning approach is based on considering that an infinitesimal change in LA is equal to the sum of infinitesimal changes in each of the underlying variables, each multiplied by the partial derivative of LA with respect to the variable (i.e. $d[\mathrm{LA}] = (\partial \mathrm{LA}/\partial n_m)dn_m + (\partial \mathrm{LA}/\partial s_m)ds_m = (\partial \mathrm{LA}/\partial n_p)dn_p + (\partial \mathrm{LA}/\partial g_p)dg_p + (\partial \mathrm{LA}/\partial s_p)ds_p + (\partial \mathrm{LA}/\partial g_s)dg_s$). Thus, an infinitesimal change in LA can be partitioned into contributions from each of the underlying variables. Similarly, for any finite difference in LA (e.g. between two genotypes), the corresponding contributions can be estimated by numerical integration of the infinitesimal contributions (Buckley & Diaz-Espejo, 2015). We applied this partitioning approach to the log transform of LA, which partitions LA into additive components; for example, $\ln(\mathrm{LA}) = \ln(n_m) + \ln(s_m) = \ln(n_p) + \ln(g_n) + \ln(s_p) + \ln(g_s)$ (from Eqn 9). The ratio of differences in the logarithm of each component to differences in ln(LA) sum to unity, for example

$$1 = \frac{\Delta \ln n_m + \Delta \ln s_m}{\Delta \ln \mathrm{LA}} \qquad \text{Eqn 10}$$

This allows us to define *% contributions of differences* (C) in n_m and s_m between two genotypes to the difference in LA between those genotypes. For partitioning LA into contributions from n_m and s_m, we have

$$C(n_m) = 100 \cdot \frac{\Delta \ln n_m}{\Delta \ln \mathrm{LA}} \qquad \text{Eqn 11}$$

$$C(s_m) = 100 \cdot \frac{\Delta \ln s_m}{\Delta \ln \mathrm{LA}} \qquad \text{Eqn 12}$$

Similarly, for partitioning LA into contributions from n_p, g_n, s_p, and g_s

$$C(n_i) = 100 \cdot \frac{\Delta \ln n_p}{\Delta \ln \mathrm{LA}} \qquad \text{Eqn 13}$$

$$C(g_n) = 100 \cdot \frac{\Delta \ln g_n}{\Delta \ln \mathrm{LA}} \qquad \text{Eqn 14}$$

$$C(s_i) = 100 \cdot \frac{\Delta \ln s_p}{\Delta \ln \mathrm{LA}} \qquad \text{Eqn 15}$$

$$C(g_s) = 100 \cdot \frac{\Delta \ln g_s}{\Delta \ln \mathrm{LA}} \qquad \text{Eqn 16}$$

To partition differences in the growth factors g_n and g_s into contributions from the rates and durations of increase in cell number and size (r_n, t_n, r_s, and t_s), we first defined *growth exponents* G_n and G_s as

$$G_n \equiv r_n t_n \qquad \text{Eqn 17}$$

$$G_s \equiv r_s t_s \qquad \text{Eqn 18}$$

and then defined the % contributions of each rate and duration trait (r_n, t_n, r_s and t_s) to differences in final LA as

$$C(r_n) \equiv \left(100 \cdot \frac{\Delta \ln r_n}{\Delta \ln G_n}\right) \cdot C(g_n) \qquad \text{Eqn 19}$$

$$C(t_n) \equiv \left(100 \cdot \frac{\Delta \ln t_n}{\Delta \ln G_n}\right) \cdot C(g_n) \qquad \text{Eqn 20}$$

$$C(r_s) \equiv \left(100 \cdot \frac{\Delta \ln r_s}{\Delta \ln G_s}\right) \cdot C(g_s) \qquad \text{Eqn 21}$$

$$C(t_s) \equiv \left(100 \cdot \frac{\Delta \ln t_s}{\Delta \ln G_s}\right) \cdot C(g_s) \qquad \text{Eqn 22}$$

Eqns 11–16 and 19–22 describe a causal partitioning framework, in which the contributions of differences in all traits to differences in mature LA add up to 100%. For example, the contributions of mature cell number and size to differences in mature LA add up to 100% ($C(n_m) + C(s_m) = 100$), and the contributions to the differences in mature LA of primordium cell number, number growth factor, primordium cell size, and size growth factor also add up to 100% ($C(n_p) + C(g_n) + C(s_p) + C(g_s) = 100$). Moreover, this framework can also be interpreted in a hierarchical sense. For example, the contributions of primordium cell number and number growth factor add up to the contribution of mature cell number ($C(n_p) + C(g_n) = C(n_m)$), and the contributions of cell proliferation rate and duration add up to the contribution of cell number growth factor ($C(r_n) + C(t_n) = C(g_n)$).

When the contribution of a given causal driver (say y) is positive, this means that, when mature LA is greater in genotype A than in genotype B, y is also greater in A than in B; that is, mature LA was greater in A partly because causal trait y was greater. Conversely, if the contribution of y is negative, this means that, if mature LA is greater in A than in B, y is smaller in

A than in B; that is, mature LA was greater in A despite causal driver y being smaller.

We used this partitioning approach to determine the causal basis for LA with respect to the developmental traits by computing the partitioning coefficients ($C(y)$, Eqns 11–16 and 19–22) for each possible pairwise comparison between genotypes, within each of four datasets: (a) 12 different species ($n = 12 \times 11/2 = 66$ pairwise comparisons), (b) all 52 Arabidopsis experiments ($n = 1326$ pairs), (c) 30 experiments on Arabidopsis WTs ($n = 435$ pairs), and (d) 22 experiments on Arabidopsis mutants for Arabidopsis ($n = 231$ pairs) (Figs S1, S2; Table S1). We then calculated median values for each partitioning coefficient across all comparisons within each dataset, computed confidence intervals for each contribution by bootstrap resampling from a total of 1747 comparisons of LA across the above four datasets separately, and compared median contributions by Mood's median test using the *rcompanion* package (Mangiafico, 2016).

Partitioning the developmental causes of shifts in LA within species associated with genetic mutations or different environmental treatments

We analyzed paired data from given studies for mutant (MU) vs WT *Arabidopsis* ($n = 24$ pairs), and for five experiments on three species ($n = 5$ pairs) in drought treatments (D) vs well-watered (WW) growing conditions, to determine the causal role of developmental traits in determining the shift in LA. In each case, we determined LA based on the control (i.e. WT or WW) trait values and then shifted one trait at a time to the mutant or drought treatment values, thus enabling determination of the contribution of each trait to the overall shift in LA from the control to the treatment value. We thus compared the 11 traits (n_p, r_n, t_n, g_n, n_m, s_p, r_s, t_s, g_s, s_m, and LA) between 24 pairs (MU vs WT) of Arabidopsis and five pairs (WW vs D) of species in drought experiments. We calculated the ratio of the mutant to WT traits or drought to well-watered traits and tested differences in developmental traits using paired t-tests.

Tests of correlations and structural equations modeling among traits and LA

To test whether the correlative structure of the traits alone can enable resolution of the causality that was established by the causal partitioning analysis, for the set of 12 eudicotyledonous species, and for the 52 Arabidopsis experiments, we calculated Pearson correlations between LA and each developmental trait, considering both untransformed data and log-transformed data (i.e. modeling both linear and power-law relationships). Furthermore, we tested graphical path models using structural equation modelling (SEM) and assessed the degree of fit between the observed and expected structures. All the variables were log-transformed to achieve linearity of the bivariate relationships, to improve normality, and to reduce heteroscedasticity before applying the SEM model.

Quantifying trade-offs among traits and the constraints that trade-offs impose on LA

For each dataset (the set of 12 eudicotyledonous species, and the set of 52 Arabidopsis experiments), we tested for general negative associations (i.e. trade-offs) between developmental traits in three ways. First, we tested for linear correlations (ordinary least squares, OLS) using $\log_{10}$-transformed data (Table S2). Second, we determined allometric relationships among developmental traits using standardized major axis (SMA) analysis and estimated the confidence intervals for the slope and elevation for (1) primordium–growth trade-offs (n_p vs g_n, n_p vs r_n, n_p vs t_n, s_p vs g_s, s_p vs r_s, and s_p vs t_s); (2) rate–duration trade-offs (r_n vs t_n, r_s vs t_s); and (3) cell number–size compensation trade-offs (r_n vs r_s, t_n vs t_s, and n_m vs s_m) (Table S3). The data were $\log_{10}$-transformed and analyzed using the R package smatr (Warton *et al.*, 2012). Third, for each tested relationship, we determined crossovers between each pair of species or genotypes (Inman-Narahari *et al.*, 2014), that is, considering the 66 species pairs from the set of 12 diverse eudicotyledonous species, and the 1326 experimental treatment pairs from the 52 Arabidopsis experiments (Fig. S3; Table S2). For each pair of species or genotypes, we quantified 'win-lose' and '-win-tie' trade-offs and 'no-crossovers'. Thus, for example, if for a given species or genotype pair (A and B), A had a higher maximum relative cell proliferation rate (r_n) than B ('win'), and additionally, A had lower duration of cell proliferation (t_n) than B ('lose'), we counted a win-lose r_n vs t_n crossover for that pair. We used the SE for each trait as the criterion to distinguish a win, a loss, or a tie; if the absolute value for the difference in r_n between species A and B was greater than the SE of r_n across the 12 species in a given dataset, we counted the difference as a win (or lose), and otherwise we counted a 'tie'. For each relationship in each database, we calculated the percentage of species or ecotype pairs with win–lose (or lose–win), win–tie (including also lose–tie and tie–tie), or no crossovers (including win–win and lose–lose). A predominance of win–lose or win–tie crossovers relative to no crossovers supports a trade-off between traits.

To quantify the degree that LA is constrained by each of the four discovered trade-offs, we simulated that the distribution of LA if that trade-off was relaxed. For the set of 12 eudicotyledonous species, for each developmental trait y (e.g. $y = t_n$), we recalculated LA 12 times for each species J – substituting the y value for species J with that from each of the 12 species – and then calculated the median of the resulting 12 values of LA. We repeated that procedure for all 12 species to produce a distribution of median modified LA for trait y. We repeated this procedure for each of the eight traits ($y = t_n$, r_n, t_s, r_s, g_n, g_s, n_p, and s_p). The spread of the resulting distributions of median modified LA for each trait, now unconstrained as compared to the distribution of observed (unmodified) LA, which is constrained by trait trade-offs, provides a measure of how much developmental trait constraints would limit variation in LA. This same analysis was implemented for the 52 *Arabidopsis* experiments. Additionally, we constructed phylogenetic trees for 12 species by using the R

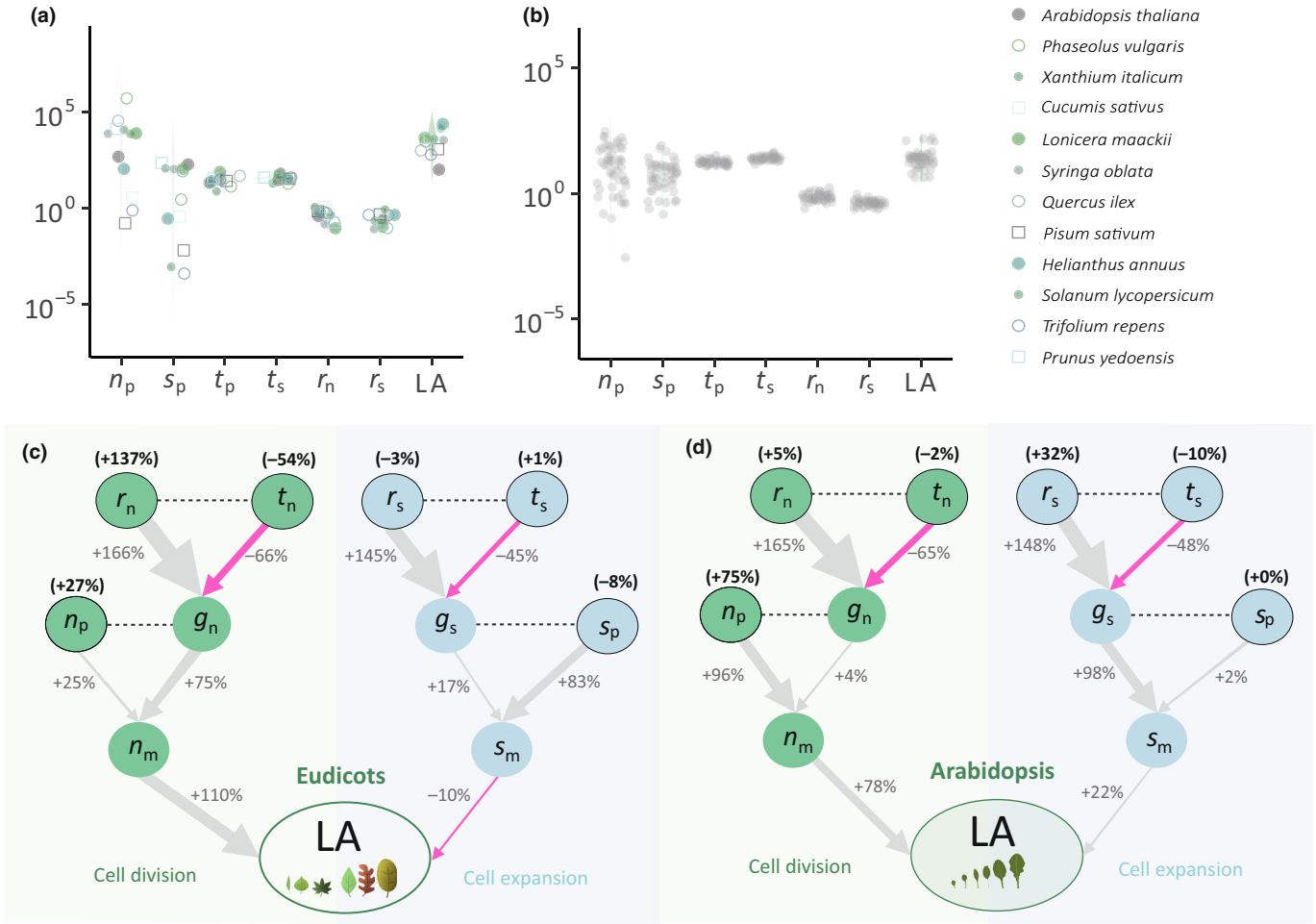

Fig. 3 Developmental basis of leaf size within and across species. Variation in developmental traits that determine mature leaf area (LA) for (a, c) 12 diverse eudicotyledon species and (b, d) 52 experiments on *Arabidopsis* genotypes; LA is a function of six developmental traits n_p, s_p, r_n, t_n, r_s, and t_s. LA is determined by epidermal cell number and mean cell size (n_m and s_m, respectively), themselves determined by leaf primordium cell number and mean cell size (n_p and s_p) and their proportional growth from primordium to mature leaf (g_n and g_s), which are in turn determined by the maximum relative rate and duration of cell proliferation (r_n and t_n) and of cell expansion (r_s and t_s). Gray numbers and arrows represent positive causal influences, magenta arrows negative causal influences, and black numbers in parentheses the overall median causal roles in determining LA of the six ultimate developmental traits (n_p, s_p, r_n, r_s, t_n, and t_s). A negative causal role signifies that larger LA was associated with trait variation that that would cause a smaller LA and that was compensated for by other traits; for example, the negative causal influence of s_m across the eudicotyledons arose because on average, larger leaves had smaller cells. Dotted lines represent trade-offs between traits that constrain the variation in LA. r_n is the mean cell size in the epidermis of the mature leaf; t_n is the duration of cell proliferation; g_n is the cell proliferation factor, that is the proportional increase in epidermal cell number from primordium to mature leaf; n_p is the mean cell size in the epidermis of the mature leaf; n_m is the cell number in the epidermis of the mature leaf; r_s is the maximum relative growth rate of leaf epidermal cell size; t_s is the duration of cell expansion; g_s is the cell expansion factor, that is the proportional increase in mean epidermal cell size from primordium to mature leaf; s_p is the mean epidermal cell size in the leaf primordium, approximated as initial cell number at time = 0; and s_m is the Mean cell size in the epidermis of the mature leaf.

'V.PHYLOMAKER2' with the GBOTB phylogeny as a backbone (Jin & Qian, 2022), and performed phylogenetic reduced major axis (RMA) regression analyses. To determine whether variation between replicate leaves of a given genotype or species would influence our overall findings, we conducted an additional bootstrap analysis by resampling data points in 1000 simulations, based on error bars provided where available, and testing the resulting inferred trade-offs (see Methods S4). We also used a bootstrap resampling procedure to assess whether the structure of our model or the fitting procedure itself could create the illusion of trade-offs where none existed in reality (see Methods S5).

Analysis of developmental traits for mesophyll cells for comparison with the epidermis

We tested whether the patterns of cell division and expansion in leaf epidermal pavement cells was representative of other leaf cell types, such as palisade mesophyll cells, during development. We focused on the three species from the single study (Ding *et al.*, 2014) that included, in addition to data for the development of the epidermis, time-series data for palisade mesophyll cell sizes and numbers. We extracted the data, fitted lines for cell sizes and numbers, and extracted the traits for palisade mesophyll cells.

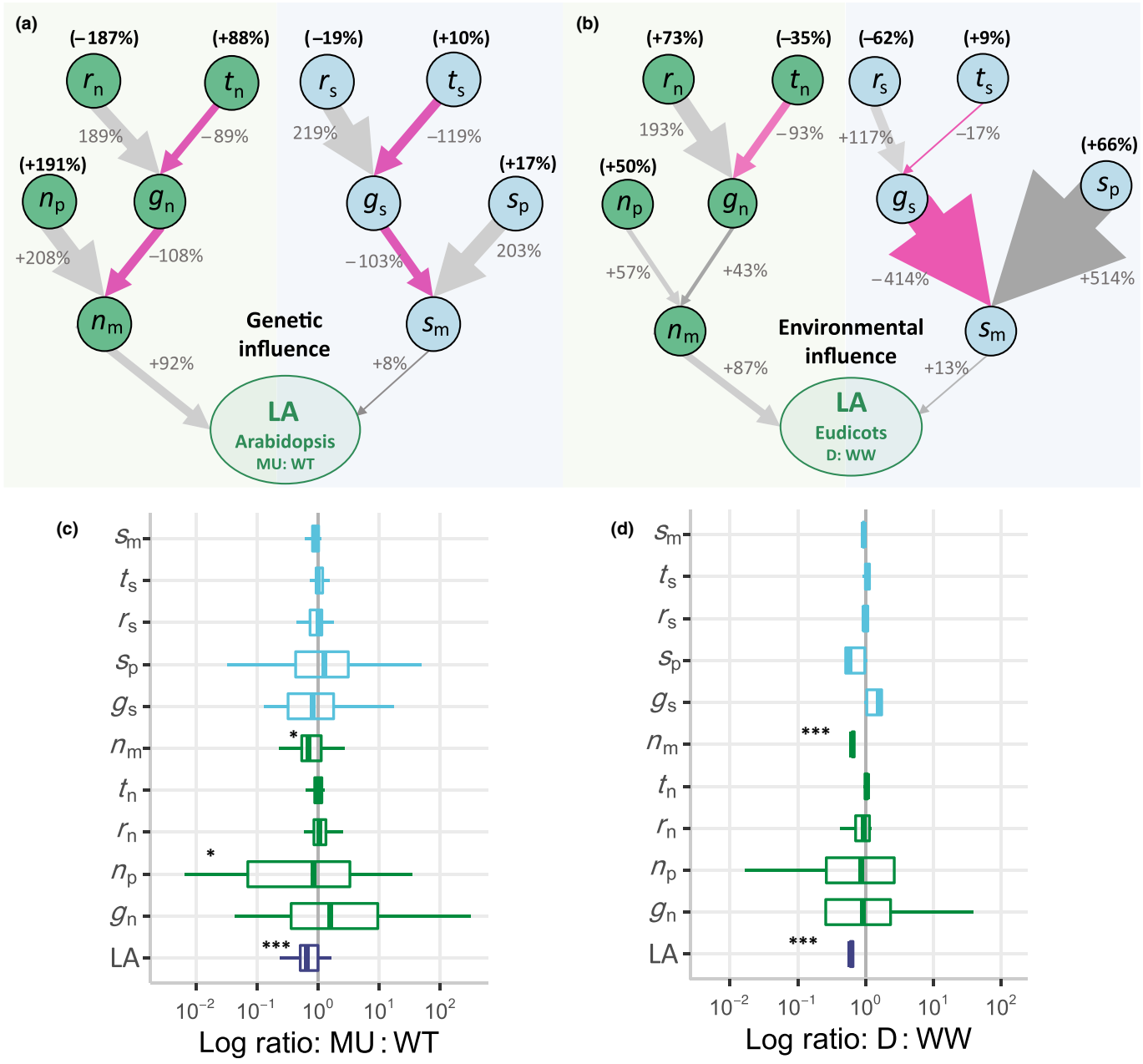

Fig. 4 Influence of cell developmental traits on the variation in leaf area (LA) between diverse experimental mutants and their background wild-type (WT) in *Arabidopsis thaliana* ('genetic influence') and on drought vs well-watered treatments in three species of eudicotyledons ('environmental influence'). (a) The partitioning of the average causal influence (%) of developmental traits on LA for drought relative to well-watered control treatment plants ($n = 5$ experiments on *Arabidopsis thaliana*, *Helianthus annuus*, and *Solanum lycopersicum*), and (b) for leaf shape and size phenotype mutants relative to their matched WT for 24-pairs Arabidopsis. (c) The ratio of values for mutant relative to their matched WT and (d) for three species grown in drought relative to a well-watered control treatment for developmental traits (n_p, s_p, r_n, r_s, t_n, and t_s; defined in Fig. 1) and final values for cell number (n_m), cell size (s_m), and LA. In (c) and (d), boxes represent 24th and 75th percentiles, centerlines are medians, and thin horizontal lines are 5th and 95th percentiles. *, $P < 0.05$; ***, $P < 0.001$ in paired *t*-test. r_n is the mean cell size in the epidermis of the mature leaf; t_n is the duration of cell proliferation; g_n is the cell proliferation factor, that is the proportional increase in epidermal cell number from primordium to mature leaf; n_p is the mean cell size in the epidermis of the mature leaf; n_m is the cell number in the epidermis of the mature leaf; r_s is the maximum relative growth rate of leaf epidermal cell size; t_s is the duration of cell expansion; g_s is the cell expansion factor, that is the proportional increase in mean epidermal cell size from primordium to mature leaf; s_p is the mean epidermal cell size in the leaf primordium, approximated as initial cell number at time = 0; and s_m is the Mean cell size in the epidermis of the mature leaf.

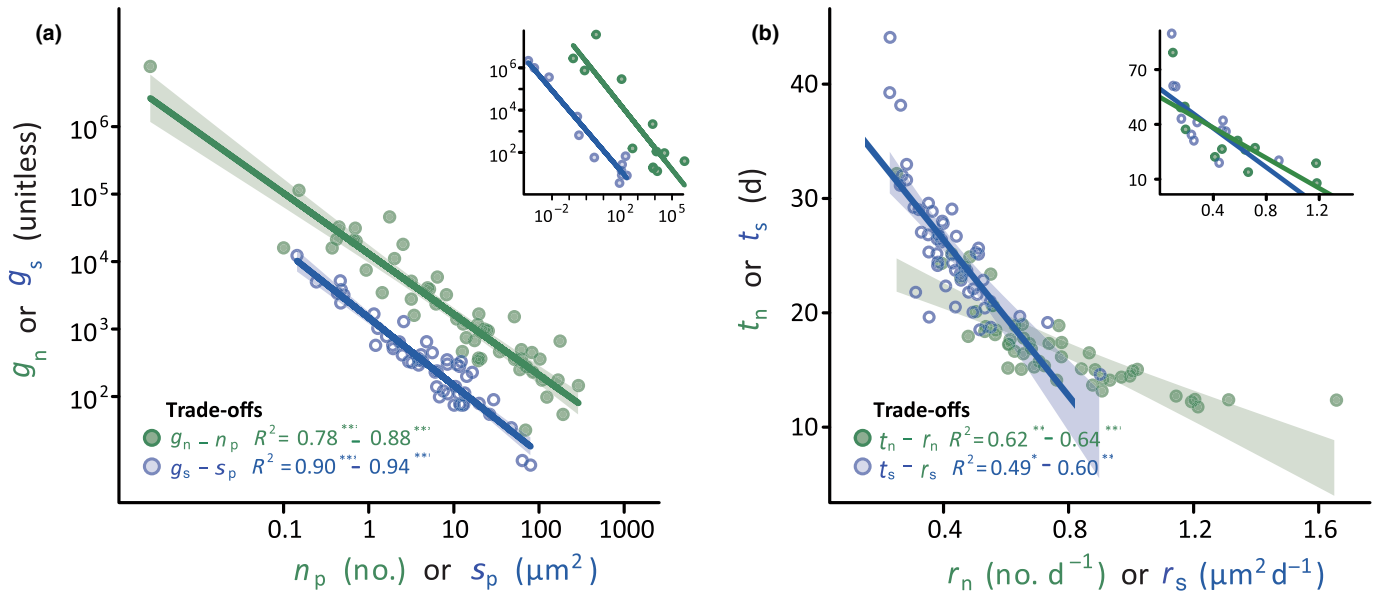

Fig. 5 Trade-offs between leaf developmental traits. (a) Trade-offs between growth in cell number vs primordium cell number, and growth in cell size vs primordium cell size (g_n vs n_p, and g_s vs s_p, in green and blue, respectively). (b) Trade-offs between cell proliferation duration vs maximum relative cell proliferation rate, and cell expansion duration vs maximum relative cell expansion rate (t_n vs r_n, and t_s vs r_s in green and blue, respectively) across 52 Arabidopsis experiments (main panels) and 12 eudicot species (insets). Shaded areas represent 95% confidence intervals. Significance: *, $P < 0.05$; **, $P < 0.05$; ***, $P < 0.001$. Detailed results in Supporting Information Tables S5 and S6. Note the logarithmic axes in this figure. g_n is the cell proliferation factor, that is the proportional increase in epidermal cell number from primordium to mature leaf; g_s is the cell expansion factor, that is the proportional increase in mean epidermal cell size from primordium to mature leaf; n_p is the mean cell size in the epidermis of the mature leaf; s_p is the mean epidermal cell size in the leaf primordium, approximated as initial cell number at time = 0; t_n is the duration of cell proliferation; t_s is the duration of cell expansion; r_n is the mean cell size in the epidermis of the mature leaf; and r_s is the maximum relative growth rate of leaf epidermal cell size.

Then, we tested whether the mesophyll cell size expansion traits correlated with those estimated for the epidermal cells.

Results

Developmental traits of LA within and across species

We determined the developmental basis of LA across 12 eudicotyledonous species, and across 52 experiments on Arabidopsis genotypes, which varied in LA by, respectively, 237-fold and 41-fold (Fig. 3a,b). The median mature leaf sizes for the 12 eudicot species ranged from 105 to 24 819 mm^2, while in the 52 Arabidopsis experiments, the leaf sizes ranged from 4.1 to 167 mm^2 (Table S1; Datasets S1, S2). The sigmoidal model, Eqn 4, fitted to the developmental trajectories of cell numbers and sizes with R^2 ranging 0.91–0.99 (0.98 on average) across the 12 eudicot species, and 0.70–0.99 (0.96 on average) across the mutant and WT genotypes of Arabidopsis (Fig. 2e,f). The developmental traits also varied strongly within and across species, from 10-fold- and 2.7-fold for t_n across eudicotyledonous species and Arabidopsis experiments, respectively, to 3×10^6 fold and 10^5-fold for n_p (Fig. 3; Table S1).

Within and across species, cell number played a major role in determining leaf size. On average across the 12 diverse species, cell number at maturity (n_m) entirely drove variation in LA, and n_m also drove most variation in LA across genotypes within *Arabidopsis*, with s_m playing an important minority role when considering all genotypes (on average 22%), and this finding was consistent when considering mutants and WTs separately (Figs 3c,d, S1; Table S4). Among the eudicotyledonous species, a higher n_m and LA were achieved through higher maximum relative cell proliferation rate (r_n), and, secondarily by higher leaf primordium cell number (n_p). Within *Arabidopsis*, variation among ecotypes in LA was driven principally by higher n_m, with a lesser role for maximum relative rate of cell expansion, and a marginal role (5%) for increase in maximum relative rate of cell proliferation (Fig. 3c,d).

Contrasting developmental regulation of LA by environmental vs genetic factors

Across 24 experiments comparing *Arabidopsis* mutants with their WT backgrounds, on average, LA was 19% smaller for the mutants (Fig. 4a,c). In comparison of drought-stressed with well-watered control plants of three species, leaves were on average 63% smaller for droughted plants (Figs 4b,d, S4). On average, respectively, 92% and 87% of these differences in LA were due to lower n_m (Fig. 4; Table S5). The smaller n_m and LA of *Arabidopsis* mutants relative to their WT backgrounds were principally due to lower n_p. The smaller n_m of drought-stressed relative to well-watered control plants was due principally to lower n_p and r_n (Fig. 4; Table S5).

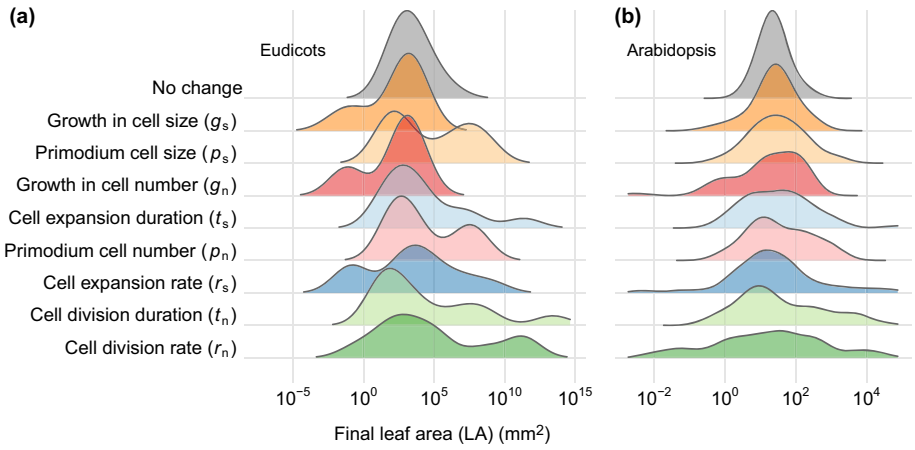

Fig. 6 Leaf size is strongly constrained by leaf developmental trait trade-offs. (a) For 12 diverse eudicotyledonous species, and (b) for 52 experiments on Arabidopsis genotypes, the observed distribution of mature leaf area (LA), and the expected distributions of LA based on simulation analyses releasing developmental trait trade-offs, that is, if growth in cell size or number (g_s and g_n, respectively) were unconstrained by primordium cell size or number (n_p and s_p, respectively), and vice versa, or if the maximum relative rates of cell proliferation or expansion (r_n and r_s, respectively) were unconstrained by their time durations (t_n and t_s, respectively), and vice versa. The range of variation of LA increases by orders of magnitude when unconstrained by developmental trait trade-offs, with the ranges of 5th–95th percentile of LA shifting from 5.3×10^5 to 2.2×10^{18} mm^2 across species and from 1.4×10^2 to 1.1×10^4 mm^2 within species, respectively.

Inability of correlation analyses to resolve the developmental causality of LA

We found that correlation tests of LA vs developmental traits, and even structural equation modeling, were not reliable for resolving the causal determination of LA by developmental traits. These tests produced relationships that did not coincide with the findings of causal analysis (Figs S2, S5; Tables S6, S7).

Developmental trait trade-offs constrain LA within and across species

We found four strong trade-offs among developmental traits. Within and across species, we found negative correlations between primordium cell number and growth in cell numbers (n_p and g_n), between primordium cell size and growth in cell size (s_p and g_s), between maximum relative cell proliferation rate and duration (r_n vs t_n), and between maximum relative cell expansion rate and duration (r_s vs t_s) (Fig. 5).

These trade-offs were additionally indicated by high frequencies of 'crossovers' in developmental trait values between species pairs for the diverse eudicots, and between genotype-pairs for Arabidopsis experiments (Fig. S3). The observation of trade-offs across numerous mutants and their WT backgrounds indicated a genetic basis for these developmental trait trade-offs in *Arabidopsis*.

These trade-offs between developmental traits explain why the causal roles of given traits in determining LA (Figs 3, 4) diverged strongly from their intrinsic individual influences in the sensitivity analysis of Eqn 3 (Box 1; Fig. 2c). Indeed, the influence of these trait trade-offs (Fig. 5) on the determination of LA (Fig. 3c, d) is highlighted by their allometric slopes (Table S7). Thus, the much greater importance of the maximum relative rates of cell proliferation and expansion (r_n and r_s) than of growth duration (t_n and t_s) in determining n_m, s_m and LA is explained by the allometric slopes of the trade-offs between t_n vs r_n, and t_s vs r_s, having magnitudes less than unity, such that across species or genotypes, a shift to a higher r_n or r_s value tends to correspond to a lesser shift to lower t_n or t_s, respectively. Phylogenetic RMA regression analyses (Fig. S6) also confirmed these trade-offs (Table S7).

Our analyses did not support general 'cell size–number compensation' trade-offs between cell number and cell size developmental traits for the 12 eudicotyledons and the 52 Arabidopsis experiments. Although crossovers were observed between r_n and r_s, or t_n and t_s, for a minority of pairs of species or *Arabidopsis* experiments (Table S3), overall, we found no statistical relationships between r_n and r_s, either across the diverse eudicotyledons or across Arabidopsis experiments, and t_n and t_s were positively related in both study groups ($r = 0.49$–0.70; $P < 0.01$ to $P < 0.05$; Tables S4, S5).

Developmental traits trade-offs constrain LA

The variation in LA within and across species was strongly constrained by the developmental trait trade-offs. In the absence of these trade-offs, our analyses found that the range of LA values achievable within and across species would be greater by many orders of magnitude (Fig. 6).

Tests of the robustness of the estimation of leaf developmental traits and trait trade-offs

In our test of the influence of considering stomata in the calculation of epidermal development traits, we found strong correlations between the development traits based on weighted average cell size with those based on epidermis pavement cell size across

Arabidopsis experiments ($r = 0.75$–0.88; $P < 0.001$; $n = 15$; Fig. S7).

In our test of epidermal developmental traits in comparison with those for palisade mesophyll cells, we found that epidermal development traits were in some cases representative (Fig. S8). While across species, initial cell size numbers, and initial mean cell sizes tended to be decoupled between epidermal pavement cells and palisade mesophyll cells ($r = 0.50$–0.77; $P = 0.08$–0.31; Figs S9, S10), the maximum relative rate of cell expansion, and the durations of cell proliferation and expansion were correlated for cells of the two types ($r = 0.87$–0.94, $P = 0.006$–0.02; Figs S9, S10).

We tested the possible influence of uncertainty in the timepoints selected for measurement of leaf cell numbers and sizes in the time series. We found perfect correlations between the leaf developmental traits determined from the complete vs the reduced datasets ($r = 1$; $P < 0.001$; Figs S11, S12).

To test the sensitivity of inferred leaf developmental trait trade-offs to uncertainty in the assumed 'time zero' for initiation of primordial growth, we conducted two analyses. First, we tested the trade-offs between developmental traits for the sets of studies individually that used given designations of time zero ($n \geq 5$), that is, for Arabidopsis, after seed stratification, after sowing, after germination, or when both cotyledons were visible; and for eudicots, after leaf primordium initiation/emergence. We found the relationships remained significant in these subsets (Table S8; $r = 0.67$–0.98; $P < 0.05$). Second, we conducted a bootstrap resampling analysis, with random shifts in the range of ±3 d in the assumed time zero; this analysis supported the developmental trait trade-offs: median r- and p-values across 1000 resampling iterations were n_p vs g_n (median $r = -0.77$; $P = 3.8 \times 10^{-11}$), for r_n vs t_n ($r = -0.79$; $P = 2.4 \times 10^{-12}$), for s_p vs g_s ($r = -0.82$; $P = 1.2 \times 10^{-13}$), and for r_s vs t_s ($r = -0.79$; $P = 3.1 \times 10^{-12}$) (Fig. S13).

In our tests of whether the model structure or fitting procedure could explain trait trade-offs, we found that this effect could not generate strong correlations between model traits where none exist in reality. We generated 1000 time-series datasets based on randomized r and t traits, added statistical noise, fitted the growth model to the simulated time series, and re-extracted r and t traits. For the randomized r and t datasets, 4.7% of r–t correlations were significant and the mean $r \pm$ SE was -0.0009 ± 0.004. After generating time-series from these traits and adding noise, re-fitting growth curves and extracting traits, the proportion of significant r-t correlations was 5.6% and the mean $r \pm$ SE was -0.036 ± 0.004 (Fig. S14). We thus conclude that the strong r-t trade-offs observed in our databases for Arabidopsis and eudicot species were not an artifact of the growth curve structure or fitting it to data.

We conducted a bootstrap resampling analysis to test the sensitivity to intragenotype variation of leaf developmental trait trade-offs (Methods S4, Figs S15, S16). Our analysis supported the developmental trait trade-offs despite intragenotypic or intraspecific variation, for n_p vs g_n ($r = -0.95$; $P = 1.1 \times 10^{-26}$), for r_n vs t_n ($r = -0.93$; $P = 1.5 \times 10^{-21}$), for s_p vs g_s ($r = -0.87$; $P = 2.1 \times 10^{-16}$), and for r_s vs t_s ($r = -0.89$; $P = 5.0 \times 10^{-18}$) (Fig. S16).

Discussion

Developmental trait determination of leaf size within and across species

Our analyses resolved epidermal developmental traits underlying the determination of LA across species. We found strong variation in the leaf developmental traits within and across species. Our further analyses validated the robustness of deriving developmental traits based on epidermal pavement cell sizes and numbers to analyze differences in mature leaf size. For the 15 Arabidopsis genotypes for which stomatal developmental time series were available, we found strong correlations of developmental traits based only on epidermal pavement cells with those that accounted for stomata by using weighted average cell sizes. Notably, when stomata differ in size from epidermal pavement cells, the developmental traits would be only slightly affected, yet we recognize there is great value for future leaf expansion studies to clearly distinguish among types of epidermal cells for even stronger precision in determining leaf developmental traits using our approach. Yet, importantly, across all measured species and genotypes, we found a strong power to predict final leaf size from the developmental traits that were determined based on epidermal pavement cells, neglecting the stomata. Additionally, we found perfect correlations of developmental traits calculated using the compiled data with datasets reduced by removing timepoints, indicating robustness to variation in timepoint selection in experiments. Furthermore, we found that developmental traits determined for the epidermal cells were in some cases representative of the development of other leaf cells, in our comparison with traits determined for palisade mesophyll cells.

We resolved general constraints on LA within and across species by developmental traits. We found a far stronger role of cell number than cell size in determining LA across contexts. This finding is consistent with organ development involving far greater increases in cell number than cell size, and the related fact that mature leaves, like other plant and animal organs, have greater variation in cell numbers than cell size by orders of magnitude within and across species (Table S1) (Korner *et al.*, 1989; Beaulieu *et al.*, 2008; Sablowski, 2016). By being largely decoupled from cell size, LA can evolve independently of other adaptive cell size-related features, including cell wall thickness, leaf thickness (John *et al.*, 2013), stomatal density (Beaulieu *et al.*, 2008; Brodribb *et al.*, 2013), and vein density (Carins Murphy *et al.*, 2012; Brodribb *et al.*, 2013), all of which can influence photosynthetic assimilation rate per unit LA (Théroux-Rancourt *et al.*, 2021). Thus, both small and large leaves can achieve high photosynthetic rates (Price *et al.*, 2014).

The strongest developmental trait determinants of leaf size were maximum relative cell proliferation rate and primordium cell number. Among the eudicotyledonous species, a higher n_m and LA were achieved principally by higher maximum relative cell proliferation rate (r_n), and, within *Arabidopsis*, principally by higher leaf primordium cell number (n_p) (Fig. 3c,d). The smaller n_m and LA of *Arabidopsis* mutants relative to their WT backgrounds were mainly due to lower n_p, and the smaller n_m of

drought-stressed relative to well-watered control plants was mainly due to lower n_p and r_n (Fig. 4; Table S5). The importance of n_p in the determination of LA variation within species highlights the major influence of mutations or growth conditions that would affect the development of the primordium from the shoot apical meristem (Figs 4a, S1) (Autran *et al.*, 2002). By contrast, both within and across species, on average, a greater LA was negligibly related to longer durations of either cell proliferation and expansion (t_n and t_s) (Fig. 3c,d). Our analyses suggested that the contradictory findings among previous studies – in which LA was in some cases linked positively with the durations of cell proliferation and expansion (Tisne *et al.*, 2008; Horiguchi & Tsukaya, 2011; Gonzalez *et al.*, 2012; Czesnick & Lenhard, 2015; Gazquez & Beemster, 2017) (Table 1) – arose from the inability to resolve causality reliably using correlation analyses or even structural equation modelling (Figs S2, S5; Tables S2, S6).

Leaf developmental trait trade-offs consistent with metabolic scaling and evolutionary theory

We found that trade-offs between developmental traits limit the variation of mature leaf size. The trade-offs we resolved were robust in tests considering differences among studies in the assumed time zero corresponding to initiation of primordial growth, the effect of heterogeneity between epidermal cells (*cf.* Le Gloanec *et al.*, 2022), and the variation among leaves of a given genotype or species. Our analyses indicate that these developmental trait trade-offs act as 'checks and balances' and constitute a mechanism for the prevention of the occurrence of extreme leaf sizes, which has remained unexplained (Grubb, 2020). Notably, these trade-offs among leaf developmental traits are analogous to a number of previously described growth rate vs body size trade-offs and growth rate vs growth duration trade-offs that constrain the sizes of diverse organisms and populations (Gillooly *et al.*, 2002; Brown *et al.*, 2004; Wright *et al.*, 2004; Savage *et al.*, 2007; Kempes *et al.*, 2012; Garland *et al.*, 2022). We hypothesize multiple, nonexclusive types of mechanisms underlying these trade-offs. First, these trade-offs may emerge from intrinsic mechanical (Trinh *et al.*, 2021) or biochemical (Brown *et al.*, 2004) constraints on metabolism and growth. For example, a larger primordium made up of more numerous and/or larger cells may be limited in its expansion by its lower surface area-to-volume ratio (Niklas & Cobb, 2017) – a mechanism that has been invoked to explain the decline in relative metabolic and growth rates with increasing size of both organisms and ecosystems (Niklas, 1994; West *et al.*, 2002; Savage *et al.*, 2007; Kempes *et al.*, 2012). The trade-off between rates of cell proliferation or expansion and their durations may arise because rapid rates cannot be sustained due to resource depletion or the accumulation of waste-products (Gillooly *et al.*, 2002; Brown *et al.*, 2004; Pantin *et al.*, 2012), mechanisms previously proposed to explain why leaves with high photosynthetic rates tend to have shorter active seasons and overall longevity (Wright *et al.*, 2004), and why speed–endurance trade-offs are general for the rates of metabolism in plants and human athletes and for industrial production (Zhang *et al.*, 2017).

A second mechanism type that may underlie developmental trait trade-offs is their reflecting extrinsic natural selection on developmental processes. For example, the duration of leaf expansion may be minimized by selection, as the developing leaf is especially vulnerable to stresses, including dehydration and herbivory (Moles & Westoby, 2000; Baird *et al.*, 2021); a greater LA may therefore be most safely achieved by increasing cell proliferation rate while reducing the duration of the vulnerable growth period (Barton *et al.*, 2019). Indeed, our findings that drought can impact on multiple developmental traits (n_p and r_n) indicates the advantage of rapid leaf expansion in between drought events. Notably, the selection of differences in developmental traits, and of LA itself, would not arise from short-term droughts but over generations under an arid climate. The question of whether leaf developmental traits can shift ontogenetically and with repeated incidences of stress requires further investigation.

Finally, the *range* of LA values itself may be under extrinsic selection, and trade-offs among developmental traits may then arise specifically to constrain variation (Fox, 2011; Fig. 6). Notably, a trade-off that arises extrinsically from stabilizing selection on LA may eventually become intrinsic, if the genes underlying these developmental traits were to form antagonistic linkages. Yet, despite their generality, these trade-offs are not absolute; extreme leaf sizes arise as outlier combinations of relatively high n_p and g_n, or of r_n and t_n. Furthermore, the trade-offs between traits were statistically similar in slope and intercept for *Arabidopsis* experiments and across diverse species for g_s vs s_p, t_n vs r_n and t_s vs r_s, suggesting strong generality. However, for g_n vs n_p, the intercept was higher for the across-species relationship (Fig. 5; Table S7), providing an example of trait divergence that breaks any single general trade-off and would enable strong shifts in LA in the evolution of diverse lineages.

Our analyses did not support previously hypothesized general 'cell size–number compensation' trade-offs between cell proliferation and either the rate or duration of cell expansion (Table S3). Notably, a compensatory interaction between cell proliferation and expansion would rely on their coordinated regulation throughout the leaf despite these processes occurring for given cells at different times (Serrano-Mislata *et al.*, 2015; Sablowski, 2016), and no mechanism for such regulation has been shown (Hisanaga *et al.*, 2015). By contrast, the observed independence of cell number and size development is consistent with their known mediation by multiple regulators, including auxin, cell turgor, vacuole function, and microtubule dynamics (Pantin *et al.*, 2012; Sablowski, 2016).

The determination of LA on the basis of developmental traits: multiple applications in plant biology

We propose that the consideration of LA on the basis of developmental traits provides new avenues to resolve new insights into the evolution of leaf size within and across species, and for the improvement of crops subject to climate change and environmental stress. First, developmental traits may be sought that underlie the adaptation of leaves to climate. Given that the leaf boundary layer is thicker in larger leaves, and major vein density

tends to be negatively related to leaf size, and these properties can potentially influence many aspects of leaf function (including leaf temperature, photosynthesis and transpiration rates, water-use efficiency, light-use efficiency, and hydraulic efficiency and safety), leaf size is adaptive under a wide range of environmental contexts (Givnish, 1979, 1987; Sack *et al.*, 2012; Baird *et al.*, 2021). Thus, in general, small leaves are associated with sunnier habitats, and colder and drier climates, and larger leaves with shaded habitats, warmer and moister climates, and more nutrient-rich soils (Givnish & Vermeij, 1976; Givnish, 1979, 1987; Leach & Givnish, 1999; Wright *et al.*, 2017; Lusk *et al.*, 2019; Smith *et al.*, 2023). Given new data on leaf developmental anatomy, the evolution of small or large leaves that provide advantages under current or novel climates can be partitioned into the influences of underlying developmental traits. New work is needed to consider whether this adaptation mirrors in its developmental basis the plastic shifts shown for several species here in different water treatments. Furthermore, future avenues of research include determining the associations of leaf anatomical developmental traits with leaf shape (He *et al.*, 2024); with compound and simple leaf types (Koch *et al.*, 2018); with plant age and growth conditions (Huang *et al.*, 2021); withother dimensions of leaf size (e.g., length and width; Schrader *et al.*, 2021), and, additionally, with other functional traits, such as leaf economics traits, including leaf mass per area and leaf nutrient concentrations.

A second set of applications of developmental traits underlying LA is a higher resolution of its genetic underpinning. Thus far, attempts to directly link given genes with LA have had limited success (Gonzalez *et al.*, 2010; Kalve *et al.*, 2014), potentially due to the multiple traits that determine LA developmentally, and thus, analyzing these traits and their genetic basis would provide a more proximate causality (Kierzkowski *et al.*, 2019). Indeed, our analyses of Arabidopsis genotypes highlighted primordium cell numbers and cell proliferation rate variation as major effects of single mutations, and we resolved a genetic basis for trade-offs between developmental traits, given these trade-offs appeared across mutants and their WT backgrounds. Our analysis provides novel insights useful for consideration in selecting or breeding for differences in leaf size. We have not mechanistically probed further into the developmental traits – aside from showing an overall genetic basis, that is the differences in these traits when comparing mutants with WTs of Arabidopsis. The ability of these traits to explain variation in LA points to this approach as an avenue for future research in molecular developmental biology. Thus, new studies of the genetic basis for LA with respect to developmental traits may provide further traction to elucidate shifts in LA within species. These studies can test the insight from our results that, given trade-offs among leaf developmental traits, it will be challenging to select or breed for increased leaf size by additive 'stacking' of leaf size promoting features (Gonzalez *et al.*, 2012). For example, if one selects for primordia with large or numerous cells, then cell expansion or proliferation rates may be low. Our findings suggest that large leaves may best be selected or bred for via the discovery of outliers from the trade-offs, for example from plants combining extreme values for primordia size, leaf maximum relative growth rates and leaf growth durations, especially through the identification of regulating genes (Dwivedi *et al.*, 2021; Vicentin *et al.*, 2024). Given that outliers from developmental trade-offs could have very small or large LA, they would provide useful material for breeding programs focused on developmental traits.

While this analysis was focused on plants, we recognize that our novel number-rate-size-time framework for analyzing the developmental basis of mature organ size could be easily adapted to analyze development in other multicellular organisms, enabling tests of the hypothesis that developmental trait trade-offs have yet greater generality in constraining organ and organism sizes across the tree of life.

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Competing interests

None declared.

Author contributions

LS and ZM developed the conceptual approach, compiled the data, and drafted the manuscript. LS, ZM and TNB developed the analytical approach and conducted the analysis.

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Data availability

The data and code are available as in Figshare: doi: 10.6084/m9.figshare.26954023.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Developmental parameters for each Arabidopsis experiment.

Dataset S2 Developmental parameters for each eudicotyledonous species.

Fig. S1 The influence of cell developmental traits on the variation in leaf area among wild-type *Arabidopsis thaliana* and among experimental mutants.

Fig. S2 Structural equation modeling of direct and indirect effects does not accurately resolve the causal influence of cellular traits during leaf development on variation in leaf size across 12 eudicotyledonous species and 52 experiments on Arabidopsis.

Fig. S3 Trade-offs between leaf developmental traits quantified using crossover analysis.

Fig. S4 Shifts in the developmental trajectory of leaf size for drought relative to well-watered treatments for three species.

Fig. S5 Testing correlations between leaf area and developmental traits for 52 experiments of Arabidopsis and 12 diverse eudicotyledonous species.

Fig. S6 Phylogenetic relationships among leaf developmental traits for 12 diverse eudicotyledonous species.

Fig. S7 Strong correlations of cell size development traits when as the weighted average cell size of epidermal pavement cells and stomata and when calculated based only on epidermal pavement cells. Across 15 Arabidopsis experiments.

Fig. S8 Developmental trajectories of cell number and cell size for palisade mesophyll cells and epidermal pavement cells across six species.

Fig. S9 Correlations matrix of key developmental traits for the palisade cell and epidermis cell across six species.

Fig. S10 Linear relationships of four key developmental traits for the palisade cell and epidermis cell across six species.

Fig. S11 Re-analyses of developmental traits extracted from the 52 Arabidopsis experiments time-series after deleting the second time measurement point.

Fig. S12 Re-analyses of developmental traits extracted from the 52 Arabidopsis experiments time-series after deleting the second, fourth-, and sixth- time measurement points.

Fig. S13 The influence of uncertainty in initial time of primordial growth across Arabidopsis experiments and 12 diverse eudicot species.

Fig. S14 In our tests of whether the model structure or fitting procedure could explain trait trade-offs.

Fig. S15 Increases of cell number and cell size with leaf development time, including the effect of intragenotypic variation, for the data of Dhondt *et al.* (2010), for Arabidopsis genotype Col-0.

Fig. S16 Testing the robustness of leaf developmental trait trade-offs to intragenotypic variation.

Methods S1 Testing the influence of uncertainty in initial time of primordial growth.

Methods S2 Testing the influence of timepoint uncertainty on the estimation of developmental traits.

Methods S3 Testing the influence of considering stomata on the estimation of epidermal developmental traits determining LA.

Methods S4 Analysis of the effect of intragenotypic variation on the finding of developmental trait trade-offs.

Methods S5 Testing whether the model structure or fitting procedure could explain trait trade-offs.

Table S1 Summary statistics for leaf developmental traits for the eudicotyledon species dataset and for Arabidopsis experiment datasets.

Table S2 Correlation matrix for relationships among 16 developmental traits across 52 experiments on Arabidopsis.

Table S3 Relationships among leaf developmental traits for 12 diverse eudicotyledonous species and 52 Arabidopsis experiments based on ordinary least squares and crossover analysis.

Table S4 Partitioning the causal influence of cell developmental traits on leaf area across 52 experiments on Arabidopsis genotypes and across 12 diverse eudicotyledonous species.

Table S5 Partitioning the causal influence of cell developmental traits on leaf area between Arabidopsis mutant and wild-type genotypes (24 pairs, within studies) and for drought vs well-watered treatments for five experiments on three eudicotyledonous species.

Table S6 Correlation matrix for relationships among 16 developmental traits across 12 species of eudicotyledons.

Table S7 Allometric relationships among leaf developmental traits for 12 diverse eudicotyledonous species and 52 Arabidopsis experiments based on standard major axes.

Table S8 Trade-offs between developmental traits for the sets of studies that used given designations of initial leaves for Arabidopsis experiments and eudicot species.

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