

Anatomical determinants of gas exchange and hydraulics vary with leaf shape in soybean

Bishal G. Tamang^{1,✉}, Yanqun Zhang³, Michelle A. Zambrano¹ and Elizabeth A. Ainsworth^{1,2,*}

¹Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA, ²Global Change and Photosynthesis Research Unit, USDA ARS, Urbana, IL, USA, and ³China Institute of Water Resources and Hydropower Research, Department of Irrigation and Drainage, Beijing, China

*For correspondence. E-mail lisa.ainsworth@usda.gov

Received: 19 May 2022 Returned for revision: 29 August 2022 Editorial decision: 9 September 2022 Accepted: 14 September 2022
Electronically published: 16 September 2022

- **Background and aims** Leaf shape in crops can impact light distribution and carbon capture at the whole plant and canopy level. Given similar leaf inclination, narrow leaves can allow a greater fraction of incident light to pass through to lower canopy leaves by reducing leaf area index, which can potentially increase canopy-scale photosynthesis. Soybean has natural variation in leaf shape which can be utilized to optimize canopy architecture. However, the anatomical and physiological differences underlying variation in leaf shape remain largely unexplored.
- **Methods** In this study, we selected 28 diverse soybean lines with leaf length to width ratios (leaf ratio) ranging between 1.1 and 3.2. We made leaf cross-sectional, gas exchange, vein density and hydraulic measurements and studied their interrelationships among these lines.
- **Key results** Our study shows that narrow leaves tend to be thicker, with an ~30 µm increase in leaf thickness for every unit increase in leaf ratio. Interestingly, thicker leaves had a greater proportion of spongy mesophyll while the proportions of palisade and paraveinal mesophyll decreased. In addition, narrow and thicker leaves had greater photosynthesis and stomatal conductance per unit area along with greater leaf hydraulic conductance.
- **Conclusions** Our results suggest that selecting for narrow leaves can improve photosynthetic performance and potentially provide a yield advantage in soybean.

Key words: Leaf shape, gas exchange, mesophyll tissue, leaf vein density, photosynthesis, leaf anatomy.

INTRODUCTION

In crops, leaf morphological characteristics can be key determinants of light interception and distribution dynamics at the whole plant and canopy scales (Hirose, 2005; Emmel *et al.*, 2020; Viridi *et al.*, 2022). In dense canopies, upper layers intercept most of the incoming solar radiation. Leaves at lower canopy levels are often starved of light, resulting in those leaves being net carbon consumers before they senesce and reallocate nitrogen to sink organs. It also appears that modern crop varieties in monoculture systems over-invest in light-harvesting organs, perhaps a carryover from evolutionary selection for competition for light at the level of an individual plant rather than a canopy (Hikosaka and Hirose, 1997; Slattery and Ort, 2021). Furthermore, over-investment in leaf tissues may be expected to increase with rising atmospheric carbon dioxide (CO₂) concentrations, which increase leaf biomass, leaf area and leaf area index (LAI, total leaf area per unit ground area) (Ainsworth and Long, 2005; Oikawa *et al.*, 2013; Kumagai *et al.*, 2015). This leads to the idea that altering leaf morphology or reducing over-investment in leaves might improve light penetration deep inside canopy layers, improving canopy photosynthesis and potentially crop yield (Tholen *et al.*, 2012; Srinivasan *et al.*, 2017).

Based on modelling and empirical studies, several traits have been proposed to improve light distribution in crop canopies.

One early suggestion was developing canopies with erect leaf morphology towards the top of the canopy and more horizontal towards the bottom for more uniform light distribution and improved canopy photosynthesis (Slattery and Ort, 2021, and references therein). The benefit of this canopy architectural trait can be observed among modern maize hybrids and biomass sorghum where it confers tolerance to higher planting density along with improved resource use efficiency, supporting current high yields (Tollenaar and Lee, 2002; Jaikumar *et al.*, 2021; Li *et al.*, 2021). Another suggestion is developing leaves with reduced chlorophyll content that can alter leaf absorbance. Reducing chlorophyll could allow greater light penetration and scattering within the canopy to improve net photosynthesis. Reducing leaf chlorophyll content in crops by 50–60 % has been shown to provide a net carbon gain via higher canopy photosynthesis and improved nitrogen use efficiency (Ort *et al.*, 2011; Song *et al.*, 2017; Walker *et al.*, 2018). More recently, making crops utilize wavelengths beyond the photosynthetically active radiation (PAR) region of the spectrum has been considered to have potential yield benefits. Expansion of this spectrum beyond the red end of the PAR spectrum (701–750 nm) could increase the number of photons available per unit area by as much as 19 % (Blankenship and Chen, 2013; Slattery and Ort, 2021), which can translate into increased net photosynthesis. Similarly, decreasing LAI in crops is another potential strategy

for improving canopy light distribution (Wells *et al.*, 1993). Reducing LAI by 40 % in soybean provided a yield advantage of up to 10 % at elevated CO₂ concentrations (550 ppm) (Srinivasan *et al.*, 2017). Such reduction in LAI could be achieved by developing crops with either reduced leaf size or number.

Soybean (*Glycine max* L. Merr.) shows tremendous diversity in its leaf shape and size that can potentially be exploited to manipulate LAI. Among wild species of the USDA soybean germplasm collection (*Glycine soja* Sieb. et Zucc.) representing maturity groups 000 to IX, leaf length can vary up to three times and length to width ratios can range between less than 2 (oval) to more than 5 (ultralinear) (Chen and Nelson, 2004). Maturity groups are based on the latitudinal zones of growth and differ in photoperiod and temperature, with 000 adapted to higher latitudes and IX adapted to lower latitudes. Genetic studies identified the *ln* locus, an abbreviation for ‘lanceolate’ or narrow leaf-type, which has been shown to pleiotropically control leaf shape and the number of seeds per pod (Domingo, 1945; Dinkins *et al.*, 2002; Sayama *et al.*, 2017). Based on fine mapping approaches, the *ln* locus was associated with a gene at Chromosome 20 (Glyma.20G116200), a homologue of the Arabidopsis JAGGED gene (Jeong *et al.*, 2011, 2012). Recently, a CRISPR-Cas9-based soybean leaf mutant was developed for the *ln* gene and field tested for two seasons (Cai *et al.*, 2021). The study reported 8–10 % yield increase with the introduction of the narrow leaf *ln* gene into a broad-leaved soybean background. Bianchi *et al.* (2020) further demonstrated that narrow-leaved isogenic lines had significantly higher red/far-red values measured at the ground level in both low and high planting densities, providing evidence for better light penetration in lower canopy layers in narrow-leaved isogenic lines. Such narrow leaf-type isogenic lines were also previously reported to have higher photosynthetic rates per unit leaf area (Egli *et al.*, 1970) and greater within-canopy profile of net radiation (Baldocchi *et al.*, 1985) compared to their broad-leaf-type isogenic counterparts. These studies support the notion that altering leaf morphology can have a significant effect on canopy light environments and soybean yield.

The anatomical and physiological differences underlying variation in leaf shape have not been thoroughly investigated. Studying such relationships can decipher trade-offs and co-variations in these components that can occur when targeting such traits for yield improvement. For instance, leaf width is strongly coupled with stomatal density and conductance, vein patterns and intrinsic water use efficiency across C₄ crop species. In field-grown sorghum, leaf width was positively associated with stomatal conductance and interveinal distance between longitudinal veins, but negatively correlated with intrinsic water use efficiency and stomatal density (Pan *et al.*, 2022). Similar observations were made among 20 different C₄ grasses in which leaf width was strongly and positively correlated with stomatal conductance and negatively with intrinsic water use efficiency (Cano *et al.*, 2019). Leaf width is also genetically coupled with vein density in rice, where mutations inducing high vein density also induced narrow leaves (Feldman *et al.*, 2014). Additionally, such high vein density has been shown to be positively correlated with leaf hydraulics and gas exchange variables (Ye *et al.*, 2021). This makes leaf width a potential morphological trait to optimize anatomical,

physiological and hydraulic traits at the leaf level, which can further impact canopy-level photosynthesis and net carbon assimilation.

In this study, we utilized natural leaf shape diversity within the USDA (United States Department of Agriculture) soybean germplasm collection planted in Urbana, Illinois, USA, to investigate differences in leaf anatomy, gas exchange variables, leaf vein density and hydraulic conductance among soybean lines with variation in leaf shape. We hypothesized that lanceolate leaf shape would be associated with greater leaf thickness, photosynthetic capacity and hydraulic conductance. We first surveyed 174 soybean accessions from four different maturity groups (MG), then selected 28 representing the diversity of leaf length to width ratios for anatomical and physiological analysis.

MATERIALS AND METHODS

Plant genetic materials

A total of 28 soybean lines with diverse leaf length to width ratios (hereafter leaf ratio) were selected for this study (Table 1). Leaf ratios of 174 soybean lines spanning MG I–IV were initially measured from the USDA germplasm collection planted in Urbana in 2020. These accessions were planted in four-row plots of 3.35 m length with 0.76 m spacing between rows. From among the 174 soybeans, 28 were selected to capture the diversity of leaf ratios among the 174 soybean lines (Table 1; Fig. 1). Fourth node leaves (sunlit and recently matured) from the top of the stem were selected, and length (from base to the tip along the midvein) and width (widest part of the leaf) of the middle leaflet were measured in triplicate from each line. The plants were at late vegetative stages during measurements and collection of leaves.

For each of the 28 selected lines, two sets of three leaves (six in total) were tagged 1 d before measurements. One set of three trifoliates was used for gas exchange measurements before collecting leaves for anatomical observations (details below). The second set of trifoliates was used for gas exchange, leaf area, leaf mass per unit area (LMA), hydraulic conductance and leaf vein density measurements (details below). Thus, there were three biological replicates per line for all measurements.

Leaf gas exchange measurements

Stomatal conductance to water vapour (g_{sw} , mol H₂O m⁻² s⁻¹), transpiration rate (E , mol H₂O m⁻² s⁻¹) and photosynthetic rate (A , μmol CO₂ m⁻² s⁻¹) were measured on the central portion of the middle trifoliolate (broadest section of the leaf) avoiding the mid-vein on three leaves per line. After gas exchange measurements, leaves were collected for anatomical observations. Three LI-6800 Portable Photosynthesis Systems (LI-COR Inc., Lincoln, NB, USA) equipped with a 6-cm² circular leaf cuvette were used. The measurements were made on a sunny day in the field between 1200 and 1400 h (solar noon at around 1300 h). Cuvette conditions during the measurements were as follows: [CO₂] = 420 ppm, leaf temperature = 32 °C, relative humidity (RH) = 70 %, flow rate = 500 μmol s⁻¹, fan speed = 10 000 rpm, PAR = 1900 μmol m⁻² s⁻¹, and overpressure = 0.1 kPa. For

TABLE I. List of selected 28 soybean lines used in the study with their PI number, cultivar name, maturity group (MG) and leaf length to width ratio (LR) with standard error (\pm s.e.) values. The soybean lines are sorted by their MG followed by LR values.

PI number	Cultivar name	MG	LR (\pm s.e.)
PI612713B	He feng 910	I	3.18 \pm 0.10
PI612713A	He feng 910	I	3.05 \pm 0.45
PI612708B	K 89-9081	I	2.94 \pm 0.09
PI612708C	K 89-9081	I	2.91 \pm 0.09
PI612709B	K 87-104	I	2.67 \pm 0.12
PI592967	Hei nong 36	I	2.57 \pm 0.08
PI593957	NEAC 593	I	2.54 \pm 0.09
PI612718	Harbin 92-1062	I	2.49 \pm 0.23
PI612735	Jiunong 21	I	2.14 \pm 0.01
PI547745	L70-4136	II	3.15 \pm 0.27
PI547778	L72D-4045	II	2.58 \pm 0.14
PI297536	Mandzsu I	II	2.19 \pm 0.16
PI547819	L74-143	III	2.32 \pm 0.10
PI628271	T361	III	1.93 \pm 0.04
PI430619	Feng shou huang tou	III	1.64 \pm 0.02
PI098243	NA	III	1.59 \pm 0.08
FC004007B	NA	III	1.50 \pm 0.03
PI424247A	NA	III	1.36 \pm 0.02
PI437690	Pin-din-guan	III	1.32 \pm 0.02
PI547800	L66-949	III	1.30 \pm 0.08
PI253665D	NA	III	1.25 \pm 0.03
PI547870	L84-2237	III	1.20 \pm 0.02
PI612594	Kottman	III	1.19 \pm 0.04
PI547858	L82-753	III	1.13 \pm 0.01
PI532462A	Fu 51	III	1.11 \pm 0.02
PI547600	L72U-4191	IV	2.70 \pm 0.09
PI547607	L73U-2774	IV	2.54 \pm 0.13
PI548191	T180	IV	1.67 \pm 0.10

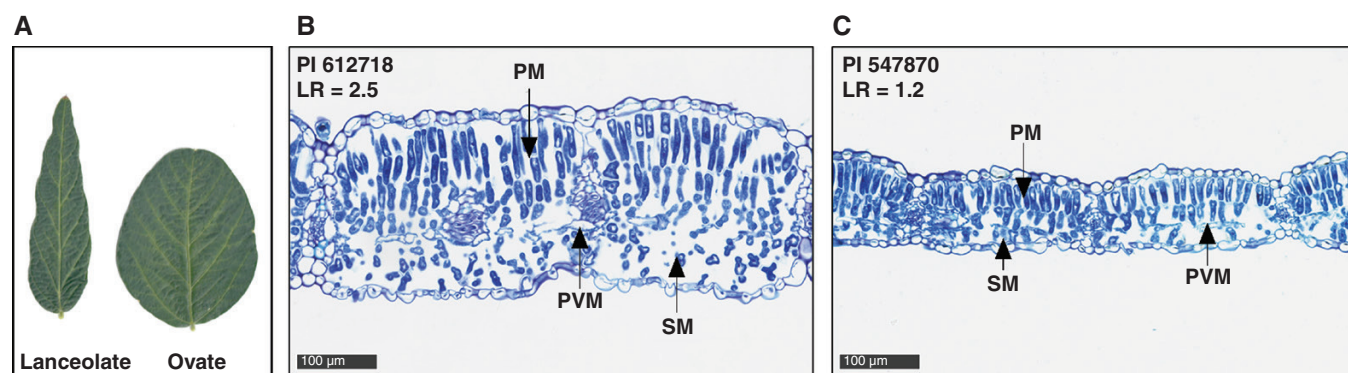


FIG. 1. Representative leaf images and cross-sections showing differences in leaf shape and anatomy. (A) Two extreme soybean leaf shapes, narrow or lanceolate on the left and broad or ovate on the right (these two leaf images are not to scale). (B) Soybean PI 612718 which has narrow leaf with leaf ratio (LR) of 2.5 and thickness of 251 μ m; (C) soybean PI 547870 which has broad leaf with leaf ratio of 1.2 and thickness of 101 μ m. Abbreviations: PM, PVM, SM and LR refer to palisade mesophyll, paraveinal mesophyll, spongy mesophyll layers and leaf length to width ratio, respectively.

each measurement, two stability thresholds were met before recording the values. The slope of Δg_{sw} had to be below 0.05 over a 15-s period and the slope of ΔA had to be below 0.7 over the same 15-s period. Relative chlorophyll content of each leaf was measured using a handheld MultispeQ instrument (PhotosynQ Inc., East Lansing, MI, USA).

Leaf anatomical observations

The same region of the middle trifoliolate leaf used for gas exchange measurements was harvested for anatomical analysis. These leaf segments spanned from blade-to-blade on both sides of the mid-vein, enabling the assessment of leaf anatomy across the leaf. For each leaf, three segments (each of ~10 mm) were cut with sharp scissors and immediately immersed in 10 % NBF (neutral buffer formalin) fixative kept in 50-mL falcon tubes. They were further cut into smaller strips and dehydrated in 70 % ethanol and processed in a Tissue-Tek VIP sixtissue processor (Sakura Finetek USA Inc., Torrance, CA, USA) for paraffin infiltration. This was followed by tissue embedding in paraffin, which was used to obtain 5- μ m paraffin sections cut with a microtome (Leica RM 2125 RTS; Leica Biosystems Inc., Buffalo Grove, IL, USA). The cut sections were dried and then deparaffinized, rehydrated and stained in 0.1 % toluidine blue solution for 10 min. Following a quick water wash, these were dehydrated through a graded ethanol series, cleared in xylene and covered with a glass cover slip.

All slides were scanned with a high-throughput slide scanning system (NanoZoomer HT model C9600-12, Hamamatsu Photonics, Tokyo, Japan) at a resolution of 460 nm with a 40 \times source lens. The image resolution of each slide was 225 nm/pixel with image size of 150 784 \times 94 720 pixels. The scanned images were saved as ndpi digital format and were analysed using NanoZoomer Digital Pathology Image (NDPI) analysis software (NDP.view2, Hamamatsu Photonics).

From each leaf cross-section, five areas were randomly selected on each side of the mid-vein (a total of ten per cross-section) spanning the entire length of the cross-section from mid-vein to the edge of the leaf (see [Supplementary Data Fig. S1](#) for details). For each of these selected areas, the following measurements were made: (1) distance of the section from mid-vein, (2) leaf thickness (LT, distance between upper and lower epidermis), (3) interveinal distance (distance between right and left vascular bundle), (4) area of the section between right and left vascular bundle excluding epidermal cells, (5) area of palisade mesophyll layer, (6) area of spongy mesophyll layer and (7) area of paraveinal mesophyll (PVM) layer (see [Lansing and Francheschi, 2000](#) for a review of PVM).

Leaf hydraulic conductance (K_{leaf}) measurements

A second set of tagged leaves was used for K_{leaf} measurements following the evaporation flux method (Licor-EFM) detailed in [Sade et al. \(2014\)](#). Briefly, gas exchange measurements were conducted (as described above) 1 d before the trifoliolates were harvested at dawn along with the petiole. The cut leaves were immediately placed inside ziplock bags with

wet paper towels so that tissues remained fully hydrated. The petioles were recut in the lab while submerged under water with the leaf blades exposed to air and kept inside a growth chamber for 30 min before further measurements. The growth chamber settings were as follows: PAR = 1200 μ mol m⁻² s⁻¹, temperature = 25 °C and RH = 60 %. After acclimation in the growth chamber (assuming the leaves achieved steady-state E), gas exchange measurements were taken with three LI-6800 Portable Photosynthesis Systems equipped with a 6-cm² circular leaf cuvette to obtain E values. The cuvette settings were set identical to the growth chamber settings as provided above. Next, the leaves were immediately sealed inside ziplock bags with a wet paper towel for 30 min and leaf water potential (ψ_1) of the leaves was measured using a pressure chamber (Model PMS EXP-1100). K_{leaf} was calculated as follows: $K_{leaf} = E / (0 - \psi_1)$

Leaf vein density, leaf area and leaf mass per unit area measurements

The same middle trifoliolate used for K_{leaf} measurements was used to analyse leaf vein architecture. Leaves were prepared following a leaf clearing procedure described by [Vasco et al. \(2014\)](#). Briefly, leaves were immersed in a 5 % NaOH solution and kept in a shallow glass container at 50 °C for a few days. After it was determined that leaves were transparent enough to visualize the veins, they were rinsed with water three times and left in a water bath at room temperature for 10 min. Next, the leaves were bleached in 5 % sodium hypochlorite for up to 5 min inside a laminar flow hood. Once the cleared leaves turned white, they were rinsed with water and kept in a water bath for 10 min. Leaf veins were stained with safranin (in 95 % ethanol) after dehydrating in a graded ethanol series of 50, 70 and 95 % for 30 min. Excessive staining was washed in twice with 100 % ethanol, each for 10 min. Finally, leaves were stored in 100 % ethanol before imaging. Imaging of the whole leaf was done with an AxioZoom V16 microscope (Zeiss Microscopy, Jena, Germany) fitted with a Zeiss AxioCam 512 colour camera. The objective magnification was set at 1 \times with 7 \times zoom while the numerical aperture was set at 0.25 with a working distance of 56 mm. The captured images were analysed with PhenoVein software ([Bühler et al., 2015](#)) for total leaf area and leaf vein density measurements.

From the remaining two side leaflets, eight leaf punches of known area (diameter of 1.75 cm) were collected and dried at 65 °C for 10 d days, leaf mass per unit area was calculated by dividing the leaf dry mass by its area.

Data analysis

From each leaf section, ten regions were randomly selected (five on each side of the midvein) for measurements of the seven anatomical variables mentioned above. Regression analysis showed that these regions did not significantly vary in their thickness ([Fig. 2C](#)). In addition, the size of these regions (interveinal distance) had no effect on the proportion of mesophyll layers ([Fig. 2D](#)). Therefore, leaf thickness and proportion of the three soybean mesophyll layers were averaged across all ten measurements at the replicate level and used for analyses.

One-way analysis of variance (ANOVA) and pairwise correlation analyses were carried out on all 15 measured or derived variables (Table 2). For statistically significant correlations, linear regression analysis was carried out. All the above-mentioned statistical analyses were conducted using PRISM 9.2.0 (GraphPad Software Inc., San Diego, CA, USA). The correlation matrix plot was generated using *corrplot* package v.0.91 (Wei and Simko, 2021) in R v.4.0.4 (R Core Team, 2021).

RESULTS

Diversity in leaf morpho-anatomical and physiological traits

Soybean genotypes showed significant genetic variation in leaf shape, from narrow or lanceolate with a leaf to width ratio (LR) of 1.1 to ovate or broad with an LR of 3.2 (Fig. 1). All anatomical and physiological traits except K_{leaf} varied significantly among the 28 soybean genotypes (Table 2). Leaf phenotypes appeared to vary with MG. Accessions in MG I

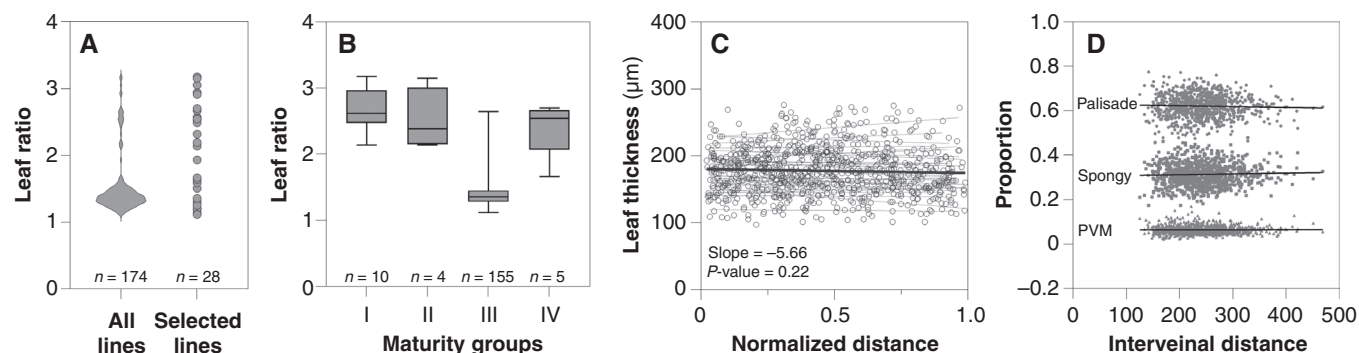


FIG. 2. (A) Leaf length to width ratio (Leaf ratio) measured from the middle trifoliate leaflet at fourth node from the top of the plant in 174 USDA soybean collection lines. The violin plot shows the distribution of leaf ratios for 174 soybean lines initially measured and 28 accessions selected for physiological analysis. (B) Box plots showing the distribution of leaf ratios by maturity groups (MG). The whiskers represent minimum and maximum values with median value represented by the horizontal bar within each box. The number of soybean lines for each MG is provided under each box. (C) The effect of distance from the midvein on leaf thickness measured on randomly selected sections across the leaf. The distance is normalized, where 1.0 is the distance from the midvein to the leaf margin so that leaves with different widths can be compared. Open circles ($n = 840$) represent all the thickness values from 28 selected lines measured across three replicates at ten sections per replicate from the midvein to the tip of the leaf. A linear regression fit line for all the datapoints is represented by a single thick black line while thin grey lines represent the linear fit for each of 28 selected lines. (D) The effect of area of the randomly selected interveinal section (expressed in interveinal distance values) on the proportion of three different mesophyll tissue types (palisade mesophyll, paraveinal mesophyll-PVM and spongy mesophyll). The linear regression fit is represented by a single thick black line for the three mesophyll layers which do not significantly deviate from zero ($P = 0.25, 0.27$ and 0.86 for palisade mesophyll, PVM and spongy mesophyll, respectively).

TABLE 2. List of 15 measured or derived leaf traits with their abbreviations and units, mean, range, sample size (n) and P-values from one-way analysis of variance (ANOVA).

Trait	Abbreviation (units)	Mean	Range	N	P-value
Morpho-anatomical					
Leaf area	LA (cm ²)	56.8	34.5–88.9	28	<0.0001
Leaf length	L (cm)	10.55	7.63–15.57	28	<0.0001
Leaf width	W (cm)	5.55	3.80–9.36	28	<0.0001
Leaf length to width ratio	LR	2.1	1.1–3.20	26	<0.0001
Leaf thickness	LT (μm)	177.3	118.5–241.1	28	<0.0001
Leaf mass per unit area	LMA (g m ⁻²)	46.9	33.9–61.4	28	<0.0001
Proportion of palisade layer	PP	0.62	0.57–0.66	28	<0.0001
Proportion of spongy layer	PS	0.31	0.27–0.36	28	<0.0001
Proportion of PVM layer	PPVM	0.06	0.05–0.08	28	<0.0001
Vein density	VD (mm mm ⁻²)	1.3	1.0–1.7	23	0.0142
Physiological					
Relative greenness	SPAD	46.8	38.6–52.9	28	<0.0001
Photosynthetic rate	A (μmol CO ₂ m ⁻² s ⁻¹)	35.1	24.3–42.8	28	<0.0001
Transpiration rate	E (mol H ₂ O m ⁻² s ⁻¹)	0.009	0.006–0.012	28	0.0048
Stomatal conductance	g_{sw} (mol H ₂ O m ⁻² s ⁻¹)	0.96	0.47–1.31	28	0.008
Hydraulic conductance	K_{leaf} (mmol m ⁻² s ⁻¹ MPa ⁻¹)	14.0	9.4–19.3	28	0.19

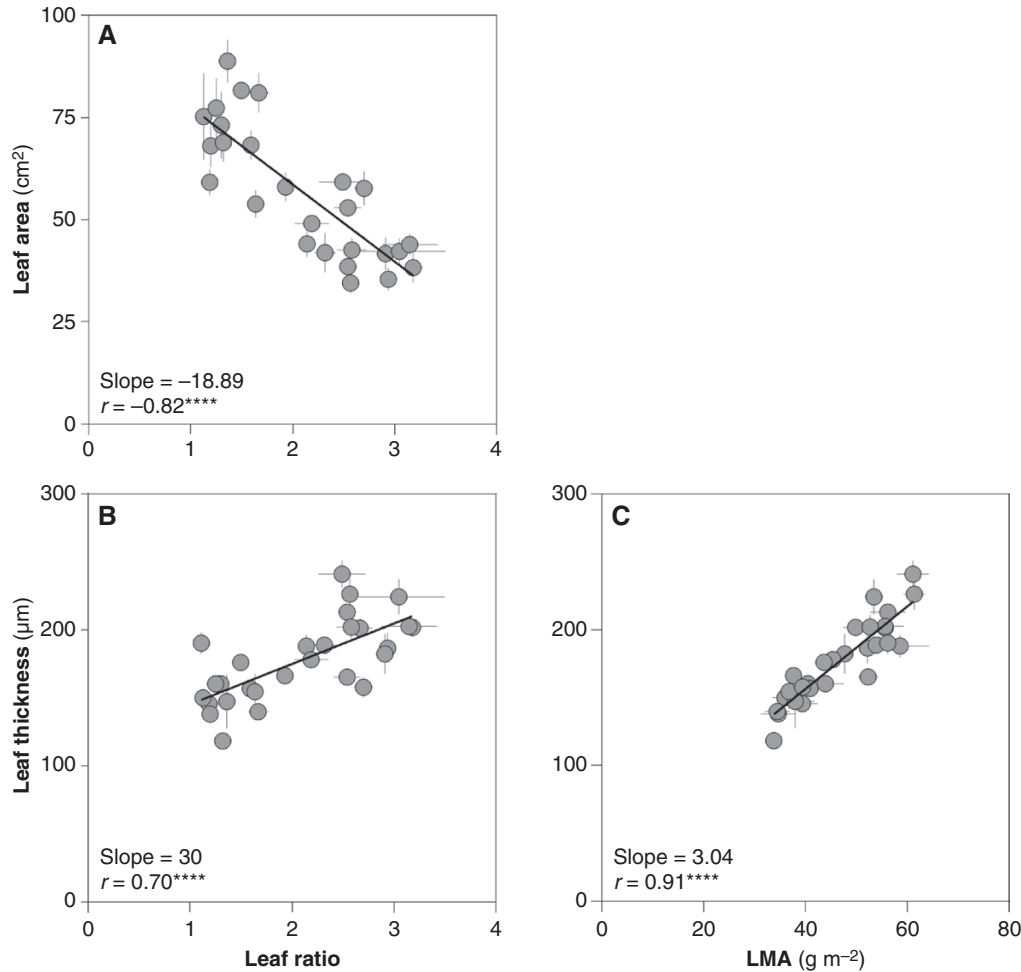


FIG. 3. Relationship between leaf ratio (LR), leaf thickness (LT) and leaf mass per unit area (LMA). (A, B) Association of LR with LA and LT, respectively. (C) Relationship between LT and LMA. In all the panels, slopes of the linear regression fit (represented by the linear line segment) and Pearson's correlation coefficient (r) along with asterisks representing significance are provided. $^{****}P < 0.0001$ ($\alpha = 0.05$). Error bars represent standard errors.

had the highest average LR values (2.72) while MG III soybeans had the lowest LR values (1.45) (Fig. 2B). However, we did not test a uniform number of accessions in each MG. Other morpho-anatomical traits also showed significant variation (Table 2). The proportion of palisade mesophyll ranged from 0.57 to 0.66 ($P < 0.0001$), while the proportions of spongy and paraveinal mesophyll varied 1.3- and 1.6-fold, respectively (Table 2). Leaf area showed the most variation, ranging from 34.5 to 88.9 cm² ($P < 0.0001$). This was closely followed by leaf mass per unit area, which showed 2-fold variation (range = 33.9 and 61.4 g m⁻², $P < 0.0001$). Leaf thickness and vein density had similar trait variations of 1.8- and 1.7-fold, respectively (Table 2).

Soybean genotypes also showed significant variation in physiological traits (Table 2). Relative chlorophyll content ranged from 38.6 to 52.9 and g_{sy} showed a 2.8-fold range, from 0.47 to 1.31 mol H₂O m⁻² s⁻¹. The other two area-based gas exchange variables, A (range = 24.3–42.8 µmol CO₂ m⁻² s⁻¹, $P < 0.0001$) and E (range = 0.006–0.012 µmol CO₂ m⁻² s⁻¹, $P < 0.005$), had 1.8- and 2-fold differences in their trait values, respectively (Table 2). K_{leaf} showed 2-fold variation (range = 9.4–19.3 mmol m⁻² s⁻¹ MPa⁻¹) but was not statistically

significant ($P = 0.19$) due to large variation among measurements within a genotype.

Relationship among leaf traits

Leaf width was significantly correlated with anatomical and physiological traits, much more so than leaf length (Supplementary Data Fig. S2). Leaves with greater LR (i.e. narrow leaves) tended to have lower leaf area (Fig. 3A). Leaves were ~30 µm thicker for every unit increase in leaf ratio (Fig. 3B). As expected, leaf thickness was highly correlated with leaf mass per unit area (Fig. 3C).

Another interesting observation made from the anatomical study of these diverse genotypes was that narrow leaves had a lower proportion of palisade mesophyll ($r = -0.59$, $P = 0.0009$) and paraveinal mesophyll ($r = -0.46$, $P < 0.0145$) which led to a higher proportion of spongy mesophyll ($r = 0.66$, $P = 0.0001$) (Fig. 4). Compared to the proportion of paraveinal mesophyll, the proportion of palisade mesophyll had a strong negative correlation with the proportion of spongy mesophyll (Supplementary Data Fig. S2, $r = -0.96$,

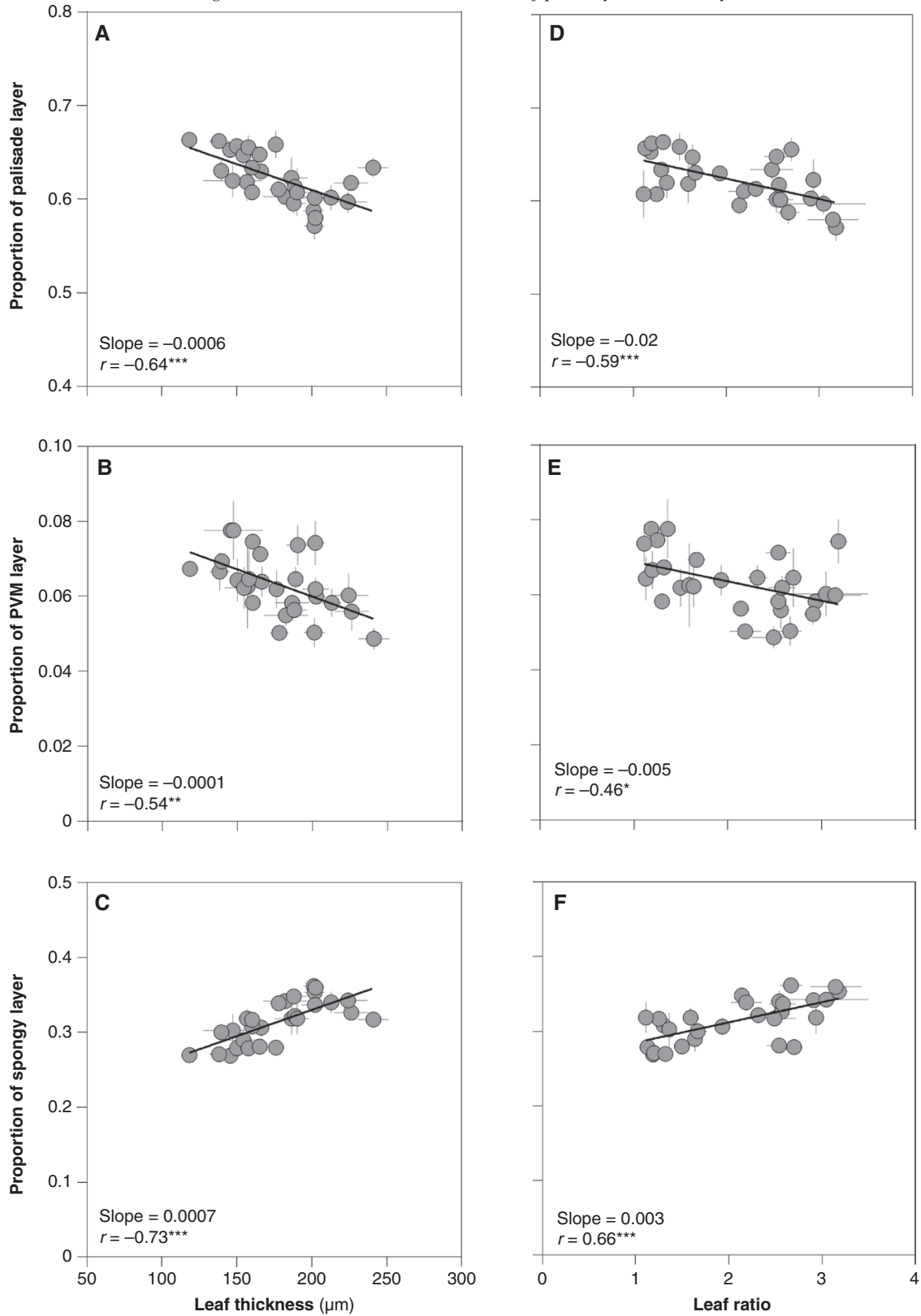


FIG. 4. Association of leaf thickness (LT) and leaf ratio (LR) with proportion of three different mesophyll tissue layers, palisade mesophyll (PP; A and D), paraveinal mesophyll (PPVM; B and E) and spongy mesophyll (PS; C and F). In all the panels, slopes of the linear regression fit (represented by the linear line segment) and Pearson's correlation coefficient (r) along with asterisks representing significance are provided. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ ($\alpha = 0.05$). Error bars represent standard errors.

$P < 0.0001$). Since leaf ratio was tightly correlated with leaf thickness (Fig. 3B), leaf thickness had a similar association with the proportion of the three mesophyll tissue layers (Fig. 4A–C).

Narrow leaves, which were thicker, had higher relative chlorophyll content (and these values were more tightly correlated with leaf thickness compared to leaf ratio (Fig. 5A, D). Leaf ratio and leaf thickness were correlated with greater A (Fig. 5B, E) and g_{sw} (Fig. 5C, F). The correlation was positive but non-significant with E (data not shown, $r = 0.34$, $P = 0.0783$ with leaf ratio and $r = 0.24$, $P = 0.2235$ with leaf thickness).

While K_{leaf} was the only trait that did not vary significantly across lines ($P = 0.19$), it was correlated with leaf thickness (Fig. 6A) and vein density (Fig. 6B). However, leaf ratio was not significantly associated with either K_{leaf} (Fig. 6C) or vein density (Fig. 6D).

DISCUSSION

In this study, we demonstrated that soybeans with different leaf shapes varied in their anatomical, physiological and hydraulic properties. A number of ecological theories have been formed to explain the diversity of leaf shape observed in nature (Nicotra *et al.*, 2011). Functionally, variation in leaf shape reflects trade-offs in water supply with high vein densities supporting high photosynthetic rates (Brodribb *et al.*, 2010). Additionally, soybean leaf shape has been correlated with seed number per pod and yield (Domingo, 1945; Dinkins *et al.*, 2002; Jeong *et al.*, 2012; Sayama *et al.*, 2017; Bianchi *et al.*, 2020). Utilizing some of the diversity within the USDA soybean collection, we selected a subset of 28 soybeans that represented a range of observable variation in soybean leaf shape. The mature, sunlit leaves of these selected soybeans were subjected to combined gas exchange, hydraulics, cross-sectional and vein architecture measurements, which allowed detailed examination of leaf properties in relation to leaf shape.

Narrow leaves tend to be small but thicker with higher leaf mass per unit area

Soybeans chosen for this study were in MG I to MG IV (Table 1). Previous examination of 6169 wild soybeans found that a narrow leaf shape was mostly observed among lines originating from higher latitudes or early maturing groups (Yan *et al.*, 2014). We also found that the narrowest leaves ($LR > 3$) were exclusively from MG I and II (Table 1, Fig. 2B), consistent with previous studies of 6169 wild soybeans (Yan *et al.*, 2014). It is possible that the narrow leaf trait evolved as a response to climatic conditions of higher latitudes to maximize light capture across the canopy layers and compensate for shorter leaf life span. In addition, narrow leaves seem to compensate for loss in leaf area and size by increasing leaf thickness and therefore leaf mass per unit area (Fig. 3B, C), which is associated with high leaf tissue density (Poorter *et al.*, 2009). This has two advantages. The first is biomechanical, where thicker leaves provide support to a slender and narrow leaf structure, perhaps a necessity to prevent those leaves from drooping or to prevent wind damage (Read and Stokes, 2006; Onoda *et*

al., 2011). The second is physiological, where a thicker leaf positively scales with cell size, cell wall and mesophyll tissue thickness (John *et al.*, 2013), the implications of which we discuss further.

Narrow leaves have higher proportions of spongy mesophyll tissues

Soybean has a reticulated leaf venation architecture with typical upper and lower epidermis, two to three layers of palisade and a layer of spongy mesophyll cells (Fig. 1B, C). Many legumes, including soybean, have an additional specialized mesophyll layer between the palisade and spongy mesophyll cells, PVM, which serves as a conduit for photosynthates from photosynthesizing cells to the phloem cells of vascular bundles (Lansing and Franceschi, 2000; Murphy *et al.*, 2005). In addition to the fact that mesophyll cell size and number increase proportionally to leaf thickness (John *et al.*, 2013), for the first time we show how three mesophyll tissues (palisade, paraveinal and spongy mesophylls, Fig. 1B, C) partition in relation to leaf shape in soybean (Fig. 4). Spongy mesophyll layers occupied a greater fraction of the total mesophyll volume in narrow leaves as the thickness increased at the expense of the space occupied by palisade and paraveinal mesophyll layers. A higher spongy layer volume changes optical properties and leads to greater light scattering within the leaf, increasing light absorption by chloroplasts (Smith *et al.*, 1997). The strong negative correlation ($r = -0.96$) observed between proportions of palisade and spongy mesophyll layers indicates how leaf construction costs are optimized between these two tissue types since they collectively occupy most of the mesophyll volume (90–95 %, Fig. 2D). Although the proportion of palisade and paraveinal mesophyll layers did not correlate with each other, the proportion of both tissues decreased with leaf thickness, suggesting their non-linear but interdependent relationship.

Narrow leaves have higher gas exchange rates

Leaf anatomy has a direct influence on internal light dynamics, internal CO_2 concentration and diffusion, surface boundary layer resistance, and leaf water relationships affecting leaf-level conductance and photosynthesis (Tholen *et al.*, 2012; Ren *et al.*, 2019). As expected, we observed a positive correlation between leaf thickness and area-based A and g_{sw} in our dataset (Fig. 5), supporting results observed in other species (Huang *et al.*, 2022). Thicker leaves have larger and/or higher numbers/layers of photosynthesizing mesophyll cells and therefore more chlorophyll content per area [higher SPAD (Soil Plant Analysis Development) reading, Fig. 5]. In addition, narrow leaves have thinner air boundary layers (Nobel, 2009) allowing less resistance to gas exchange between internal leaf air spaces and the external atmosphere. This shows that narrow leaves compensate for loss in total leaf area with rates of A and greater g_{sw} in addition to increased leaf thickness and leaf mass per unit area, a trait adaptation that increases fitness. Interestingly, E did not vary with leaf thickness or leaf ratio, indicating there are virtually non-existent trade-offs between leaf shape and water use.

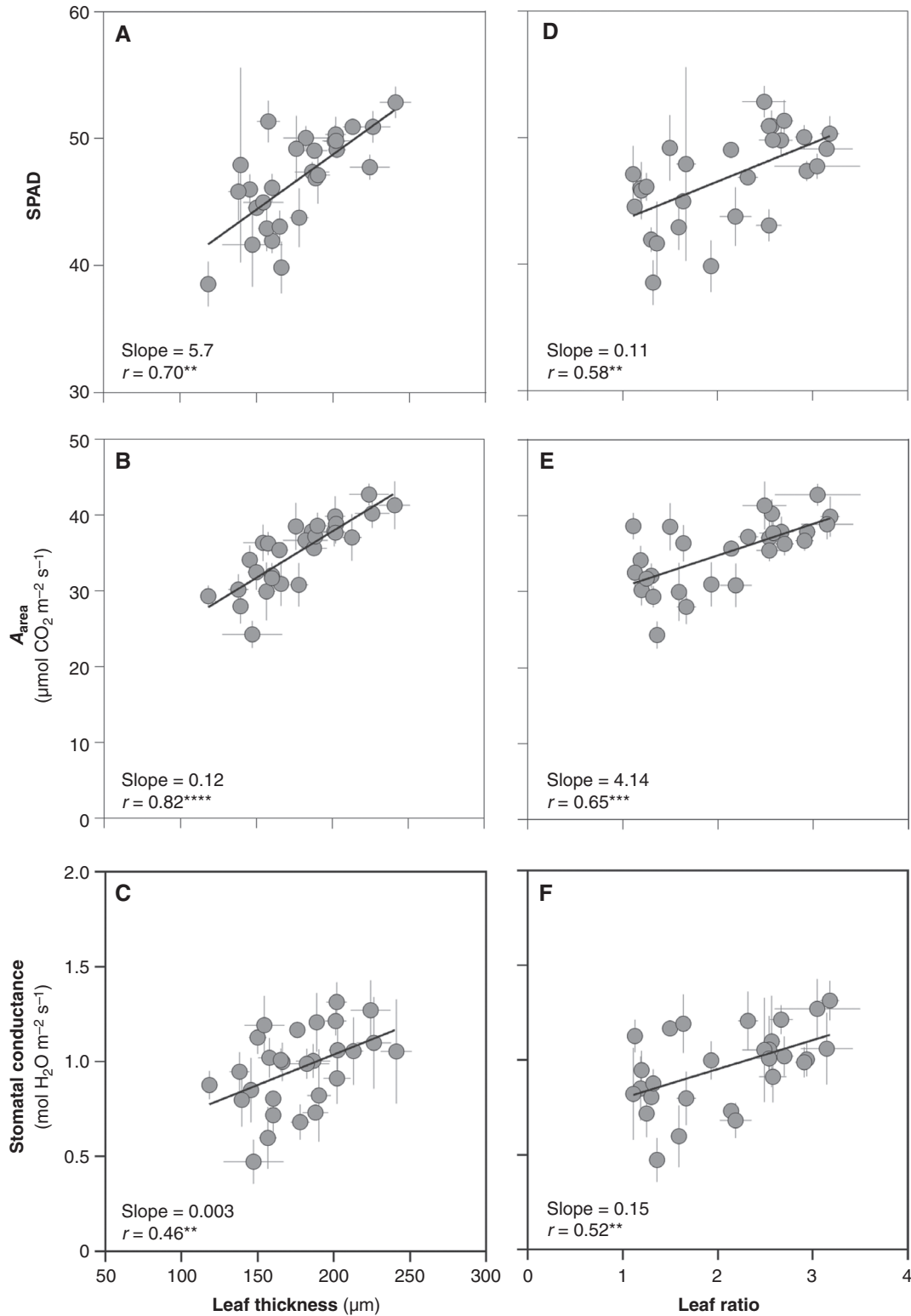


FIG. 5. Correlation of leaf thickness (LT) and leaf ratio (LR) with relative greenness (SPAD; A and D), area-based photosynthetic rate (A_{area} ; B and E) and stomatal conductance (g_{sw} ; C and F). In all the panels, slopes of the linear regression fit (represented by the linear line segment) and Pearson's correlation coefficient (r) along with asterisks representing significance are provided. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$ ($\alpha = 0.05$). Error bars represent standard errors.

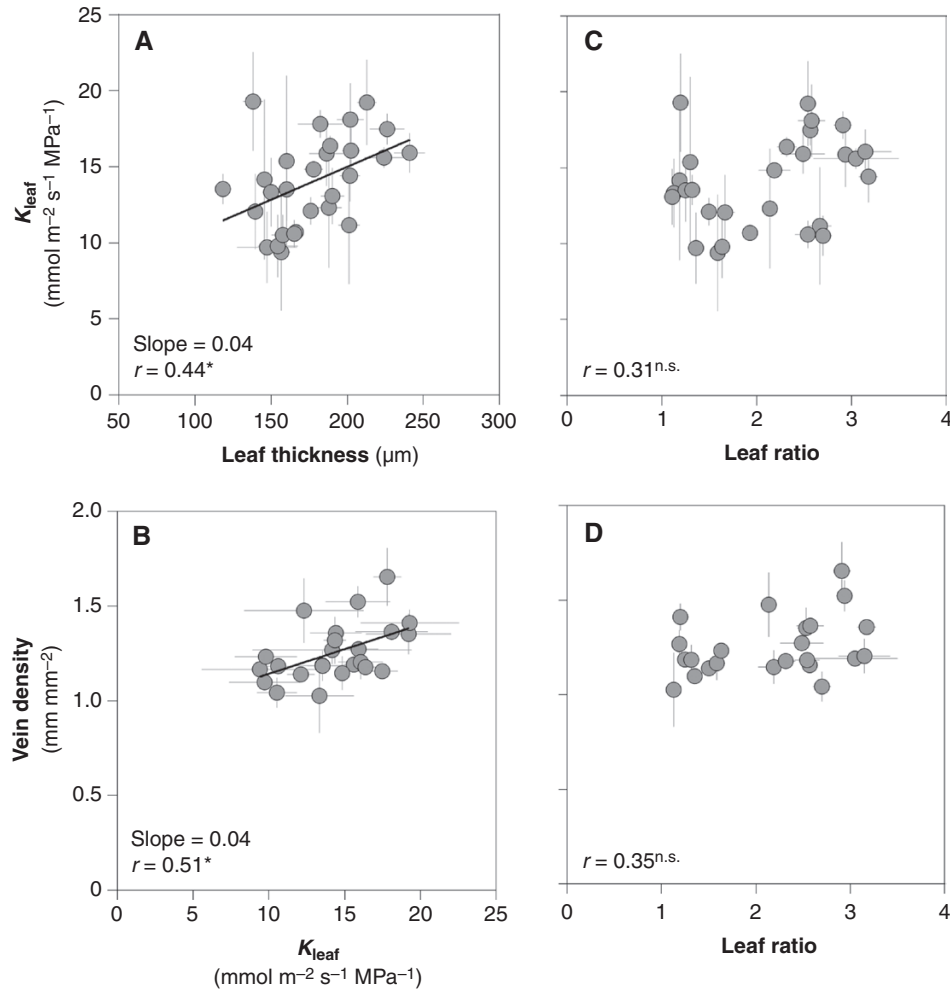


FIG. 6. Correlation between leaf vein density (VD), leaf hydraulics (K_{leaf}), leaf thickness (LT) and leaf ratio (LR). (A–C) Correlation of K_{leaf} with LT, VD and LR, respectively. (D) Correlation between VD and LR. In all the panels, slopes of the linear regression fit (represented by the linear line segment) and Pearson's correlation coefficient (r) along with asterisks representing significance are provided. * $P < 0.05$ and $^{\text{n.s.}}P > 0.05$ ($\alpha = 0.05$). Error bars represent standard errors.

Leaf hydraulics (K_{leaf}) is driven by vein density and leaf thickness

K_{leaf} positively scaled with leaf thickness (Fig. 6A), which potentially contributed to the measured higher A and g_{sw} in narrow leaves, although we did not observe significant genotypic variation in K_{leaf} . Vein density, which is loosely correlated with K_{leaf} (Fig. 6B), is more than 3-fold lower than what has been documented in some studies in soybean (e.g. 1–1.7 mm mm^{-2} in our measurements vs. 4.23–4.95 mm mm^{-2} in Tanaka and Shiraiwa, 2010). One possible reason is the difference in the resolution of images (40 \times in our study vs. 100 \times in Tanaka and Shiraiwa, 2010). Higher image resolution is required to resolve minor veins which largely contribute to the overall vein density values (Sack et al., 2012; Sack and Scoffoni, 2013). This probably limited the images to capture only major veins and therefore led to lower vein density values. This also possibly led to a non-significant association between vein density and leaf thickness or leaf ratio, or a weak correlation between K_{leaf} and vein density because vein density has been documented to strongly drive K_{leaf} and gas exchange variables (Brodribb et al., 2007; Boyce et al., 2009). However, a lower imaging resolution was

necessary in our study to cover the whole leaf surface while achieving reasonable imaging speed and file size that could later be handled by the imaging software used (see Materials and Methods).

CONCLUSION

Modelling and experimental studies indicate that the narrow leaf trait in soybean can potentially contribute to better canopy light distribution and yield. Here, we examined the anatomical and physiological traits correlated with leaf shape. There is extreme variation in leaf shape in the USDA soybean germplasm collection that is strongly linked to different anatomical and physiological traits. Narrow leaves have lower leaf area, but greater vein density and leaf thickness, supporting higher gas exchange and carbon capture per unit area. The distribution of palisade and spongy mesophyll also varies with leaf thickness. Selecting for the narrow leaf trait in combination with other agronomically important traits could potentially provide yield advantages in soybean.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. **Figure S1.** Representative leaf cross-section of soybean line PI 592967 showing the anatomical measurement method. **Figure S2.** Correlation matrix of the traits considered in the study.

ACKNOWLEDGMENTS

We thank Esther Peregrine and the USDA ARS Soybean/maize Germplasm, Pathology, and Genetics Research Unit for facilitating access to the USDA germplasm field plots, Chris Moller for help with gas exchange measurements, and Karen Doty for help in preparing microscope slides of leaf cross-sections. Any opinions, findings and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the U.S. Department of Agriculture. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer. B.G.T., Y.Z. and E.A.A. designed the experiments; B.G.T. and M.A.Z. carried out leaf clearing, imaging and anatomical measurements; B.G.T. and Y.Z. conducted leaf hydraulic conductance and gas exchange measurements; B.G.T. led the manuscript writing, and all authors edited it. The authors declare no competing interests.

FUNDING

This research was supported by a subaward from the University of Illinois as part of the research project Realizing Increased Photosynthetic Efficiency (RIPE) funded by the Bill & Melinda Gates Foundation, the Foundation for Food and Agriculture Research, and the UK Government's Foreign, Commonwealth & Development Office under Grant OPP1172157.

LITERATURE CITED

- Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO₂ enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO₂. *New Phytologist* **165**: 351–372.
- Baldocchi DD, Verma SB, Rosenberg NJ, Blad BL, Specht JE. 1985. Microclimate–plant architectural interactions: influence of leaf width on the mass and energy exchange of a soybean canopy. *Agricultural and Forest Meteorology* **35**: 1–20.
- Bianchi JS, Quijano A, Gosparini CO, Morandi EN. 2020. Changes in leaflet shape and seeds per pod modify crop growth parameters, canopy light environment, and yield components in soybean. *The Crop Journal* **8**: 351–364. doi:10.1016/j.cj.2019.09.011.
- Blankenship RE, Chen M. 2013. Spectral expansion and antenna reduction can enhance photosynthesis for energy production. *Current Opinion in Chemical Biology* **17**: 457–461. doi:10.1016/j.cbpa.2013.03.031.
- Boyce CK, Brodribb TJ, Field TS, Zwieniecki MA. 2009. Angiosperm leaf vein evolution was physiologically and environmentally transformative. *Proceedings of the Royal Society B* **276**: 1771–1776.
- Brodribb TJ, Feild TS, Jordan GJ. 2007. Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiology* **144**: 1890–1898. doi:10.1104/pp.107.101352.
- Brodribb TJ, Feild TS, Sack L. 2010. Viewing leaf structure and evolution from a hydraulic perspective. *Functional Plant Biology* **37**: 488–498. doi:10.1071/fp10010.
- Bühler J, Rishmawi L, Pflugfelder D, et al. 2015. phenoVein – a tool for leaf vein segmentation and analysis. *Plant Physiology* **169**: 2359–2370. doi:10.1104/pp.15.00974.
- Cai Z, Xian P, Cheng Y, et al. 2021. CRISPR/Cas9-mediated gene editing of GmJAGGED1 increased yield in the low-latitude soybean variety Huachun 6. *Plant Biotechnology Journal* **19**: 1898–1900. doi:10.1111/pbi.13673.
- Cano FJ, Sharwood RE, Cousins AB, Ghannoum O. 2019. The role of leaf width and conductances to CO₂ in determining water use efficiency in C₄ grasses. *New Phytologist* **223**: 1280–1295. doi:10.1111/nph.15920.
- Chen Y, Nelson RL. 2004. Evaluation and classification of leaflet shape and size in wild soybean. *Crop Science* **44**: 671–677. doi:10.2135/cropsci2004.0671.
- Dinkins RD, Keim KR, Farno L, Edwards LH. 2002. Expression of the narrow leaflet gene for yield and agronomic traits in soybean. *Journal of Heredity* **93**: 346–351.
- Domingo WE. 1945. Inheritance of number of seeds per pod and leaflet shape in the soybean. *Journal of Agricultural Research* **70**: 251–268.
- Egli DB, Pendketon JW, Peters DB. 1970. Photosynthetic rate of three soybean communities as related to carbon dioxide levels and solar radiation. *Agronomy Journal* **62**: 411–414.
- Emmel C, D'Odorico P, Revill A, et al. 2020. Canopy photosynthesis of six major arable crops is enhanced under diffuse light due to canopy architecture. *Global Change Biology* **26**: 5164–5177. doi:10.1111/gcb.15226.
- Feldman AB, Murchie EH, Leung H, Baraoidan M, Coe R, Yu S-M, Lo S-F, Quick WP. 2014. Increasing leaf vein density by mutagenesis: laying the foundations for C₃ rice. *PLoS One* **9**: e94947.
- Hikosaka K, Hirose T. 1997. Leaf angle as a strategy for light competition: optimal and evolutionarily stable light-extinction coefficient within a leaf canopy. *Ecoscience* **4**: 501–507.
- Hirose T. 2005. Development of the Monsi–Saeki theory on canopy structure and function. *Annals of Botany* **95**: 483–494. doi:10.1093/aob/mci047.
- Huang G, Shu Y, Peng S, Li Y. 2022. Leaf photosynthesis is positively correlated with xylem and phloem areas in leaf veins in rice (*Oryza sativa*) plants. *Annals of Botany* **129**: 619–631. doi:10.1093/aob/mcac020.
- Jaikumar NS, Stutz SS, Fernandes SB, et al. 2021. Can improved canopy light transmission ameliorate loss of photosynthetic efficiency in the shade? An investigation of natural variation in *Sorghum bicolor*. *Journal of Experimental Botany* **72**: 4965–4980. doi:10.1093/jxb/erab176.
- Jeong N, Moon J-K, Kim HS, Kim C-G, Jeong S-C. 2011. Fine genetic mapping of the genomic region controlling leaflet shape and number of seeds per pod in the soybean. *Theoretical and Applied Genetics* **122**: 856–874.
- Jeong N, Suh SJ, Kim M-H, et al. 2012. *Lt1* is a key regulator of leaflet shape and number of seeds per pod in soybean. *The Plant Cell* **24**: 4807–4818. doi:10.1105/tpc.112.104968.
- John GP, Scoffoni C, Sack L. 2013. Allometry of cells and tissues within leaves. *American Journal of Botany* **100**: 1936–1948. doi:10.3732/ajb.1200608.
- Kumagai E, Aoki N, Masuya Y, Shimono H. 2015. Phenotypic plasticity conditions the response of soybean seed yield to elevated atmospheric CO₂ concentrations. *Plant Physiology* **169**: 2021–2029. doi:10.1104/pp.15.00980.
- Lansing AJ, Franceschi VR. 2000. The paraveinal mesophyll: a specialized path for intermediary transfer of assimilates in legume leaves. *Australian Journal of Plant Physiology* **27**: 757–767.
- Li R, Zhang G, Liu G, et al. 2021. Improving the yield potential in maize by constructing the ideal plant type and optimizing the maize canopy structure. *Food and Energy Security* **10**: e312.
- Murphy KA, Kuhle RA, Fischer AM, Anterola AM, Grimes HD. 2005. The functional status of paraveinal mesophyll vacuoles changes in response to altered metabolic conditions in soybean leaves. *Functional Plant Biology* **32**: 335–344.
- Nicotra AB, Leigh A, Boyce CK, et al. 2011. The evolution and functional significance of leaf shape in the angiosperms. *Functional Plant Biology* **38**: 535–552. doi:10.1071/fp11057.
- Nobel PS. 2009. *Physicochemical and environmental plant physiology*, 4th edn. Saint Diego: Academic Press.
- Oikawa S, Okada M, Hikosaka K. 2013. Effects of elevated CO₂ on leaf area dynamics in nodulating and non-nodulating soybean stands. *Plant and Soil* **373**: 627–639. doi:10.1007/s11104-013-1826-6.

- Onoda Y, Westoby M, Adler PB, et al. 2011.** Global patterns of leaf mechanical properties. *Ecology Letters* **14**: 301–312.
- Ort DR, Zhu X, Melis A. 2011.** Optimizing antenna size to maximize photosynthetic efficiency. *Plant Physiology* **155**: 79–85.
- Pan L, George-Jaeggli B, Borrell A, et al. 2022.** Coordination of stomata and vein patterns with leaf width underpins water-use efficiency in a C₄ crop. *Plant, Cell & Environment* **45**: 1612–1630. doi:10.1111/pce.14225.
- Poorter H, Niinemets U, Poorter L, Wright IJ, Villar R. 2009.** Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytologist* **182**: 565–588. doi:10.1111/j.1469-8137.2009.02830.x.
- R Core Team. 2021.** *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Consulting. <https://www.R-project.org/>.
- Read J, Stokes A. 2006.** Plant biomechanics in an ecological context. *American Journal of Botany* **93**: 1546–1565. doi:10.3732/ajb.93.10.1546.
- Ren T, Weraduwage SM, Sharkey TD. 2019.** Prospects for enhancing leaf photosynthetic capacity by manipulating mesophyll cell morphology. *Journal of Experimental Botany* **70**: 1153–1165.
- Sack L, Scoffoni C, McKown AD, et al. 2012.** Developmentally based scaling of leaf venation architecture explains global ecological patterns. *Nature Communications* **3**: 837.
- Sack L, Scoffoni C. 2013.** Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. *New Phytologist* **198**: 983–1000. doi:10.1111/nph.12253.
- Sade N, Shatil-Cohen A, Attia Z, et al. 2014.** The role of plasma membrane aquaporins in regulating the bundle sheath-mesophyll continuum and leaf hydraulics. *Plant Physiology* **166**: 1609–1620. doi:10.1104/pp.114.248633.
- Sayama T, Tanabata T, Saruta M, et al. 2017.** Confirmation of the pleiotropic control of leaflet shape and number of seeds per pod by the Ln gene in induced soybean mutants. *Breeding Science* **67**: 363–369. doi:10.1270/jsbbs.16201.
- Slattery RA, Ort DR. 2021.** Perspectives on improving light distribution and light use efficiency in crop canopies. *Plant Physiology* **185**: 34–48. doi:10.1093/plphys/kiab006.
- Smith WK, Vogelmann TC, DeLucia EH, Bell DT, Shepherd KA. 1997.** Leaf form and photosynthesis. *Bioscience* **47**: 785–793. doi:10.2307/1313100.
- Song Q, Wang Y, Qu M, Ort DR, Zhu X-G. 2017.** The impact of modifying photosystem antenna size on canopy photosynthetic efficiency – Development of a new canopy photosynthesis model scaling from metabolism to canopy level process. *Plant, Cell & Environment* **40**: 2946–2957. doi:10.1111/pce.13041.
- Srinivasan V, Kumar P, Long SP. 2017.** Decreasing, not increasing, leaf area will raise crop yields under global atmospheric change. *Global Change Biology* **23**: 1626–1635. doi:10.1111/gcb.13526.
- Tanaka Y, Fujii K, Shiraiwa T. 2010.** Variability of leaf morphology and stomatal conductances in soybean [*Glycine max* (L.) Merr.] cultivars. *Crop Science* **50**: 2525–2532. doi:10.2135/cropsci2010.02.0058.
- Tholen D, Boom C, Zhu X-G. 2012.** Prospects for improving photosynthesis by altering leaf anatomy. *Plant Science* **197**: 92–101. doi:10.1016/j.plantsci.2012.09.005.
- Tollenaar M, Lee EA. 2002.** Yield potential, yield stability and stress tolerance in maize. *Field Crops Research* **7**: 161–169.
- Vasco A, Thadeo M, Conover M, Daly DC. 2014.** Preparation of samples for leaf architecture studies, a method for mounting cleared leaves. *Applications in Plant Sciences* **2**: 1400038. doi:10.3732/apps.1400038.
- Walker BJ, Drewry DT, Slattery RA, VanLoocke A, Cho YB, Ort DR. 2018.** Chlorophyll can be reduced in crop canopies with little penalty to photosynthesis. *Plant Physiology* **176**: 1215–1232. doi:10.1104/pp.17.01401.
- Wei T, Simko V. 2021.** *R package 'corrplot': Visualization of a Correlation Matrix (version 0.91)*. Available from <https://github.com/taiyun/corrplot>.
- Wells R, Burton JW, Kilen TC. 1993.** Soybean growth and light interception: response to differing leaf and stem morphology. *Crop Science* **33**: 520–524. doi:10.2135/cropsci1993.0011183x003300030020x.
- Yan X, Zhao H, Liu X, et al. 2014.** Phenotypic traits and diversity of different leaf shape accessions of the wild soybean (*Glycine soja* Sieb. Et Zucc.) in China. *Canadian Journal of Plant Science* **94**: 397–404.
- Ye M, Wu M, Zhang H, Zhang Z, Zhang Z. 2021.** High leaf vein density promotes leaf gas exchange by enhancing leaf hydraulic conductance in *Oryza sativa* L. plants. *Frontiers in Plant Science* **12**: 693815. doi:10.3389/fpls.2021.693815.