

MicroRNA156-mediated changes in leaf composition lead to altered photosynthetic traits during vegetative phase change

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Summary

- Plant morphology and physiology change with growth and development. Some of these changes are due to change in plant size and some are the result of genetically programmed developmental transitions. In this study we investigate the role of the developmental transition, vegetative phase change (VPC), on morphological and photosynthetic changes.
- We used overexpression of microRNA156, the master regulator of VPC, to modulate the timing of VPC in *Populus tremula* × *alba*, *Zea mays*, and *Arabidopsis thaliana* to determine its role in trait variation independent of changes in size and overall age.
- Here, we find that juvenile and adult leaves in all three species photosynthesize at different rates and that these differences are due to phase-dependent changes in specific leaf area (SLA) and leaf N but not photosynthetic biochemistry. Further, we found juvenile leaves with high SLA were associated with better photosynthetic performance at low light levels.
- This study establishes a role for VPC in leaf composition and photosynthetic performance across diverse species and environments. Variation in leaf traits due to VPC are likely to provide distinct benefits under specific environments; as a result, selection on the timing of this transition could be a mechanism for environmental adaptation.

Introduction

As plants age they go through developmental transitions that impact their form and function. One of these transitions occurs as plants shift between juvenile and adult vegetative growth phases. This developmental transition is known as vegetative phase change (VPC) and has been observed across phylogenetically diverse groups of plants from mosses to angiosperms. This transition is controlled by expression levels of the highly conserved microRNA156 (miR156) and in some species the closely related miR157 (Willmann & Poethig, 2007; Axtell & Bowman, 2008; Zhang *et al.*, 2015). miR156 and 7 are expressed at high levels in leaves produced early in development, and negatively regulate the expression of their targets, the Squamosa Promoter Binding Protein-Like (*SPL*) transcription factors. Expression of miR156 and 7 declines later in development, alleviating the transcriptional and translational repression of these *SPL* genes. This increase in *SPL* expression promotes adult vegetative traits, leading to VPC (Wu & Poethig, 2006; Wu *et al.*, 2009; Wang *et al.*, 2011; Xu *et al.*, 2016; He *et al.*, 2018). The traits that change during VPC are species dependent, but they broadly include changes in leaf morphology, growth rate, growth form, and reproductive competence (Poethig, 1990; Bassiri *et al.*, 1992; Bongard-Pierce *et al.*, 1996; Telfer *et al.*, 1997; Wang *et al.*, 2011; Feng *et al.*, 2016; Leichty & Poethig, 2019; Silva *et al.*, 2019).

How plants respond to dynamic challenges in their environment varies with age (Cavender-Bares & Bazzaz, 2000; Niinemets, 2010; Kitajima *et al.*, 2013; Hahn & Orrock, 2016) and has important implications for plant community composition, the competitive ability of different species, and their response to future climate change (Parish & Bazzaz, 1985; Lamb & Cahill, 2006; Moll & Brown, 2008; Piao *et al.*, 2013; Spasojevic *et al.*, 2014; Kerr *et al.*, 2015; Lasky *et al.*, 2015). For example, seedlings are particularly vulnerable to factors such as shading, drought, disturbance, and herbivory (Kabrick *et al.*, 2015; Charles *et al.*, 2018), and they often experience a high rate of mortality (Grossnickle, 2012). Species that are able to transition to a more resilient phase for their environment are likely to have a competitive advantage. Although it is reasonable to assume that VPC plays an important role in this process, how VPC affects the response of plants to various biotic and abiotic stresses is still poorly understood.

Defining the role of VPC in plant physiology is difficult because this transition occurs concurrently with changes in plant size and age. Juvenile leaves and branches are produced on smaller plants than adult organs and, thus, are exposed to different amounts and types of endogenous factors (e.g. hormones, carbohydrates). Furthermore, light, temperature, and humidity vary according to position within the canopy and proximity to the ground (Evans & Coombe, 1959; Waggoner & Reifsnnyder, 1968; Shuttleworth *et al.*, 1985; Canham, 1988; Canham *et al.*,

1994; Still *et al.*, 2019), meaning that juvenile and adult organs exist in different microclimates. Finally, the temporal separation between the production of juvenile and adult organs means that seasonal changes in environmental conditions also contribute to age-dependent differences in physiological traits. These problems can only be addressed by varying the timing of VPC under controlled environmental conditions, independent of shoot growth.

The importance of various leaf traits – including photosynthetic traits, specific leaf area (SLA), leaf nitrogen (N) content, and gas exchange – for plant growth and survival have been well documented (Lusk & Del Pozo, 2002; Poorter & Bongers, 2006; Modrzyński *et al.*, 2015). Previous studies have shown that photosynthetic genes are differentially expressed in juvenile and adult maize (*Zea mays*) leaves (Strable *et al.*, 2008; Beydler, 2014), but comparisons of various photosynthetic traits in these leaves have produced inconclusive and sometimes conflicting results (Bond, 2000; Steppe *et al.*, 2011; Sun *et al.*, 2018; Kuusk *et al.*, 2018a, b). The basis for these inconsistencies is unclear, but the compounding effects of variation in plant size, leaf age, environment, and time of year are possibilities (Bauer & Bauer, 1980; Bond, 2000; Ishida *et al.*, 2005; Velikova *et al.*, 2008; Steppe *et al.*, 2011). Although these effects can be minimized through techniques such as grafting, *in vitro* rejuvenation, and pruning (Hutchison *et al.*, 1990; Huang *et al.*, 2003; Kubien *et al.*, 2007; Jaya *et al.*, 2010), these methods do not completely distinguish the effect of VPC from other factors that may contribute to these differences. For example, grafting old shoots to young roots is often used to determine if a trait is dependent on plant size. However, miR156 is a mobile microRNA and can move across a graft junction (Marin-Gonzalez & Suarez-Lopez, 2012; Bhogale *et al.*, 2014; Ahsan *et al.*, 2019; Fouracre & Poethig, 2019), so this approach does not necessarily eliminate the effect of this key regulator of vegetative identity. Similarly, the methods that are typically used to induce vegetative rejuvenation (*in vitro* culture, pruning) affect both the level of miR156 (Irish & Karlen, 1998; Li *et al.*, 2012) and plant size.

We used overexpression of miR156 in three species – *Populus tremula* × *alba*, *Z. mays*, and *Arabidopsis thaliana* – to delay the timing of VPC, allowing us to differentiate traits associated with this developmental transition from those regulated by plant size or age. Our results demonstrate that juvenile leaves are photosynthetically distinct from adult leaves and that this difference can be attributed primarily to the morphological differences between these leaves, not to a fundamental difference in the biochemistry of photosynthesis.

Materials and Methods

Plant material

Populus tremula × *alba* line 717-1B4 and two independent miR156 overexpressor lines, 40 and 78, described in Lawrence *et al.* (2020), were obtained by *in vitro* propagation and hardened on propagation media as described in Meilan & Ma (2006). Plants were then transplanted to Fafard-2 growing mix (Sun Gro Horticulture, Agawam, MA, USA) in 0.3 l pots in a glasshouse at

the University of Pennsylvania (39.9493°N, 75.1995°W, 22.38 m above sea level (asl)) and kept in plastic bags for increased humidity for 2 wk. Plants were transferred to 4.2 l pots with Fafard-52 growing mix 3 wk later and fertilized with Osmocote 14-14-14 (The Scotts Company, Marysville, OH, USA). Plants were additionally fertilized once a week with Peters 20-10-20 (ICL Fertilizers, Dublin, OH, USA). Glasshouse conditions consisted of a 16 h photoperiod with temperatures between 22 and 27°C. Light levels were based on natural light and supplemented with 400 W metal halide lamps (P.L. Light Systems, Lincoln, ON, Canada) with daily irradiances between 300 to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ across the day. All settings were controlled by Priva (Vineland Station, ON, Canada) and Microgrow (Temecula, Canada) glasshouse systems.

Populus tremula × *alba* seeds from Sheffield's Seed Company (Locke, NY, USA) were germinated on a layer of vermiculite on top of Fafard-2 growing mix in 0.64 l pots in a glasshouse under the conditions already described herein. Seedlings were transplanted to 1.76 l pots with Fafard-52 growing mix with Osmocote 14-14-14 at 1 month after germination and were then transplanted to 4.2 l pots 3 months following the previous transplant.

Zea mays seeds with the *Corngrass 1* (*Cg1*) mutation (stock 310D) – which consists of a tandem duplication of miR156b/c primary sequences described in Chuck *et al.* (2007) – and the W22 inbred line were obtained from the Maize Genetics Cooperation Stock Center (Urbana, IL, USA). Plants heterozygous for *Cg1* were crossed to W22 to produce the *Cg1/+* and *+/+* siblings used in this study. Seeds were planted in 9.09 l pots with Fafard-52 growing mix and fertilized with Osmocote 14-14-14 in a glasshouse under growing conditions already described herein.

Arabidopsis thaliana (Col) and the 35S:miR156 overexpressor line described in Wu & Poethig (2006) were planted in 0.06 l pots with Fafard-2 growing mix as described by Flexas *et al.* (2007). Beneficial nematodes (*Steinernema feltiae*; BioLogic, Willow Hill, PA, USA), Marathon® 1% granular insecticide and diatomaceous earth were added to the growing mix to control insects. Planted seeds were placed at 4°C for 3 d before being grown at 22°C in Conviron growth chambers under short days (10 h : 14 h, light : dark) at 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light to obtain leaves large enough to fit in the gas-exchange chamber. Plants were fertilized with Peters 20-10-20 every other week.

Individuals from genotypes of all species were positioned in a randomized fashion in the glasshouse and rotated frequently. Planting was staggered across two, three and five months for *Arabidopsis*, *P. tremula* × *alba*, and *Z. mays*, respectively.

Leaf samples

All measurements and sampling were conducted on the uppermost fully expanded leaf. In *P. tremula* × *alba* 717-1B4 and miR156 overexpressor lines, leaves 10, 15, 20, and 25 were measured. Leaves 10 and 15 in the wild-type 717-1B4 line were juvenile and leaves 20 and 25 were adult, as determined by petiole shape and abaxial trichome density as described in Lawrence *et al.* (2020). All leaves measured in the miR156 overexpressor

lines were juvenile. Leaves 1–52 were measured in poplar plants germinated from seed. The juvenile-to-adult transition in these plants occurred between leaves 20 and 30, as determined by the change in petiole shape and trichome density (Lawrence *et al.*, 2020). In *Z. mays*, leaves 2–11 were measured with leaves 1–5 juvenile in wild-type plants and all leaves juvenile in *Cg1* mutants. Developmental stage in maize was determined by the presence or absence of epicuticular wax and trichomes as described in Poethig (1988). In *A. thaliana*, leaves 5 and 10 were measured. In wild-type plants, leaf 5 was juvenile and leaf 10 was adult, as determined by the presence or absence of abaxial trichomes. All leaves were juvenile in miR156 overexpressors. Throughout this paper, ‘juvenile’ and ‘adult’ refer to naturally juvenile or adult leaves in wild-type lines, and ‘juvenilized’ refers to leaves in miR156 overexpressor lines that have a juvenile phenotype at leaf positions that would normally be adult. The phenotypes of fully expanded adult, juvenile, and juvenilized leaves of *P. tremula* × *alba*, *A. thaliana*, and maize are shown in Fig. 1.

Gas-exchange measurements

All gas-exchange measurements were made using an Li-6400 portable photosynthesis machine (Li-Cor Environmental, Lincoln, NE, USA) at a leaf temperature of 25°C following acclimatization to starting chamber conditions. Photosynthetic capacity in *A. thaliana* was measured using steady-state net photosynthesis (A_{net})–intercellular $[\text{CO}_2]$ (C_i) curves by measuring A_{net} at reference $[\text{CO}_2]$ of 400, 200, 50, 100, 150, 200, 250, 300, 600, 800, 1000, and 1200 ppm, at an air flow rate of 300 $\mu\text{mol s}^{-1}$, minimum wait time of 2 min, and light level of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. *Zea mays* $A_{\text{net}}-C_i$ curves measured A_{net} at reference $[\text{CO}_2]$ of 400, 350, 300, 250, 200, 150, 100, 50, 400, 500, 600, 700, 800, 1000, 1200 ppm, at an air flow rate of 400 $\mu\text{mol s}^{-1}$, minimum wait time of 2 min, and light level of 1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Photosynthetic capacity in *P. tremula* × *alba* was measured using rapid $A_{\text{net}}-C_i$ response (RACiRTM) curves as described in Lawrence *et al.* (2019). Briefly, A_{net} was measured from reference

$[\text{CO}_2]$ of 300 to 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 60 $\mu\text{mol mol}^{-1} \text{min}^{-1}$ CO_2 and a light level of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. This technique was used to expedite measurements after development of the RACiR technique for the Li-6400 showed no significant differences from steady-state $A_{\text{net}}-C_i$ curves.

Light-response curves were performed in all three species at a reference $[\text{CO}_2]$ of 400 ppm. A_{net} was measured at light levels of 1000, 800, 600, 300, 200, 150, 100, 75, 50, 25, and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in *A. thaliana*, 1800, 1500, 1200, 1000, 800, 600, 300, 200, 150, 100, 75, 50, 25 and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in *Z. mays*, and 1500, 1200, 1000, 800, 600, 300, 200, 150, 100, 75, 50, 25, 10 and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in *P. tremula* × *alba*. Flow rate, leaf temperature, and minimum wait times were the same as for $A_{\text{net}}-C_i$ curves.

The low-light photosynthetic rates $A_{\text{low light}}$ shown in Fig. 7 were obtained by averaging photosynthetic rates over a 2 min period at light levels approximately two to three times the light compensation point. These values were 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in *P. tremula* × *alba* and *A. thaliana* and 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in *Z. mays*. All leaves were acclimated to the chamber conditions before measurements began, and flow rate and leaf temperature were consistent with previously described measurements.

Daytime respiration rates were determined by averaging A_{net} at 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance over a 1 min period after the leaves were dark adapted for 1 h.

Leaf fluorescence

Light and dark-adapted fluorescences were determined using an Li-6400 equipped with fluorometer head. Light-adapted measurements were taken using a multiphase flash with a 250 ms phase 1, 500 ms phase 2 with a 20% declining ramp and 250 ms phase 3 after leaves acclimated to saturating light values of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for *A. thaliana*, *Z. mays* and *P. tremula* × *alba*, respectively. Dark-adapted fluorescence measurements were taken using an 800 ms saturating rectangular flash after dark-adapting leaves for 1 h.

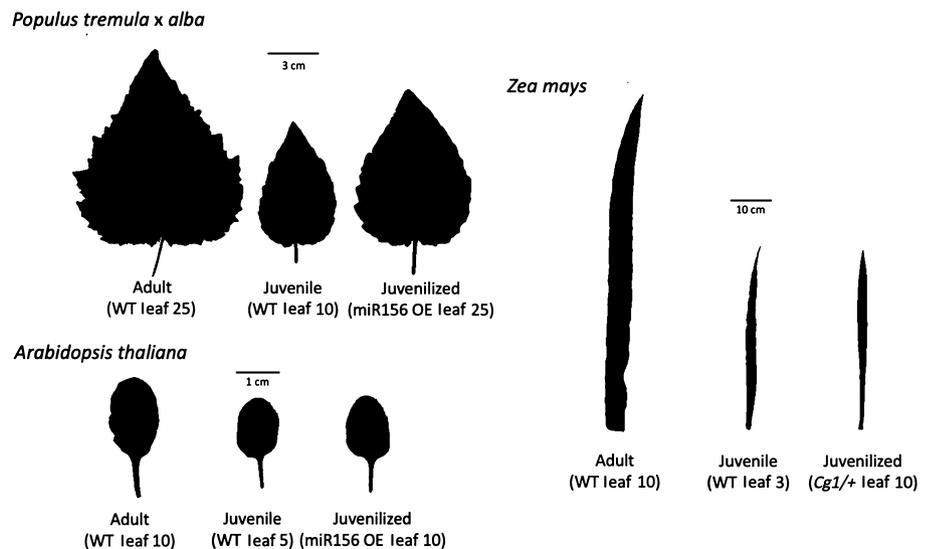


Fig. 1 Leaf shape phenotypes of fully expanded adult, juvenile, and juvenilized leaves of *Populus tremula* × *alba*, *Arabidopsis thaliana*, and *Zea mays*. *Cg1*, *Corngress 1*; OE, overexpressor; WT, wild-type.

Leaf nitrogen, Chl, and specific leaf area

Leaf tissue was sampled after gas exchange; one subsample for each leaf was dried at 60°C until constant mass to determine SLA. Dried tissues were ground using a mortar and pestle. Leaf N was measured in the dried samples using an ECS 4010 CHNSO Analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA). A second subsample was frozen and used for Chl quantification. Chl was extracted using 80% acetone and quantified using a spectrophotometer according to equations found in Porra *et al.* (1989).

Leaf cross-sections

Fresh leaf tissue from the middle of fully expanded leaves at positions 5 and 10 of *A. thaliana*, 10 and 25 of *P. tremula* × *alba*, and 4 and 11 of *Z. mays* in both wild-type and miR156 overexpressor lines was cut and fixed with a 10× formalin–propionic acid–glycerol–95% ethanol solution overnight. Samples were then washed with 50% ethanol and dehydrated through an ethanol/*tert*-butyl alcohol (TBA) series with 2 h incubations at room temperature for each step. Sections in 100% TBA were subsequently transferred to Paraplast Plus embedding medium at 60°C and incubated for 48 h. Embedded samples were set in molds and cut into 12 µm sections using a microtome. Samples were floated on 0.01% Sta-on on glass slides and dried at 40°C. Samples were then deparaffinized in xylenes and rehydrated through an ethanol series for staining with 1% Safranin O in 50% ethanol and subsequent dehydrating for staining with 0.1% Fast green in 95% ethanol. Once fully stained and dehydrated, sections were mounted in permount and visualized and photographed using an Olympus BX51 light microscope and DP71 digital camera.

Curve fitting

The PLANTECOPHYS package in Duursma (2015) was used for fitting $A_{\text{net}}-C_i$ curves to determine V_{cmax} and J_{max} using the bilinear function for *A. thaliana* and *P. tremula* × *alba*. The C_4 photosynthesis estimation tool presented in Zhou *et al.* (2019) based on Yin *et al.* (2011) was used for fitting $A_{\text{net}}-C_i$ curves for *Z. mays*.

Light-response curves were analyzed using the AQ_CURVES curve-fitting script in R (Tomeo, 2019), which uses equations based on a standard nonrectangular hyperbola model fit described in Lobo *et al.* (2013).

Data analysis

All statistical analyses were performed in JMP[®] PRO v.14.0.0 (SAS Institute Inc., Cary, NC, USA). Gas exchange and leaf composition traits between adult, juvenile, and juvenilized leaves were compared by one-way ANOVA, where developmental stage was the main effect and *P*-values were corrected for multiple testing within a species using false-discovery rate (FDR). When ANOVA results were significant ($P < 0.05$) a Student's *t*-test was

performed to determine differences between developmental groups. Traits were considered to be affected by developmental phase when adult leaves were significantly different from both juvenile and juvenilized leaves with the same trend. The effect of leaf position on measured traits was determined by two-way ANOVA with leaf position and genotype as the main effects and *P*-values corrected using FDR. Because developmental phase and leaf position are coordinated in wild-type plants, many traits affected by development showed significant leaf position effects ($P < 0.05$). Of these traits, those that showed no significant interaction between leaf position and genotype, indicating there were no significant differences between wild-type and miR156 overexpressor plants that do not produce adult leaves, are affected by leaf position independent of leaf developmental stage. Photosynthetic N use efficiency was determined using least-squares linear regression analysis across all leaves and was compared by ANCOVA with developmental stage as the covariate. The relationships between A_{sat} and leaf N or SLA were determined using least-squares linear regression analysis.

Results

Photosynthetic rates differ between juvenile and adult leaves

The rate of light-saturated area-based photosynthesis $A_{\text{sat Area}}$ was significantly different in juvenile and adult leaves of *P. tremula* × *alba* and *A. thaliana* but was not significantly different in maize (Fig. 2a–c). Adult leaves in *P. tremula* × *alba* had a 26% greater $A_{\text{sat Area}}$ ($15.56 \mu\text{mol m}^{-2} \text{s}^{-1}$) than their juvenile counterparts did ($12.28 \mu\text{mol m}^{-2} \text{s}^{-1}$), whereas the adult leaves in *A. thaliana* had a 57% greater $A_{\text{sat Area}}$ ($8.13 \mu\text{mol m}^{-2} \text{s}^{-1}$) than juvenile leaves did ($5.18 \mu\text{mol m}^{-2} \text{s}^{-1}$). The phase dependence of this difference was confirmed by the phenotype of lines overexpressing miR156. The $A_{\text{sat Area}}$ of adult leaves in *P. tremula* × *alba* was, respectively, 104% and 105% greater than the $A_{\text{sat Area}}$ of the corresponding juvenilized leaves in lines 40 ($7.57 \mu\text{mol m}^{-2} \text{s}^{-1}$) and 78 ($7.54 \mu\text{mol m}^{-2} \text{s}^{-1}$), whereas the $A_{\text{sat Area}}$ of adult leaves in *Arabidopsis* was 42% higher than that of juvenilized leaves ($5.73 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Mean mass-based photosynthetic rates $A_{\text{sat Mass}}$ were lower in adult leaves than in juvenile leaves in all three species, although this difference was only statistically significant in maize (Fig. 2d–f). In maize, juvenilized leaves had essentially the same $A_{\text{sat Mass}}$ as normal juvenile leaves, suggesting that the difference in $A_{\text{sat Mass}}$ between juvenile and adult leaves is phase dependent. However, the $A_{\text{sat Mass}}$ of juvenilized leaves in *P. tremula* × *alba* and *A. thaliana* was significantly lower than that of juvenile leaves and was more similar to that of adult leaves.

Leaf morphology and composition is phase dependent

Inconsistencies in the relationship between leaf identity and A_{sat} on an area or mass basis across species suggests that leaf-to-leaf variation in the rate of photosynthesis is either determined by variation in the leaf area/mass relationship or by

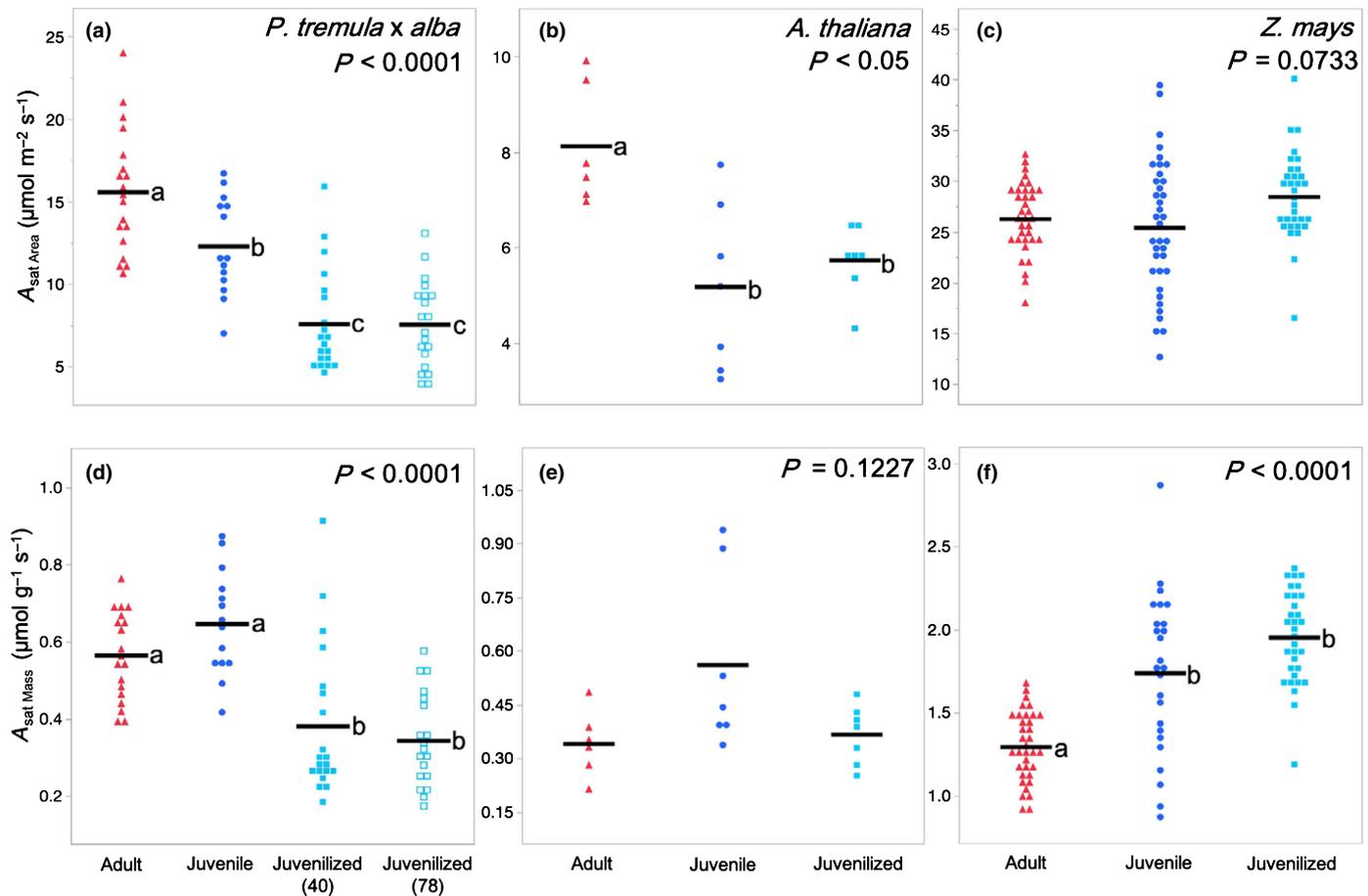


Fig. 2 Photosynthetic rates of adult (red triangles), juvenile (blue circles), and juvenalized (light blue closed or open squares) leaves of (a, d) *Populus tremula* × *alba*, (b, e) *Arabidopsis thaliana*, and (c, f) *Zea mays*. Traits depicted are (a–c) area-based light-saturated net photosynthetic rate ($A_{\text{sat Area}}$) and (d–f) mass-based light-saturated net photosynthetic rate ($A_{\text{sat Mass}}$). Means presented as black horizontal lines. P -values from one-way ANOVA with development as the main effect. For traits with significant ANOVA results ($P < 0.05$), different lower-case letters indicate means of developmental stage are significantly different according to Student's t -test ($P < 0.05$).

variation in the photosynthetic biochemistry in these species. *Populus tremula* × *alba* and *A. thaliana* both undergo C_3 photosynthesis, whereas maize is a C_4 plant, so it is reasonable to assume that the factors contributing to developmental variation in photosynthesis in these species could be quite different. To address this issue, we measured morphological, chemical, and physiological traits in adult, juvenile, and juvenalized leaves of these species.

SLA represents the amount of area per unit of leaf mass and is a proxy for the thickness or density of the leaf blade; in general, leaves with a high SLA are thinner than leaves with a low SLA. Adult leaves of all three species had a significantly lower SLA than juvenile leaves (Fig. 3a–c). Furthermore, the SLA of juvenalized leaves was significantly higher than that of adult leaves and was similar, if not identical to, the SLA of juvenile leaves in both *P. tremula* × *alba* and maize. This result suggests that SLA is phase dependent in all three species. Although we did not conduct a detailed quantitative analysis of the basis for the difference in SLA between juvenile and adult leaves, cross-sections of the leaf blade suggest that this difference is attributable to a combination of variation in both leaf thickness and tissue density, with

the relative importance of these traits differing in different species (Fig. 4). Consistent with their SLA, the anatomy of juvenalized leaves of *P. tremula* × *alba* and *Arabidopsis* appeared to be intermediate between that of juvenile and adult leaves, whereas juvenalized leaves in maize more closely resembled normal juvenile leaves than adult leaves.

The relationship between leaf N and phase identity varied depending on whether this trait was measured on an area or mass basis and was similar to the results obtained for photosynthetic rates. Measured on a mass basis, leaf N was not significantly different in juvenile and adult leaves of *P. tremula* × *alba* or *A. thaliana* and was not significantly different between juvenalized and adult leaves of these species. However, in maize, leaf N/mass was significantly lower in adult leaves than in either juvenile or juvenalized leaves (Fig. 3d–f). Thus, leaf N/mass is a phase-dependent trait in maize but not in *P. tremula* × *alba* or *A. thaliana*. The opposite result was obtained when leaf N was measured as a function of leaf area. The leaf N/area in both *P. tremula* × *alba* and *A. thaliana* was significantly higher in adult leaves than in juvenile or juvenalized leaves, implying that it is phase dependent in these species. However, there was no

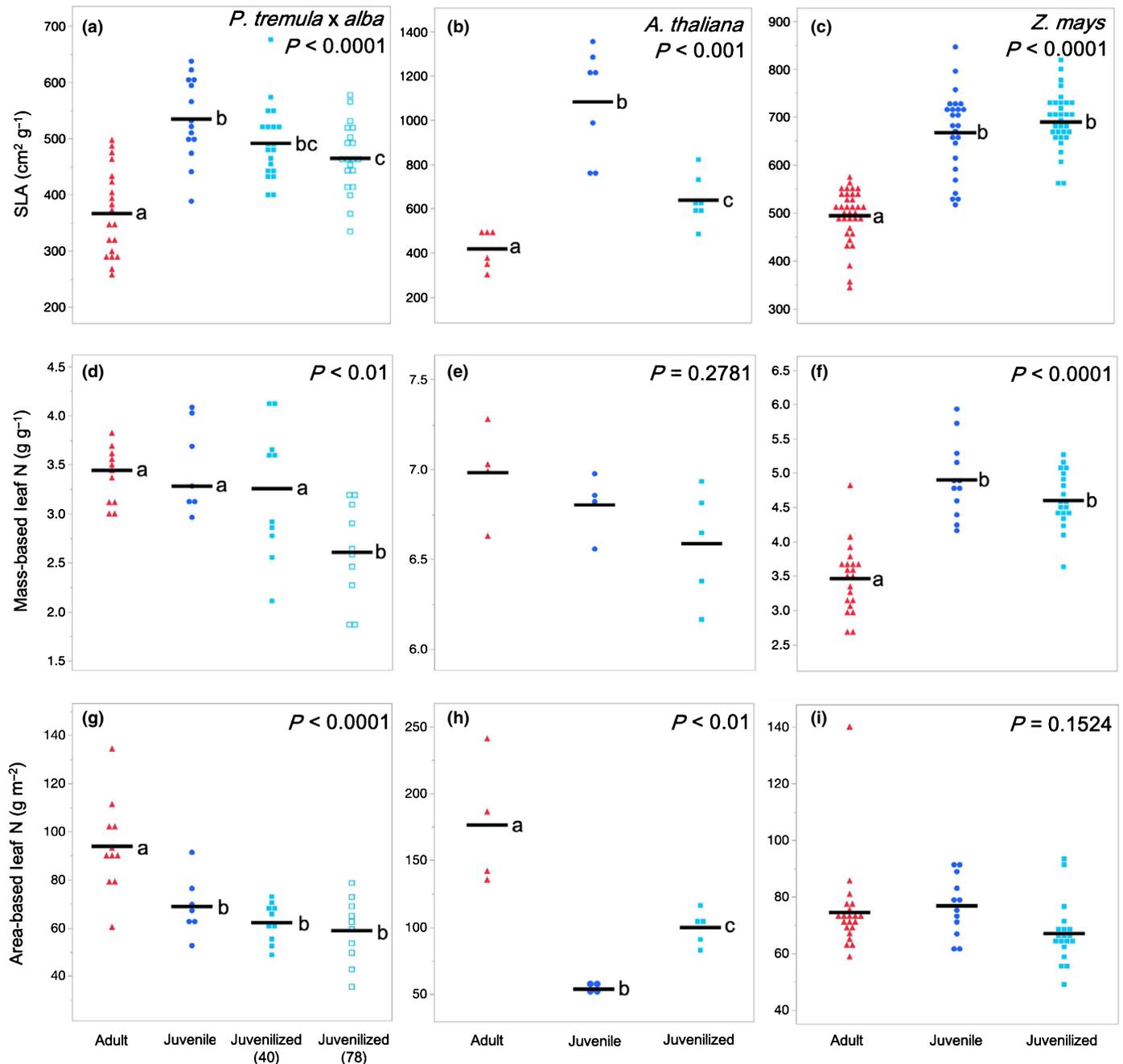


Fig. 3 Leaf morphological and compositional traits of adult (red triangles), juvenile (blue circles), and juvenilized (light blue closed or open squares) leaves of (a, d, g) *Populus tremula* × *alba*, (b, e, h) *Arabidopsis thaliana*, and (c, f, i) *Zea mays*. Traits depicted are (a–c) specific leaf area (SLA), (d–f) mass-based leaf nitrogen (N) content, and (g–i) area-based leaf N content. Means presented as black horizontal lines. *P*-values from one-way ANOVA with development as the main effect. For traits with significant ANOVA results ($P < 0.05$) different lower-case letters indicate means of developmental stage are significantly different according to Student's *t*-test ($P < 0.05$).

significant difference in the leaf N/area of adult, juvenile, or juvenilized leaves in maize (Fig. 3g–i).

SLA and leaf N were significantly correlated with phase-dependent photosynthetic rates ($A_{\text{sat Area}}$ in *P. tremula* × *alba* and *A. thaliana*; $A_{\text{sat Mass}}$ in maize) in all three species (Fig. 5; Supporting Information Table S1). SLA was negatively correlated with $A_{\text{sat Area}}$ in *P. tremula* × *alba* and *A. thaliana* and positively correlated with $A_{\text{sat Mass}}$ in *Z. mays*. This negative relationship

was weaker in *P. tremula* × *alba* ($r^2 = 0.167$) likely because of low g_s in the juvenilized leaves (Table S2) leading to lower than wild-type A_{sat} . When juvenilized leaves were excluded from the analysis, a stronger but similar linear fit existed among the adult and juvenile wild-type leaves ($A_{\text{sat}} = 24.11 - 0.02255 \times \text{SLA}$, $r^2 = 0.436$, $P < 0.0001$). Leaf N is positively correlated with $A_{\text{sat Area}}$ in *P. tremula* × *alba* and *A. thaliana* and $A_{\text{sat Mass}}$ in *Z. mays*. However, photosynthetic N use efficiency (PNUE),

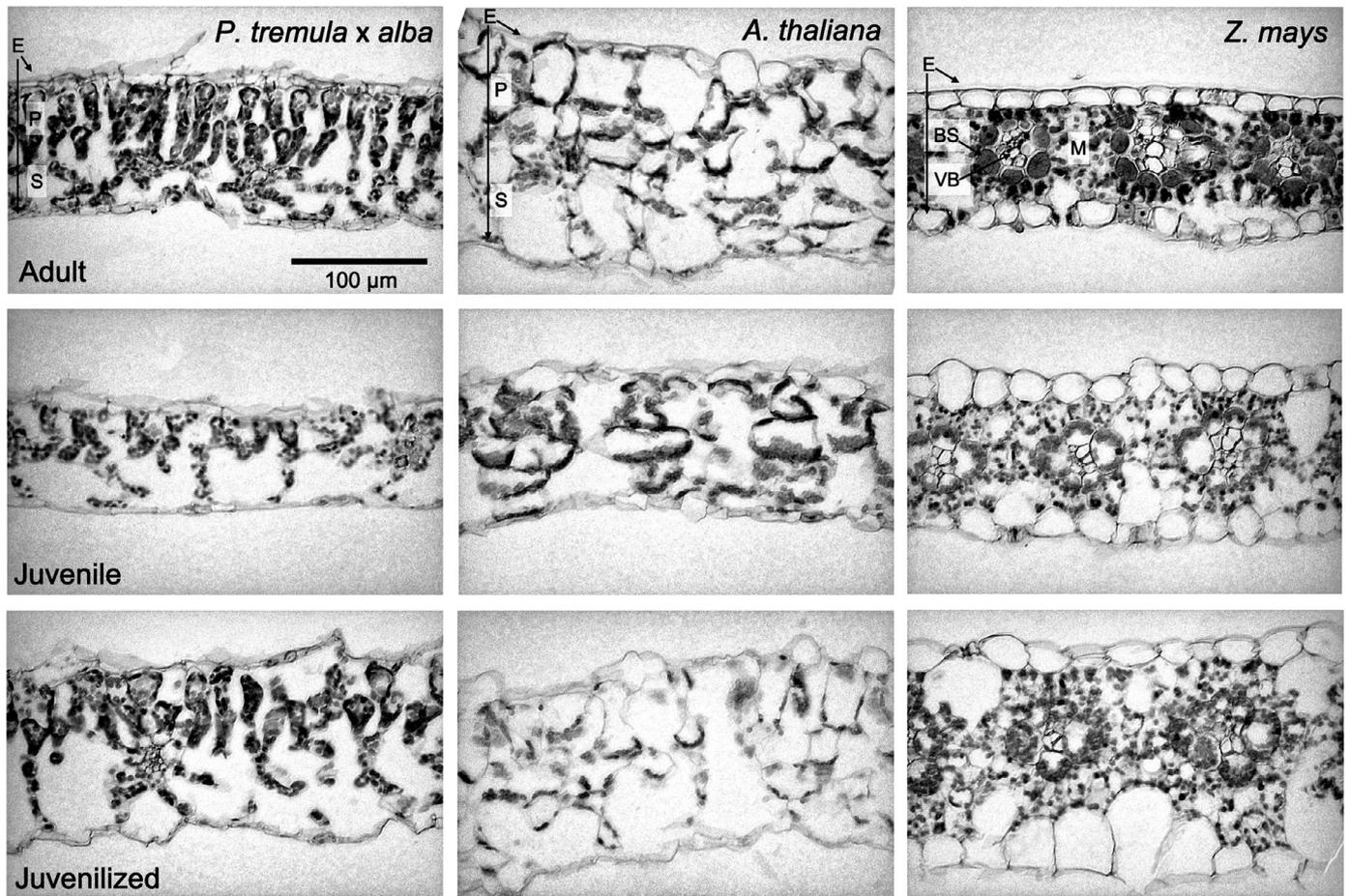


Fig. 4 Leaf cross-sections of *Populus tremula* × *alba*, *Arabidopsis thaliana*, and *Zea mays* adult, juvenile, and juvenilized leaves stained with safranin-O and fast green. E, epidermis; P, palisade tissues; S, spongy tissues; BS, bundle sheath; VB, vascular bundle; M, mesophyll.

calculated as the relationship between A_{sat} and leaf N, did not vary based on leaf developmental phase (Table 1).

We also compared Chl*a* and *b* (Chl*a*+*b*) levels and ratios between adult, juvenile, and juvenilized leaves. Chl*a* + *b* was not significantly different across leaves of different developmental phases. However, the Chl*a* : Chl*b* ratio was phase dependent in *P. tremula* × *alba* and *Z. mays* (Table S2). Changes in Chl*a* : Chl*b* ratios followed the same trends as leaf N, with lower ratios in juvenile and juvenilized leaves than in adult leaves of *P. tremula* × *alba* and the opposite in *Z. mays*. As Chl*a* is associated with more proteins than Chl*b* is, these data support one another.

There were no phase-dependent differences in stomatal conductance g_s or daytime respiration R_{day} between adult and juvenile or juvenilized leaves in any of the test species (Table S2).

To determine if phase-dependent variation in A_{sat} is attributable to variation in the biochemistry of photosynthesis, we examined traits modeled from $A_{\text{net}}-C_i$ curves ($V_{\text{cm}_{\text{max}}}$, maximum Rubisco carboxylation rate; J_{max} , maximum electron transport rate for ribulose-1,5-bisphosphate regeneration; Fig. 6), traits modeled from light-response curves (Φ , quantum yield; LCP, light compensation point), and traits modeled from dark and light-adapted

fluorescence (F_v/F_m , maximum quantum efficiency of photosystem II (PSII); F'_v/F'_m , maximum operating efficiency; Φ_{PSII} , quantum yield of PSII; NPQ, nonphotochemical quenching; ETR, electron transport rate). With one exception, none of these traits was significantly different between adult and juvenile/juvenilized leaves. The sole exception was F_v/F_m in *P. tremula* × *alba*, which was 6.3% higher in adult leaves than in juvenile leaves (Table S2). When $V_{\text{cm}_{\text{max}}}$, J_{max} , and ETR were converted to mass-based measures using SLA, ETR_{Mass} in *Z. mays* was the only trait determined to be phase dependent with juvenile and juvenilized leaves, having rates of $2.24 \mu\text{mol g}^{-1} \text{s}^{-1}$ and $3.18 \mu\text{mol g}^{-1} \text{s}^{-1}$ faster than adult leaves, respectively (Table S2). It is possible these differences in ETR contribute to the phase-dependent differences observed in $A_{\text{sat Mass}}$. However, the lack of phase dependence in Φ_{PSII} and J_{max} suggest otherwise.

The observation that phase-dependent variation in A_{sat} is correlated with SLA and leaf N but not with most measures of photosynthetic or physiological efficiency suggests that phase-dependent aspects of leaf anatomy, as well as phase-dependent variation in leaf composition (e.g. protein content), are the primary determinants of variation in the rate of photosynthesis during shoot development.

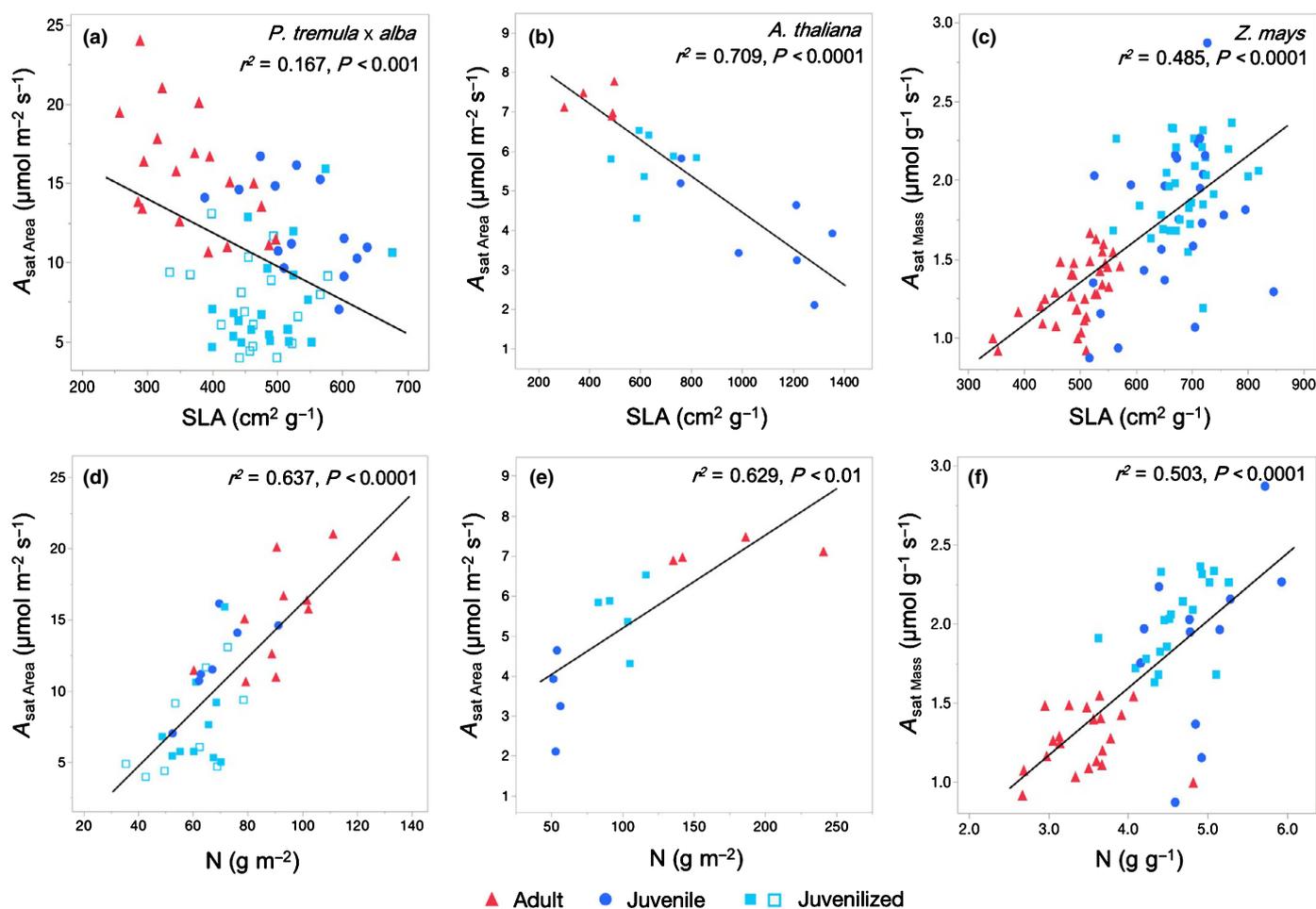


Fig. 5 Phase-dependent light-saturated photosynthetic rates A_{sat} are significantly correlated with the leaf composition traits specific leaf area (SLA) and leaf nitrogen (N) in (a, d) *Populus tremula* × *alba*, (b, e) *Arabidopsis thaliana*, and (c, f) *Zea mays*. *Populus tremula* × *alba* and *A. thaliana* show significant differences between developmental phase in area-based measures, whereas *Z. mays* shows phase dependence in mass-based measures. Leaf developmental phase represented as adult (red triangles), juvenile (blue circles), and juvenitized (light blue closed or open squares). P -values and r^2 from linear regression analysis displayed in upper right corner of each panel. Linear fits for panels (a)–(f) are as follows: (a) $A_{\text{sat Area}} = 20.41 - 0.02134(\text{SLA})$; (b) $A_{\text{sat Area}} = 9.049 - 0.004591(\text{SLA})$; (c) $A_{\text{sat Mass}} = 0.01188 + 0.002678(\text{SLA})$; (d) $A_{\text{sat Area}} = -2.948 + 0.1911(\text{N}_{\text{area}})$; (e) $A_{\text{sat Area}} = 2.867 + 0.02324(\text{N}_{\text{area}})$; (f) $A_{\text{sat Mass}} = -0.1081 + 0.4253(\text{N}_{\text{mass}})$.

Table 1 Photosynthetic nitrogen use efficiency represented by the slope of the linear relationship between mass-based light-saturated photosynthetic rate $A_{\text{sat Mass}}$ and mass-based leaf nitrogen N_{mass} .

Species	Effect	Slope	y-intercept	r^2	P -value
<i>Populus tremula</i> × <i>alba</i>	All stages	0.25	−0.30	0.55	< 0.0001
	$\text{N}_{\text{mass}} \times \text{Developmental stage}$				0.8654
<i>Arabidopsis thaliana</i>	All stages	ns	ns	ns	ns
	$\text{N}_{\text{mass}} \times \text{Developmental stage}$				ns
<i>Zea mays</i>	All stages	0.42	−0.11	0.50	< 0.0001
	$\text{N}_{\text{mass}} \times \text{Developmental stage}$				0.1626

Variation in leaf nitrogen was too low to find any significant relationship in *Arabidopsis thaliana* (ns, not significant). P -values determined by least-squares linear regression analysis across all leaves and by ANCOVA with developmental stage as the covariate.

Low-light photosynthetic traits

Under low light conditions ($\leq 50 \mu\text{mol m}^{-2} \text{s}^{-1}$), adult and juvenile/juvenitized leaves of *P. tremula* × *alba* and *A. thaliana* showed no differences in area-based photosynthetic rates

$A_{\text{low light Area}}$, whereas adult leaves of *Z. mays* had a slightly, but significantly lower $A_{\text{low light Area}}$ than juvenile or juvenitized leaves (Fig. 7). This is in contrast to the relative rates of photosynthesis we observed at saturating light levels, where adult leaves of *P. tremula* × *alba* and *A. thaliana* had a significantly higher

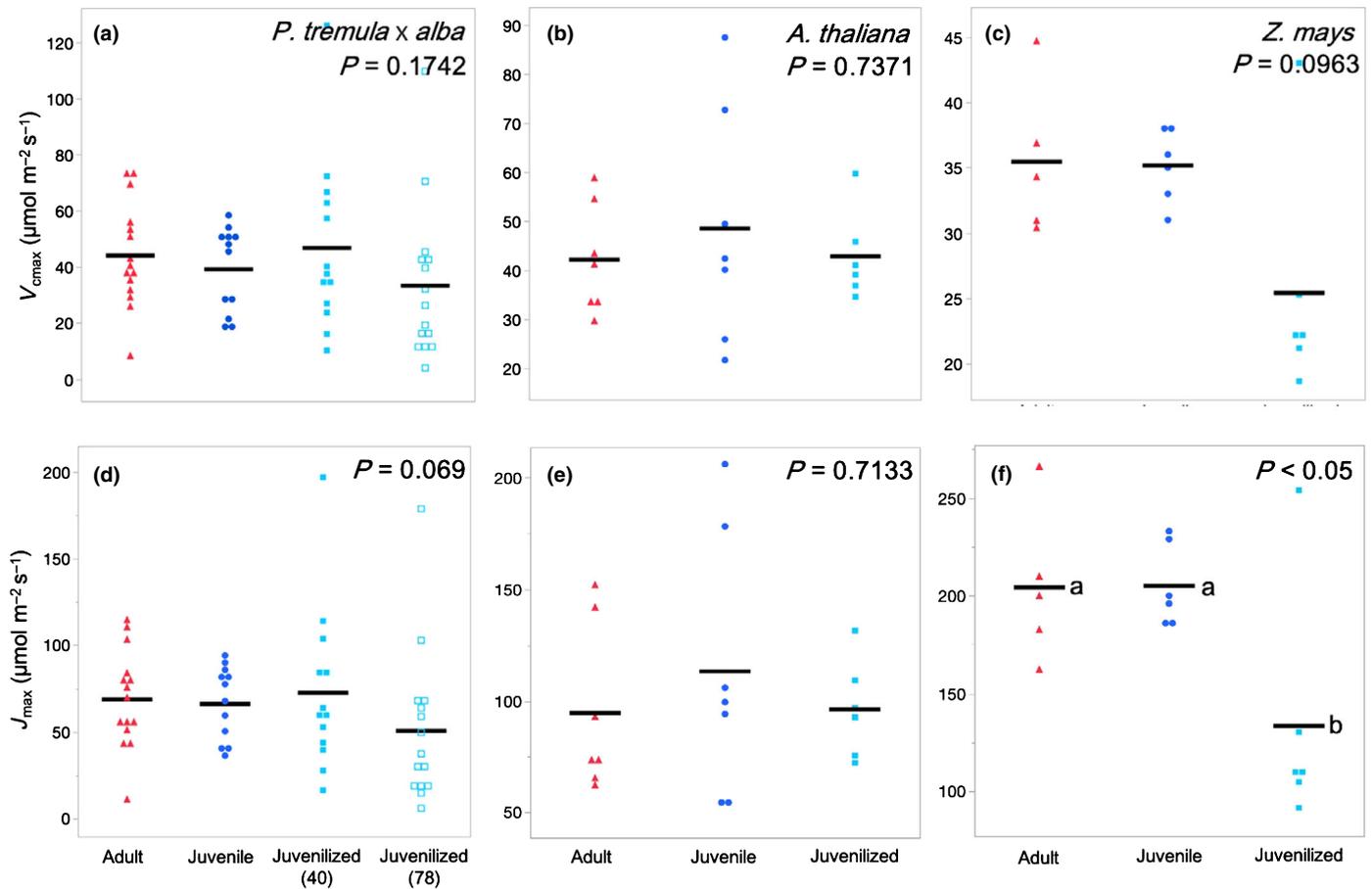


Fig. 6 Maximum Rubisco carboxylation rate V_{cmax} and maximum electron transport rate for ribulose-1,5-bisphosphate regeneration J_{max} of adult (red triangles), juvenile (blue circles), and juvenilized (light blue closed or open squares) leaves of (a, d) *Populus tremula* × *alba*, (b, e) *Arabidopsis thaliana*, and (c, f) *Zea mays*. Means presented as black horizontal lines. P -values from one-way ANOVA with development as the main effect. For traits with significant ANOVA results ($P < 0.05$), different lower-case letters indicate means of developmental stage are significantly different according to Student's t -test ($P < 0.05$).

$A_{sat Area}$ than juvenile leaves, and the $A_{sat Area}$ in maize was not significantly different in these leaf types. The relative advantage of juvenile leaves under low light conditions was even more pronounced when photosynthesis was measured on a mass basis ($A_{low light Mass}$): in low light, juvenile and juvenilized leaves of all three species had a significantly higher $A_{low light Mass}$ than adult leaves did. These results suggest that juvenile leaves are better adapted for photosynthesis under low light conditions than adult leaves are.

Role of leaf position on phase-dependent traits

To determine whether there was an effect of leaf position – independent of phase identity – on various traits, we looked across all measured leaf positions in wild-type and miR156 overexpressors of *P. tremula* × *alba* and *Z. mays*. Traits that varied with leaf number but were not significantly different between wild-type and mutant plants were considered to be affected by leaf position independently of their phase identity. This is because wild-type plants had juvenile leaves at low nodes and adult leaves at high

nodes, whereas miR156 overexpressors had juvenile leaves at all nodes. The traits that showed a leaf position effect, independent of developmental phase, were $A_{sat Area}$ and $A_{low light Mass}$ in *Z. mays* and J_{max} in *P. tremula* × *alba* (Table S3).

Photosynthetic traits in *Populus tremula* × *alba* grown from seed

The analyses of *P. tremula* × *alba* already described herein were conducted with cuttings of the 717-1B4 clone propagated *in vitro*. We considered the first-formed leaves on these plants to be juvenile leaves because they differed morphologically from later-formed leaves, and because the leaves of transgenic plants overexpressing miR156 closely resembled these first-formed leaves. To determine how closely these plants resemble normal *P. tremula* × *alba*, we examined a variety of traits in successive leaves of plants grown from seeds. Consistent with the results obtained with plants propagated *in vitro*, SLA, $A_{sat Area}$, $A_{low light Area}$ and F_v/F_m all showed significant differences between juvenile and adult leaves (Table 2). All other gas-exchange and

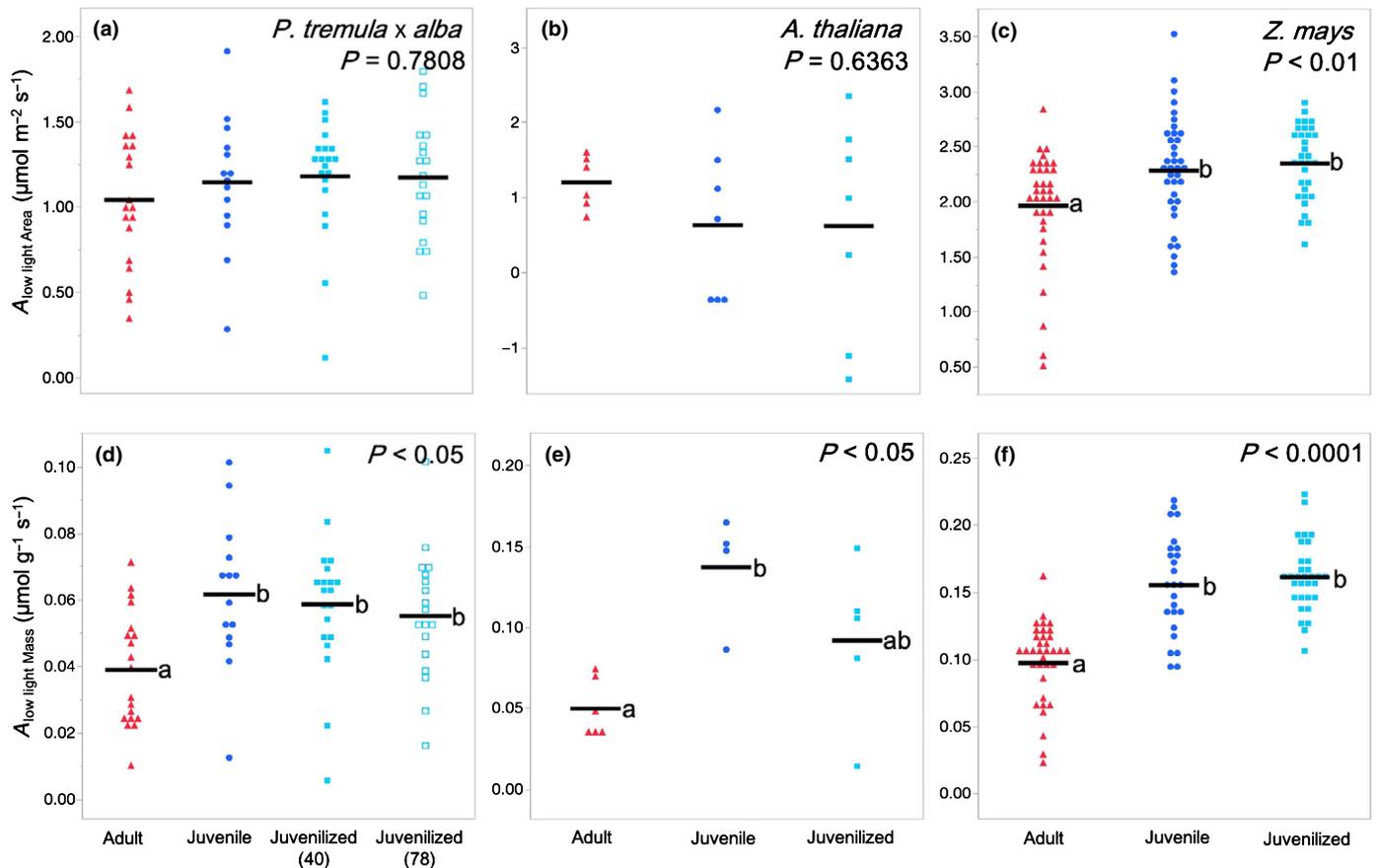


Fig. 7 Low-light photosynthetic rates for (a, d) *Populus tremula* × *alba*, (b, e) *Arabidopsis thaliana*, and (c, f) *Zea mays*. Light levels were approximately two to three times the light compensation point at $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ for *P. tremula* × *alba* and *A. thaliana* or $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ for *Z. mays*. Traits depicted are (a–c) area-based net photosynthetic rate at low light $A_{\text{low light Area}}$ and (d–f) mass-based net photosynthetic rate at low light $A_{\text{low light Mass}}$. Leaf developmental phase represented as adult (red triangles), juvenile (blue circles), and juvenitized (light blue closed or open squares). Means presented as black horizontal lines. *P*-values from one-way ANOVA with development as the main effect. For traits with significant ANOVA results ($P < 0.05$), different lower-case letters indicate means of developmental stage are significantly different according to Student's *t*-test ($P < 0.05$).

fluorescence traits did not display phase-specific differences, consistent with the results we obtained with 717-1B4 plants. These results demonstrate that photosynthetic physiology associated with VPC in *P. tremula* × *alba* plants regenerated *in vitro* is similar, if not identical, to VPC in seed-derived plants.

Discussion

Numerous studies have shown that leaves produced at different times in plant development often have different rates of photosynthesis (Bond, 2000). Here, we investigated whether this phenomenon can be attributed to the transition between juvenile and adult phases of vegetative development, a process called VPC. Previous studies have described differences in photosynthetic efficiency between juvenile and adult leaves of strongly heteroblastic species of *Eucalyptus* (Cameron, 1970; Velikova *et al.*, 2008) and *Acacia* (Brodrigg & Hill, 1993; Hansen, 1996; Yu & Li, 2007). However, it is difficult to know if these studies are generally relevant because of the large anatomical differences between juvenile and adult leaves in these species, and because these studies did not control for the effect of leaf position.

We characterized how VPC impacts photosynthesis independent of other confounding factors by manipulating the expression of miR156, the master regulator of this process. The miR156 overexpressors used in this study delay VPC, causing the plants to produce leaves with juvenile identity at positions that are normally adult. This made it possible to distinguish miR156-regulated photosynthetic traits from photosynthetic traits that vary as a function of leaf position or plant age.

In all three of the species we examined (*P. tremula* × *alba*, *A. thaliana* and *Z. mays*) the rate of light-saturated photosynthesis was phase dependent, although this relationship differed between species depending on whether area or mass-based measures were used (Fig. 2). Previous studies have revealed significant differences in the expression of photosynthetic genes in juvenile and adult leaves of *Z. mays* (Strable *et al.*, 2008; Beydler, 2014) and *Malus domestica* Borkh. (Gao *et al.*, 2014), suggesting that phase-dependent variation in the rate of photosynthesis might be attributable to differences in the biochemistry of photosynthesis in different leaves. However, multiple measures of photosynthetic capacity and light-use efficiency provided no evidence of this. Instead, we found that the difference in the rate of photosynthesis

Table 2 Photosynthetic and leaf morphological traits for juvenile and adult leaves of *Populus tremula* × *alba* grown from seed.

Trait	Developmental stage	Mean ± SE	n	Effect	df	P-value
SLA (cm ² mg ⁻¹)	Adult	0.20 ± 0.01	20	Developmental stage	1	< 0.001
	Juvenile	0.25 ± 0.01	34	Leaf position	1	< 0.05
A _{sat Area} (μmol m ⁻² s ⁻¹)	Adult	13.81 ± 0.69	20	Developmental stage	1	< 0.05
	Juvenile	10.32 ± 0.71	39	Leaf position	1	< 0.001
A _{sat Mass} (μmol m ⁻² s ⁻¹)	Adult	0.026 ± 0.002	20	Developmental stage	1	0.6039
	Juvenile	0.020 ± 0.001	33	Leaf position	1	< 0.1126
V _{cmax} (μmol m ⁻² s ⁻¹)	Adult	52.48 ± 4.03	20	Developmental stage	1	0.3886
	Juvenile	35.71 ± 2.56	37	Leaf position	1	< 0.01
J _{max} (μmol m ⁻² s ⁻¹)	Adult	84.34 ± 6.74	20	Developmental stage	1	0.3918
	Juvenile	56.91 ± 3.87	37	Leaf position	1	< 0.01
g _s high light (mol m ⁻² s ⁻¹)	Adult	0.206 ± 0.017	20	Developmental stage	1	0.4312
	Juvenile	0.181 ± 0.011	39	Leaf position	1	0.8840
A _{low light Area} (μmol m ⁻² s ⁻¹)	Adult	0.79 ± 0.10	20	Developmental stage	1	0.5738
	Juvenile	1.04 ± 0.05	39	Leaf position	1	0.9723
A _{low light Mass} (μmol m ⁻² s ⁻¹)	Adult	0.002 ± 0.0003	20	Developmental stage	1	0.9623
	Juvenile	0.002 ± 0.0002	33	Leaf position	1	0.8233
g _s low light (mol m ⁻² s ⁻¹)	Adult	0.102 ± 0.012	20	Developmental stage	1	0.6891
	Juvenile	0.139 ± 0.011	39	Leaf position	1	0.9723
Φ	Adult	0.057 ± 0.003	20	Developmental stage	1	0.4110
	Juvenile	0.053 ± 0.002	39	Leaf position	1	0.1068
LCP (μmol m ⁻² s ⁻¹)	Adult	14.46 ± 1.40	20	Developmental stage	1	0.6686
	Juvenile	8.61 ± 0.63	39	Leaf position	1	0.1916
F _v /F _m	Adult	0.78 ± 0.008	20	Developmental stage	1	< 0.05
	Juvenile	0.77 ± 0.007	39	Leaf position	1	< 0.01
ΦPSII	Adult	0.120 ± 0.010	20	Developmental stage	1	0.1680
	Juvenile	0.092 ± 0.008	39	Leaf position	1	< 0.01
NPQ	Adult	4.90 ± 1.03	20	Developmental stage	1	0.6039
	Juvenile	5.75 ± 0.64	39	Leaf position	1	0.8400
ETR (μmol m ⁻² s ⁻¹)	Adult	78.58 ± 6.63	20	Developmental stage	1	0.1680
	Juvenile	60.48 ± 5.42	39	Leaf position	1	< 0.01
R _{day} (μmol m ⁻² s ⁻¹)	Adult	-1.09 ± 0.16	20	Developmental stage	1	0.3886
	Juvenile	-0.78 ± 0.12	39	Leaf position	1	< 0.05

P-values from one-way ANOVA with developmental stage or leaf position as the effect variable. A_{low light}, low-light net photosynthesis; A_{sat}, light-saturated photosynthetic rate; ETR, electron transport rate; g_s, stomatal conductance; J_{max}, maximum electron transport rate for ribulose-1,5-bisphosphate regeneration; LCP, light compensation point; NPQ, non-photochemical quenching; PSII, photosystem II; R_{day}, day respiration; SLA, specific leaf area; V_{cmax}, maximum rate of Rubisco carboxylation.

in juvenile and adult leaves was most highly correlated with differences in the SLA and N content of these leaves (Fig. 5). This observation suggests that phase-dependent differences in photosynthetic rates are attributable to differences in leaf anatomy and leaf composition, rather than differences in the biochemistry of photosynthesis.

Leaf morphology and composition have robust relationships with photosynthesis across species and environments (Niinemets & Tenhunen, 1997; Reich *et al.*, 1998, 1999, 2003; Meziane & Shipley, 2001). Leaf thickness and tissue density – the structural changes that determine SLA – modulate intra-leaf light dynamics, CO₂ diffusion, and the distribution of leaf N (Parkhurst, 1994; Epron *et al.*, 1995; Terashima & Hikosaka, 1995; Reich *et al.*, 1998; Terashima *et al.*, 2006; Evans *et al.*, 2009). Specifically, variation in SLA changes the way light moves within the leaf, as path length and scattering are altered. This leads to leaves with low SLA absorbing more light per area as path length increases, ultimately leading to higher A_{sat Area} (Terashima & Hikosaka, 1995). However, leaves with low SLA face the

challenge of increased CO₂ diffusion resistance, as CO₂ must travel farther from stomata and through denser tissue to reach carboxylating enzymes (Parkhurst, 1994; Terashima *et al.*, 2006). SLA further impacts photosynthesis through the distribution of leaf N, as leaves with low SLA are associated with more cytoplasmic volume per leaf area, and therefore more N. The relationship between leaf N and photosynthesis results from the well-established relationship between N, Rubisco, and other photosynthetically important proteins (Field & Mooney, 1986; Evans, 1989; Ellsworth & Reich, 1993; Makino *et al.*, 1994; Bond *et al.*, 1999; Chmura & Tjoelker, 2008). These mechanisms describe how the physical properties of a leaf can alter photosynthetic rates independent of changes in biochemistry, supporting what we found in the developmentally distinct leaves of three species.

It is currently unclear why phase dependence in A_{sat} and leaf N are observed in area-based measures for *P. tremula* × *alba* and *A. thaliana* but in mass-based measures for *Z. mays* (although the fact that only one form of measurement correlates with SLA and leaf N is expected) (Westoby *et al.*, 2013). These three species all

Table 3 Linear fit between mass-based low-light photosynthetic rates ($A_{\text{low light Mass}}$) and specific leaf area (SLA).

Species	Traits	Slope	y-intercept	r^2	P-value
<i>Populus tremula</i> × <i>alba</i>	$A_{\text{low light Mass}}$ vs SLA	1.52×10^{-4}	−0.017	0.408	< 0.0001
<i>Arabidopsis thaliana</i>	$A_{\text{low light Mass}}$ vs SLA	9.851×10^{-5}	0.02	0.382	< 0.05
<i>Zea mays</i>	$A_{\text{low light Mass}}$ vs SLA	2.89×10^{-4}	−0.04	0.576	< 0.0001

have relatively high SLA, and no differences in PNUE between juvenile and adult leaves (Table 1), which would suggest differences in the A_{sat} –N slope due to SLA (Reich *et al.*, 1998) do not contribute to this phenomenon. Other potential explanations include differences in photosynthetic pathway (C_3 vs C_4), developmental form (dicot vs monocot), or variation in the morphological contributors to SLA (leaf thickness vs cell density). Because the relationships between SLA and photosynthetic rate are conserved across data sets that include both C_3 and C_4 species as well as both monocots and dicots, these traits are unlikely to explain the differences between species in this study (Reich *et al.*, 1999, 2003; Meziane & Shipley, 2001). Though density and thickness each contribute to variation in SLA, the degree to which they alter the photosynthetically important properties of a leaf vary. Because of this, Niinemets (1999) found that changes in leaf thickness are more closely correlated with area-based photosynthetic rates and changes in density are correlated with mass-based rates. As to be expected, changes in both leaf thickness and density have been associated with changes in SLA across all three study species (Bongard-Pierce *et al.*, 1996; Chuck *et al.*, 2011; Wang *et al.*, 2011; Coneva & Chitwood, 2018) and can be observed in cross-sections of adult, juvenile, and juvenilized leaves in this study (Fig. 4). Further studies are needed to determine the extent to which density and thickness contribute to phase-dependent changes in SLA and the mass or area-based correlations observed in this study.

Juvenile leaf morphology and photosynthetic properties may contribute to better survival in low-light environments, such as those frequently experienced by juvenile tissues at the bottom of a canopy. High SLA, found in juvenile leaves of all three species, is strongly correlated with higher photosynthetic rates under light-limited conditions and shade tolerance (Givnish, 1988; Niinemets & Tenhunen, 1997; Walters & Reich, 1999; Reich *et al.*, 2003). In support of this hypothesis, the juvenile leaves in each species had higher mass-based photosynthetic rates at low light levels ($A_{\text{low light Mass}}$) than adult leaves did (Fig. 7d–f; Table 3). Even in area-based measures of *P. tremula* × *alba* and *A. thaliana*, where adult leaves have higher A_{sat} , this photosynthetic advantage is lost under light-limited conditions (Fig. 7a–c). Further, variation in photosynthesis and SLA have been associated with tolerance to additional environmental factors, including drought and herbivory, and with changes in growth strategy, such as leaf life-span and growth rate (Poorter, 1999; Wright & Cannon, 2001; Reich *et al.*, 2003; Poorter *et al.*, 2009; Niinemets, 2010; Dayrell *et al.*, 2018). Because these traits are phase dependent, it is likely that, during a plant's lifetime, VPC contributes to variation in biotic and abiotic stress tolerance through changes in leaf morphology and photosynthesis, in addition to previously identified mechanisms (Stief *et al.*, 2014; Cui

et al., 2014; Arshad *et al.*, 2017; Ge *et al.*, 2018; Leichty & Poethig, 2019; Visentin *et al.*, 2020).

Our observation that SLA and the rate of photosynthesis change in a coordinated and phase-dependent fashion in three phylogenetically diverse species suggests that the phase dependence of these traits is widespread in flowering plants. The observation that these traits are independent of plant size and age also suggests that natural selection on the rate of photosynthesis may play an important role in the morphological adaptation of plants to their environment. In particular, conditions that favor juvenile or adult patterns of photosynthesis could drive changes in the timing of VPC, which would in turn lead to changes in numerous other phase-dependent vegetative traits. Determining how photosynthesis and other phase-dependent processes interact to influence plant performance is crucial for understanding plant ecology and evolution.

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Author contributions

EHL, CJS, BRH and RSP planned and designed the research. EHL performed the experiments. EHL performed statistical analyses and wrote the manuscript. EHL, CJS, BRH and RSP revised and provided comments on the manuscript.

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Supporting Information

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

Table S1 Linear fit between photosynthetic rates and leaf composition traits depicted in Fig. 5.

Table S2 Additional photosynthetic leaf traits for adult, juvenile and juvenilized leaves of *P. tremula* × *alba*, *A. thaliana* and *Z. mays*.

Table S3 Statistical results for leaf traits depicted in Figs 2, 3, 6 and 7.

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