Unknown

Notes

In preamble: - short codes for commonly used symbols - ?

Terminology to standardize:

- the native light habitat or environment: the SPLASH paper refers to what they calculated as 'habitat' PPFD, so I am going to use that terminology
- growth condition versus measurement conditions
- ?

Main

[introduction] Stomata are important

Stomatal ratio is important unsolved problem

Amphi leaves are more common in sunny habitats and confer a great benefit. (this pattern is also true in Solanum? could we analyze herbarium data here?)

[maybe make distinction between amphi leaves and var in SR here since all tomato species are amphi]

Leaves with greater stomatal density ratio are more in open, sunny habitats because they deliver the greatest benefit in those circumstances. An amphistomatous leaf increases photosynthetic carbon gain compared to an otherwise identical hypostomatous leaf by increasing conductance through the leaf intercellular airspaces and boudary layers; the additional water loss through a second boundary layer is typically small [cites]. We quantify this benefit as the amphistomy advantage (AA = log(Aamphi/Ahypo). Why would AA be greater in sun than shade? There are three nonmutually exclusive hypotheses that we classify as 'acclimatory', 'plastic', and 'constitutive'.

Acclimatory hypothesis: Leaves acclimated to high light intensity typically increase total leaf stomatal conductance (increased CO$_2$ supply) and upregulate Rubisco activation (increased CO$*2$* demand). A one-dimensional circuit model using the Farquhar-von Caemmerer-Berry biochemical model

of C$_3$ photosynthesis shows that both increased stomatal conductance and Rubisco activity should increase AA, all else being equal (Supporting Information). If the acclimatory hypothesis is correct, we predict that AAhigh > AAlow for all species regardless of native habitat or growth environment. Plants adapted to sunny, open habitats will evolve greater stomatal density ratio to take advantage of regular exposure to high light intensity.

Plastic hypothesis: Individuals of the same genotype often develop dramatically different leaves in sun and shade conditions (cite). Plastic responses are likely adaptations to optimize photosynthesis at different light intensities (cites), but changes in leaf anatomy and biochemistry could modulate AA as a byproduct. Thicker or less porous leaves, both of which are associated with high leaf mass per area (LMA), will have lower g_ias; leaves with increased total stomatal density and Rubisco concentration have greater potential CO2 supply and demand. Under the plastic hypothesis, we predict that AAsun > AAshade for all species and light intensities. AAsun and gsmax,sun should be positively associated with native light habitat. [transition] We assume that genotypes adapted to sunny, open habitats will express a phenotype best adapted to that environment when leaves develop under high light intensity; genotypes adapted to shaded closed habitats may be plastic, but suboptimal for light intensities they do not regularly experience in nature.

[conceptual figure could also show differential benefit of amphi leaves in sun/shade]

to plants growing there. in those It has been hypothesized that amphistomy increases photosynthesis more in sunny places. Three nonmutually exclusive adaptive explanations for why amphi leaves:

Conceptual figure explaining each hypothesis

- acclimation: greater demand, higher gs increase AA
- developmental plasticity: light-induced changes in leaf anatomy modulate AA
- constitutive: genetic differences in leaves adapted to different light habitats

We distinguished among these hypotheses by comparing AA among wild tomato species from different native light habitats, grown under simulated sun and shade light treatments, and measured under contrasting light intensity (Figure of hypotheses and predictions). We measured AA on 600 individual plants from 30 accessions (average of 10 replicates per light treatment) using a recently developed method (*1*). With this method, we directly compare the photosynthetic rate of an untreated amphistomatous leaf to that of the same leaf with gas exchange blocked through the adaxial (upper) surface by transparent plastic, which we refer to as 'pseudohypostomy'. To compare amphi- and pseudohypostomatous leaves at identical whole-leaf g_{sc}, we measure A over a range of g_{sc}, inducing stomatal opening and closure by modulating humidity (see Materials and Methods for further details).

Table of directional predictions (with table summarizing results? part of conceptual figure? in supplement?)

caption: Directional predictions associated with each hypothesis explaining why amphistomy advantage (AA) might be greater for leaves in sunny, open habitats. For each hypothesis, we make predictions for how native light habitat, light treatment, and light intensity would affect AA.

Table 2: Solanum accessions

hypothesis	native light habitat	light treatment	light intensity
acclimatory	cor(PAR, AA) = 0	AAsun = AAshade	AA2000 > AA150
plastic	cor(PAR, AAsun) > 0	AAsun > AAshade	AA2000 = AA150
constitutive	cor(PAR, AA) > 0	AAsun = AAshade	AA2000 = AA150

[add rows for when multiple hypotheses are supported simultaneously? or put that in SI?]

[results] Amphistomy increases A in all accessions, in both sun and shade leaves, and light intensities. We infer this from the fact that blocking gas exchange in pseudohypostomatous leaves reduced A by X-X% depending on the accession, light treatment, and light intensity (Table/figure). The AA is equivalent to an X-X% change in total g_{sc} (see SI section gs equivalency). But whereas increasing g_{sc} would increase water loss as a necessary by-product, amphistomy can increase A without any appreciable affect on transpiration.

Sun leaves from high light habitats [not sure if htis result is true yet] benefit the most from amphistomy because of a both developmental plasticity and constitutive differences among accessions. [quantify difference in AA and contribution of different affects]. Surprisingly, light intensity had a little effect… (should this be in this paragraph, or it's own?).

[discussion] - sun/shade has long been appreciated and this shows new trait that should be considered and that CO2 diffusion becomes major limitation

Materials and Methods

[this will be moved to SI eventually]

Accessions

We compared AA among 30 ecologically diverse accessions of wild tomato, including representatives of all described species of *Solanum* sect. *Lycopersicon* and sect. *Lycopersicoides* (*2*) and the cultivated tomato *S. lycopersicum* var. *lycopersicum* (tab:accessions). Due to constraints on growth space and time, we spread out measurements over 80 weeks from May 1, 2022 to October 31, 2023. Replicates within accession were evenly spread out over this period to prevent confounding of temporal variation in growth conditions with accession. [anything else to say here? maybe explain accession selection and phylogeny?]

Plant growth conditions

In all growth spaces, we recorded PPFD using full spectrum quantum sensors (SQ-500-SS, Apogee Instruments, Logan, Utah, USA); we recorded temperature, RH, and [CO_2] using an EE894 sensor (E+E Elektronik, Engerwitzdorf, Austria) protected by a radiation shield. All environmental measurements were taken every 10 minutes from the middle of plants racks at approximately the same height as the leaves we measured. We measured leaf temperature of focal leaves prior to measurement using an infrared radiometer (SI-111-SS, Apogee Instruments, Logan, Utah, USA).

Germination and seedling stage

Seeds provided by the Tomato Genetics Resource Center germinated on moist paper in plastic boxes after soaking for 30-60 minutes in a 50% (volume per volume) solution of household bleach and water, followed by a thorough rinse. We transferred seedlings to cell-pack flats containing Pro-Mix BX potting mix (Premier Tech, Rivière-du-Loup, Quebec, Canada) once cotyledons fully emerged, typically within 1-2 weeks of sowing. We grew seeds and seedlings for both sun and shade treatments under the same environmental conditions (12:12 h, 25:20 °C, 40:60 RH day:night cycle). LED light provided PPFD $=$ 200 µmol m^{-2} s^{-1} (Fluence RAZRx, Austin, Texas, USA).

Light treatments

Seedlings were randomly assigned in alternating order within accession to the sun or shade treatment during transplanting. After seedlings established in cell-pack flats for $\approx$ 2 weeks, we transplanted them to 3.78 L plastic pots containing 60% Pro-Mix BX potting mix, 20% coral sand (Pro-Pak, Honolulu, Hawai‘i, USA), and 20% cinders (Niu Nursery, Honolulu, Hawai‘i, USA). Percentage composition is on a volume basis. The soil mixture contained slow release NPK fertilizer following manufacturer instructions (Osmocote Smart-Release Plant Food Flower & Vegetable, The Scotts Company, Marysville, Ohio, USA). We determined pot field capacity one week after transplanting using a scale (Ohaus V12P15 Valor 1000, Parsippany, New Jersey, USA) and watered to field capacity three times per week to prevent drought stress.

We assigned sun and shade treatment to lower and upper racks of a $1.22\,\text{m} \times 2.44\,\text{m}$ shelving unit in a climate-controlled growth room. We assigned the sun treatment to the lower rack to limit diffuse light from reaching the shade treatment. The average daytime PPFD was PPFD $= 800\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ (800 µmol m^{-2} s^{-1}) and PPFD $= 125\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ (125 µmol m^{-2} s^{-1}) for sun and shade treatments, respectively. To isolate the effect of light intensity from quality, we used the same LED model with the the same spectrum (Fluence SPYDR 2i, Austin, Texas, USAS), but dimmed the lights in the shade treatment. To maintain homogeneous environmental conditions other than light, we mixed air within the growth room using an air circulator (Vornado 693DC, Andover, Kansas, USA) and within racks using a miniature oscillating air circulator (Vornado Atom 1, Andover, Kansas, USA). Despite these efforts, the air in the sun treatment was on average 1 °C warmer and the average RH was consequently 5 lower. However, because of evaporative cooling, the leaves in the sun treatment were only 1 °C on average ($n = 600$ leaves).

Leaf trait measurements

We selected a fully expanded, unshaded leaf at least six leaves above the cotyledons during early vegetative growth. This typically meant that plants had grown in light treatments for ≈ 4 weeks, ensuring they had time to sense and respond developmentally to the light intensity of the treatment rather than the seedling conditions (*3*). Shade plants grew slower than sun plants, hence leaves at the same developmental stage were measured on chronologically older plants in the shade treatment. In some sun plants, we had to use leaves higher on the stem because short internodes made lower leaves inaccessible with the gas exchange equipment. We measured terminal leaflets in 70.0% of cases, but used the lateral leaflet closest to the terminal leaflet when it was damaged or difficult to clamp into the gas exchange chamber. When a leaflet was damaged during gas exchange measurements, we collected anatomical data from the nearest leaflet on the same leaf (10.0)%. We measured chlorophyll concentration index (CCI) using a chrolophyll concentration meter (MC-100, Apogee Instruments, Logan, Utah, USA) on the lamina of focal leaflets before gas exchange measurements at the same time we measured leaf temperature.

Amphistomy advantage

We estimated 'amphistomy advantage' (AA) *sensu* (*4*), but with modifications previously described in (*1*). AA is calculated as the log-response ratio of A compared at the same total g_{sw}:

$$\text{AA} = \log(A_{\text{amphi}}/A_{\text{hypo}})$$

We measured the photosynthetic rate of an untreated amphistomatous leaf (A_{amphi}) over a range of g_{sw} values. We refer to this as an A–g_{sw} curve. We compared the A–g_{sw} curve of the untreated leaf to the photosynthetic rate of pseudohypostomatous leaf (A_{hypo}), which is the same leaf but with gas exchange through the upper surface blocked by a neutral density plastic (propafilm).

We measured A–g_{sw} curves using a portable infrared gas analyzer (LI-6800PF, LI-COR Biosciences, Lincoln, Nebraska, USA). Light-acclimated plants were placed under LEDs dimmed to match their light treatment during gas exchange measurements. We estimated the photosynthetic rate (A) and stomatal conductance to CO_2 (g_{sw}) at ambient CO_2 ($C_{\text{a}} = 415\,\mu\text{mol}\,\text{mol}^{-1}$) and $T_{\text{leaf}} = 25.0\,^{\circ}\text{C}$. The irradiance of the light source in the pseudohypo leaf was higher because the propafilm reduces transmission. To compensate for reduced transmission, we increased incident PPFDfor pseudohypo leaves by a factor 1/0.91, the inverse of the measured transmissivity of the propafilm. We also set the stomatal conductance ratio, for purposes of calculating boundary layer conductance, to 0 for pseudohypo leaves following manufacturer directions.

We collected four A–g_{sw} curves per leaf, an amphi (untreated) curve and a pseudohypo (treated) curve at high light-intensity (PPFD $= 2000\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$; 97.7:2.3 red:blue) and low light-intensity (PPFD $= 150\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$; 87.0:13.0 red:blue). We always measured high light-intensity curves first because photosynthetic downregulation is faster than upregulation in these species. To control for order effects, we alternated between starting with amphi or pseudohypo leaf measurements. Unlike (*1*), preliminary

experiments with *Solanum* indicated a strong order effect in that A declined in the second curve. Therefore, we made measurements over two days. On the first day, we measured high and low light-intensity curves for either amphi or pseudohypo leaves; on the second day, we measured high and low light-intensity curves on the other leaf type.

In all cases, we acclimated the focal leaf to high light (PPFD $= 2000\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$) and high relative humidity (RH $= 70\%$) until A and g_{sw} reach their maximum. After that, we decreased RH to $\approx 10\%$ to induce rapid stomatal closure without biochemical downregulation. Hence, A_{amphi} and A_{hypo} were both measured at low chamber humidity after the leaf had acclimated to high humidity. All other environmental conditions in the leaf chamber remained the same. We logged data until g_{sw} reached its nadir. We then acclimated the leaf to low light (PPFD $= 150\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$) and RH $= 70\%$ before inducing stomatal closure with low RHand logging data as described above.

Light-response curves

In 90.0% of plants, we measured light-response curves on the same leaflets as A–g_{sw} curves. However, when a leaflet was damaged during A–g_{sw} curves, we used the next closest leaflet for light-response curves. Leaves acclimated to high light-intensity (PPFD $= 2000\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$), ambient CO_2 ($C_{\text{a}} = 415\,\mu\text{mol}\,\text{mol}^{-1}$), RH $= 50\%$, and $T_{\text{leaf}} = 25\,°\text{C}$. After A and g_{sw} stabilized, we measured A at 20 light-intensity levels between 0 and $2000\,\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$ in descending order.

Stomatal anatomy

We estimated the stomatal density and size on ab- and adaxial leaf surfaces from all leaves, using guard cell length as a proxy for stomatal size since it proportional to maximum conductance (*5*). We made surface impressions of leaf lamina from the same area used for gas exchange measurements using a-silicone impression material (Zhermack elite HD+, light body, fast set, Rovigo, Italy). We applied clear nail polish to make positive replicas of the impression. After nail polish dried, we mounted replicas on a microscope slide using transparent tape (*6*). We digitized a portion of each leaf surface replica using a brightfield microscope (Leica DM2000, Wetzlar, Germany). We counted and measured guard cell length on all stomata using the FIJI implementation of ImageJ2 version 2.3.0 (*7*), then divided the count by the visible leaf area ($0.890\,\text{mm}^2$) to estimate stomatal density.

Internal leaf anatomy

Add information on internal leaf anatomy, if using

Leaf mass per area

Leaf mass per area (LMA) is the dry mass divided by the leaflet area. We scanned fresh leaflets on a flat bed scanner (Epson V600, Los Alamitos, California. USA) and measured leaflet area from digital images using the FIJI implementation of ImageJ2 version 2.3.0 (*7*). We dried leaves for 72 hours at

74 °C in a food dehydrator (Cosori CP267-FD, Vesync Co., Anaheim, California, USA) and weighed using a benchtop analytical balance (Ohaus PR64 Analytical Balance, Parsippany, New Jersey, USA). In 5.0% we measured LMA on the adjacent leaflet because the focal leaflet was damaged or wilted while making surface impressions and we could not reliably estimate area. LMA data are missing from 3.0% of individuals because the area or mass was not recorded at all or recorded incorrectly.

References

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