EVALUATING THE LINK BETWEEN PHOTOSYNTHETIC CAPACITY AND LEAF VASCULAR ORGANIZATION WITH PRINCIPAL COMPONENT ANALYSIS

by

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Evaluating the link between photosynthetic capacity and leaf vascular organization with principal component analysis

Thesis directed by Professors Barbara Demmig-Adams and William W. Adams III

Principal component analysis was used to investigate variation in the anatomical features of the leaf minor veins among several summer annual crops (four symplastic versus four apoplastic phloem loaders) and three ecotypes of *Arabidopsis thaliana* (winter annual apoplastic loader) grown under multiple environmental conditions. The relationship between photosynthetic capacity and the first two principal components emerging from each analysis was then evaluated to identify the primary minor vein features underpinning photosynthetic capacity. Significant linear relationships between photosynthetic capacity and a principal component loaded by tracheary element cross-sectional areas and volumes per unit leaf area (water flux capacity proxy) was present for all species, emphasizing the importance of water delivery to the leaf in support of photosynthesis regardless of phloem loading mechanism. Significant linear relationships were also found between photosynthetic capacity and principal components loaded by phloem cell numbers and tracheary elements per minor vein as well as the latter two normalized for vein density (proxy for apoplastic phloem loading capacity involving membrane transporters) for all apoplastic loaders (summer annuals and winter annual *Arabidopsis thaliana*). Lastly, a significant linear relationship between photosynthetic capacity and a principal component loaded by phloem cell cross-sectional areas and volumes per unit leaf area (proxy for symplastic loading capacity involving cytosolic enzymes for companion cells) was revealed for summer annual symplastic loaders as well as for *A. thaliana* (in the case of sieve elements, a proxy for sugar export capacity from the leaves).

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CONTENTS

TABLES

Table

FIGURES

Figure

INTRODUCTION

The leaf vascular system is responsible for the concomitant import of water via xylem tissues and export of sugars via phloem tissues. In addition to water and sugar transport, the smallest (minor) veins of some species facilitate the movement of sugars from the photosynthesizing leaf mesophyll tissue into the vascular system through the process of active phloem loading (Slewinski *et al.* 2013). Some species load sugars apoplastically through H^+-sucrose (or H^+-sugar alcohol; *see*, *e.g.*, Klepek *et al.* 2005, Ramsperger-Gleixner *et al.* 2004) symporters embedded in the cell membranes of phloem cells (companion cells [CCs] and, in some species, sieve elements [SEs]) fueled by cell membrane ATPases (in CCs and phloem parenchyma cells [PPCs]) that pump protons into the apoplastic space of the phloem cell walls. Other species load sugars symplastically by converting smaller sugars (sucrose and galactose) into larger sugars (raffinose-family oligosaccharides) via enzymes localized to the cytosol of specialized CCs (Rennie and Turgeon 2009, Slewinski *et al.* 2013).

We have suggested that a leaf's light- and CO_2-saturated capacity for photosynthesis depends on the capacity for sugar export from that leaf, but that the particular phloem features supporting sugar-export capacity vary depending on the phloem-loading strategy employed by a particular species. While our previous studies used regression analysis to compare individual vascular parameters against photosynthetic capacity, we here use principal component analysis to compare the contributions of multiple vascular features to variation in the data and to the relationship with photosynthetic capacity. Based on our previous regression analyses, we had proposed that photosynthetic capacity among symplastic loaders depends on the size of phloem-loading cells and foliar vein density (as proxies for the capacities to synthesize and export raffinose-family sugars; Adams *et al.* 2013,

Muller *et al*. 2014a,b), whereas photosynthetic capacity among apoplastic loaders depends on the number of phloem cells per minor vein and vein density (as proxies for the total number of membrane-bound transport proteins involved in active loading and the capacity to export sugar out of the leaf; Adams *et al*. 2013, 2016; Cohu *et al*. 2013a, 2014; Muller *et al*. 2014a,b; Stewart *et al*. 2016, 2017a). However, each of the above studies focused on one or several species and/or growth habits and considered only one or a few light and temperature growth regimes. The present study applies principal component analyses to comprehensively evaluate which foliar vascular metrics likely support and underlie photosynthetic capacity by pooling results from most of the above-mentioned studies with new data for some of the species grown under additional conditions.

We compare the results from several summer annual crop species to those of the model organism *Arabidopsis thaliana* to relate our findings to the substantial body of knowledge on the biology of *A. thaliana* (Koornneef and Meinke 2010, Provart *et al.* 2016) and to also emphasize the features of this widely used model species. In contrast to many major crop species such as sunflower, *A. thaliana*, as a winter annual, germinates in the autumn, overwinters in a vegetative state, and reproduces in the spring. Winter annuals exhibit considerable anatomical and physiological adjustments in response to cold temperatures, whereas summer annuals exhibit little responsiveness to growth at low temperature (Adams *et al.* 2013, 2014, 2016; Cohu *et al*. 2013a,b, 2014; Dumlao *et al*. 2012, Gorsuch *et al.* 2010, Stewart *et al.* 2016, 2017a; Strand *et al.* 1997, 1999).

Principal component analysis (PCA) is a powerful and widely used statistical technique that reduces dimensionality and identifies relationships among variables (for an overview on PCA, *see* Abdi and William 2010). When PCA is performed on parameters of a similar nature, the principal components (PCs) can be thought of as composite parameters (*e.g*., multiple leaf phloem features) that can

subsequently be compared to other parameters of interest (*e.g.*, photosynthetic capacity). For example, Fedriani *et al.* (2015) applied PCA to multiple top-soil metrics, including pH and nitrogen content, and found that the first principal component provided an estimate of soil fertility at their sampling sites. These authors then evaluated the relationship between soil quality and seedling performance by plotting various measures of seedling growth and development at those sites against the first principal component. Furthermore, PCA allows researchers to identify and explain variation among groups in a large dataset through simultaneous consideration of multiple parameters. PCAs are, therefore, especially valuable in comparisons of groups that subtly but consistently differ across parameters of interest. In such cases, whereas collective differences between groups might be underestimated by multiple analyses of individual parameters or traits (*e.g.*, *t*-tests), a more accurate estimate of differences between groups can be provided by PCA (*i.e.*, a single analysis of multiple parameters).

In the present study, PCA was used to investigate variation in the physical features of the functional cell types of leaf minor veins among multiple summer annual crop species and three ecotypes of the model plant *A. thaliana* grown under various combinations of different light intensities, photoperiod lengths, and air temperatures. The relationship between photosynthetic capacity and the two most prominent (first two) principal components emerging from each analysis was then evaluated to identify the primary minor vein features underpinning photosynthetic capacity for summer annual symplastic loaders, summer annual apoplastic loaders, and the winter annual apoplastic loader *A. thaliana*. This analysis revealed both universal relationships among species and relationships that differed with growth habit and phloem loading mechanism.

MATERIALS AND METHODS

Plant material and growth conditions: Three ecotypes of the model plant *Arabidopsis thaliana* (L.) Heynhold (Col-0 wild type from Poland, as well as lines from Italy and Sweden; Ågren and Schemske 2012, Stewart *et al.* 2015), an apoplastic phloem loader, were compared with summer annual crop species with well-documented phloem loading strategies (*see* Muller *et al.* 2014a). Apoplastic loaders included cotton (*Gossypium hirsutum* L.), sunflower (*Helianthus annuus* L. cv. Soraya), tomato (*Solanum lycopersicum* L. cv. Brandywine), and tobacco (*Nicotiana tabacum* L.), while symplastic loaders included cucumber (*Cucumis sativus* L. cv. Straight Eight), pumpkin (*Cucurbita pepo* L. cv. Autumn Gold), squash (*Cucurbita pepo* L. cv. Italian Zucchini Romanesco), and watermelon (*Citrullus lanatus* L. cv. Faerie Hybrid).

All plants were grown from seed in soil (*Fafard Canadian Growing Mix 2*, *Conrad Fafard Inc.*, Agawam, MA, USA) in large pots with volumes of approximately 2.9 L for *A. thaliana*, 9.6 L for cotton, and 6.3 L for the other summer annuals, and received water daily with nutrients added every other day. To elicit a wide array of anatomical and physiological responses, plants were grown in climate-controlled growth chambers (*E15* and *PGR15*, *Conviron*, Winnipeg, Canada) under several light and temperature regimes previously described in detail by Cohu *et al.* (2013b, 2014), Muller *et al.* (2014a), and Stewart *et al.* (2017a). Final growth conditions (light/dark air temperature, photoperiod length, and light intensity) were as follows: 25°C/20°C, 9-h of 400 μmol(photon) m^{-2} s^{-1}; 25°C/20°C, 9-h of 1000 μmol(photon) m^{-2} s^{-1}; 25°C/20°C, 14-h of 1000 μmol(photon) m^{-2} s^{-1}; 8°C/12°C, 9-h of 400 μmol(photon) m^{-2} s^{-1}; 8°C/12°C, 9-h of 1000 μmol(photon) m^{-2} s^{-1}; 20°C/12°C, 9-h of 100 μmol(photon) m^{-2} s^{-1}; 12°C/12°C, 9-h of 1000 μmol(photon) m^{-2} s^{-1}. In addition, cucumber, pumpkin, tomato, and a wild sunflower (*Helianthus annuus* L.

Ames 17939; obtained from USDA Germplasm Resources Information Network, accession = PI 586873) were grown under 12-h photoperiods of low [100 μmol(photon) m^{-2} s^{-1}] and high [750 μmol(photon) m^{-2} s^{-1}] light intensities with respective air temperatures of 28°C and 27°C during the photoperiods, which resulted in similar leaf temperatures across all species and growth conditions (*i.e.*, no significant differences between groups; one-way ANOVA), and a common air temperature of 22°C during the dark periods.

Leaf minor vein anatomy and photosynthetic capacity: Leaf minor vein cell numbers and cross-sectional areas, minor vein density, and photosynthetic capacity were quantified as previously described (Amiard *et al.* 2005, Cohu *et al.* 2013b, Dumlao *et al.* 2012, Muller *et al.* 2014b, Stewart *et al.* 2017a). Leaf segments used to prepare minor vein cross-sections for quantification of leaf minor vein anatomy were fixed with a glutaraldehyde solution and embedded in Spurr resin (Spurr 1969), and leaf segments used for quantification of minor vein density [mm(minor vein length) mm^{-2}(leaf area)] were chemically cleared with 70% (v/v) ethanol and then 5% (w/v) NaOH. Leaf minor vein metrics were quantified with *ImageJ* (*Rasband WS*, *National Institute of Health*, Bethesda, MD, USA) from images taken with a light microscope (*Axioskop 20*, *Carl Zeiss AG*, Oberkochen, Germany) fitted with a digital camera system (*OptixCam OCView*, *The Microscope Store, LLC*, Roanoke, VA, USA). Photosynthetic capacity was assessed as light- and CO_2-saturated rates of photosynthetic oxygen evolution at 25°C (Delieu and Walker 1981; for additional explanation, *see* Adams *et al.* 2016) using leaf-disc oxygen electrode chambers (*LD2/2*, *Hansatech Instruments*, King's Lynn, Norfolk, UK).

Statistical analyses: Principal component analysis (PCA) was conducted on the leaf minor vein anatomical parameters (*see* Tables 1 and 2 for the parameters) of all

species included in the current study. For both PCAs, only the first two principal components (PCs) were featured, as they were the only PCs that explained more than 10% of the variation of the data (data not shown). Linear regression analyses were then conducted to assess the relationship between the coordinates of the first two PCs (representing composite vascular metrics) and photosynthetic capacity. Mean values for each group (*i.e.*, one genotype from one growth condition; n = 3–4 plants) were used for these linear regressions since, for species with smaller leaves (*e.g.*, *A. thaliana*), leaf segments used for photosynthetic capacity came from different leaves than the leaf segments used for anatomical measurements. A *t*-test was used for the comparison of two means, while a one-way analysis of variance (ANOVA) and post-hoc Tukey-Kramer test for Honestly Significance Differences were used for the comparison of multiple means. All statistical tests were conducted with *JMP* software (*Pro 13.0.0*, *SAS Institute Inc.*, Cary, NC, USA).

RESULTS

Due to the inclusion of multiple species and ecotypes acclimated to a wide range of growth conditions, photosynthetic capacities varied by an order of magnitude [from 10 to 108 $\mu mol(O_2)\ m^{-2}\ s^{-1}$], as did the total volumes per leaf area of all vascular cells (from 545 to 7356 $mm^3\ m^{-2}$), the volumes of sugar-exporting sieve elements (SEs; from 15 to 264 $mm^3\ m^{-2}$), and the volumes of sugar-loading companion cells and phloem parenchyma cells (CCs and PPCs; from 407 to 4525 $mm^3\ m^{-2}$). The means of photosynthetic capacity and the latter three volumes per leaf area did not differ significantly between the two summer annual groups and *A. thaliana*. The volume of water-transporting tracheary elements (TEs) per leaf area ranged from 94 to 1751 $mm^3\ m^{-2}$ among all species and growth conditions. In contrast to the means of the other vascular parameters, the mean TE volume per

unit leaf area (357 ± 17 mm^3 m^{-2}) in the winter annual *A. thaliana* was significantly lower than the corresponding mean for summer annuals (644 ± 36 mm^3 m^{-2}). This lower tracheary element volume in *A. thaliana* relative to that of the summer annuals resulted from a combination of significantly fewer veins per unit leaf area (Fig. 1*A*) with TEs that were significantly more numerous per minor vein (mean of 5.2 ± 0.1 compared to 2.1 ± 0.1 in the summer annuals) but with significantly smaller cross-sectional area per TE (mean of 24 ± 1 compared to 47 ± 2 μm^2 in the summer annuals).

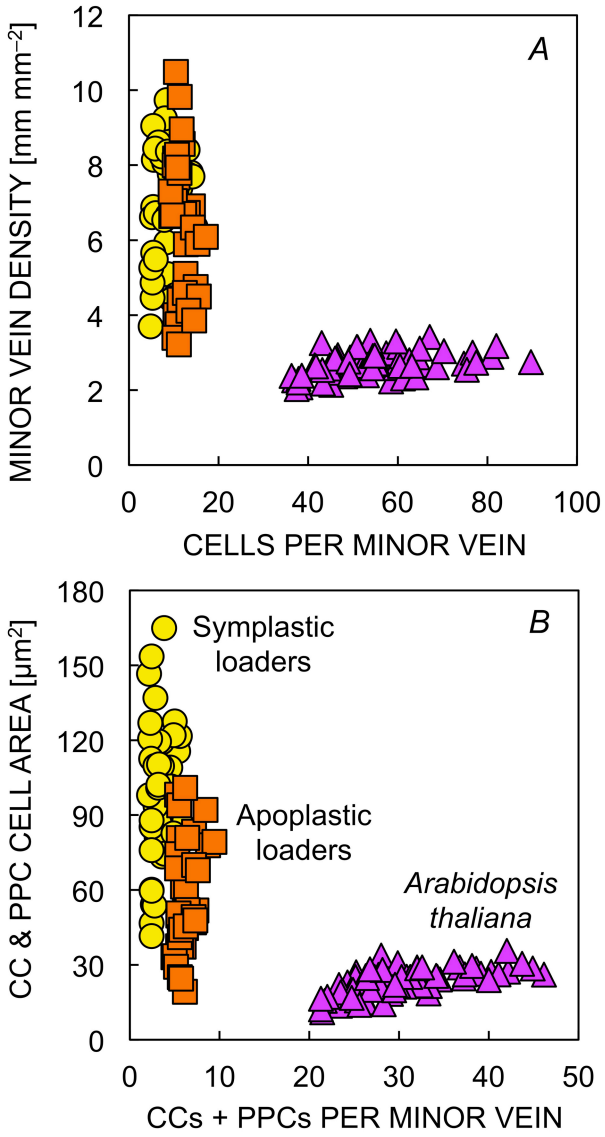

Figure 1. Relationships between (*A*) minor vein density and cell number per minor vein and (*B*) companion (CC) and phloem parenchyma (PPC) cell cross-sectional area and number per minor vein in the leaves of several summer annual symplastic (*circles*; $n = 39$) and apoplastic (*squares*; $n = 45$) loaders, as well as the winter annual apoplastic loader *A. thaliana* (*triangles*; $n = 72$).

Foliar minor vein density was low (mean of 2.7 ± 0.3 mm mm^{-2}), ranging between 2.0 and 3.4 mm mm^{-2}, among the ecotypes of *A. thaliana* under the different growth conditions included (Fig. 1*A*). In contrast, vein density spanned a

much larger range (3.2 to 10.5 mm mm^{-2}) among summer annuals (Fig. 1*A*). The number of vascular cells per minor vein, on the other hand, varied little among summer annuals (Fig. 1*A*), with means of 8.8 ± 0.4 cells (symplastic loaders) and 11.9 ± 0.3 cells (apoplastic loaders). In contrast, vascular cell number per minor vein was significantly greater, and spanned a considerable range from 36 to 90 cells per minor vein, among the ecotypes of *A. thaliana* (Fig. 1*A*). This latter pattern of numerous cells per vein was also seen for the subset of phloem cells (CCs and PPCs) facilitating the movement and loading of sugars from mesophyll cells to the conduits (SEs) for transport of those sugars out of the leaf (as it was for the SEs; not shown). While CC + PPC number per vein was relatively high and spanned a large range from 21 to 46 in *A. thaliana*, this number was significantly lower and spanned a smaller range from 2 to 9 cells among summer annuals (Fig. 1*B*). The number of CCs + PPCs per minor vein was, furthermore, significantly lower among summer annual symplastic loaders (mean of 3.4 ± 0.2) compared to summer annual apoplastic loaders (mean of 6.2 ± 0.2). In contrast, the sizes of these phloem cells (*i.e.*, the cross-sectional area of each cell) in *A. thaliana* spanned a narrow range (Fig. 1*B*) and, at a mean of 23.2 ± 0.6 μm^2, was significantly smaller on average than those of summer annual apoplastic loaders (ranging between 19 and 101 μm^2) or symplastic loaders (ranging between 42 and 165 μm^2). These vascular parameters, as well as others pertaining to the SEs of the phloem and TEs of the xylem, were subjected to principal component analyses as detailed in the following.

The first two principal components (PCs) from the PCA of leaf minor vein phloem features of all plants in the present study, $PC1_{Phloem}$ and $PC2_{Phloem}$ (Fig. 2), accounted for 91.1% of variation in the data. $PC1_{Phloem}$ was negatively loaded by phloem cell cross-sectional areas and positively loaded by phloem cell numbers per vein as well as the product of vein density and both phloem cell numbers per vein and cross-sectional areas (Fig. 2*B*, Table 1). As a consequence, larger cross-sectional

areas of minor-vein phloem cells of summer annual leaves (compared to *A. thaliana*) coupled with a greater number of phloem cells per vein of *A. thaliana* (compared to the summer annuals) provided a total separation (P<0.0001) between the $PC1_{Phloem}$ scores for the winter annual *A. thaliana* versus the summer annuals (Fig. 2*A*). $PC1_{Phloem}$ also provided a partial (albeit significant; P=0.001) separation between summer annual symplastic and apoplastic loaders, which was due to slightly larger phloem cell cross-sectional areas in symplastic loaders and slightly greater numbers of phloem cells per vein in apoplastic loaders (Fig. 2*A*). Although there was no significant difference between the two summer annual loading types in $PC2_{Phloem}$ scores, those of *A. thaliana* were significantly lower than those of either group of summer annuals (P<0.01), which was due to smaller phloem cell cross-sectional areas and volumes per unit leaf area (Fig. 2*A*). Plotting photosynthetic capacity versus $PC1_{Phloem}$ scores (Fig. 3*B,C*) yielded a significant linear correlation for summer annual apoplastic loaders as well as the winter annual apoplastic loader *A. thaliana*; increasing photosynthetic capacity was associated primarily with increasing phloem cell numbers per minor vein (Fig. 2, Table 1). In contrast, there was no correlation between photosynthetic capacity and $PC1_{Phloem}$ scores (Fig. 3*A*) for the summer annual symplastic loaders. In addition, plotting photosynthetic capacity versus the scores of $PC2_{Phloem}$, which were primarily driven by phloem cell cross-sectional areas and especially the volume of phloem cells per leaf area (Fig. 2*B*, Table 1), resulted in significant positive linear correlations for the summer annual symplastic loaders (Fig. 3*D*) as well as the winter annual apoplastic loader *A. thaliana* (Fig. 3*F*), but not for the summer annual apoplastic loaders (Fig. 3*E*). The differences between the two groups of summer annuals lie in the somewhat more numerous cells per minor vein in apoplastic loaders and somewhat larger cells in symplastic loaders (*see* also Fig. 1). For summer annual symplastic loaders (that synthesize larger sugars in their loading cells), photosynthetic capacity was not

correlated with $PC1_{Phloem}$ (Fig. 3*A*), but was positively correlated with $PC2_{Phloem}$ (Fig. 3*D*); *i.e.*, the component driven primarily by loading-cell cross-sectional areas and volumes per leaf area. In contrast, photosynthetic capacity of apoplastic loaders (that load sugars via membrane-bound transporters) was not correlated with $PC2_{Phloem}$ (Fig. 3*E*) but was positively correlated with $PC1_{Phloem}$ (Fig. 3*B*), *i.e.*, the component driven primarily by phloem cell numbers (per minor vein and normalized by vein density; Fig. 2*B*, Table 1).

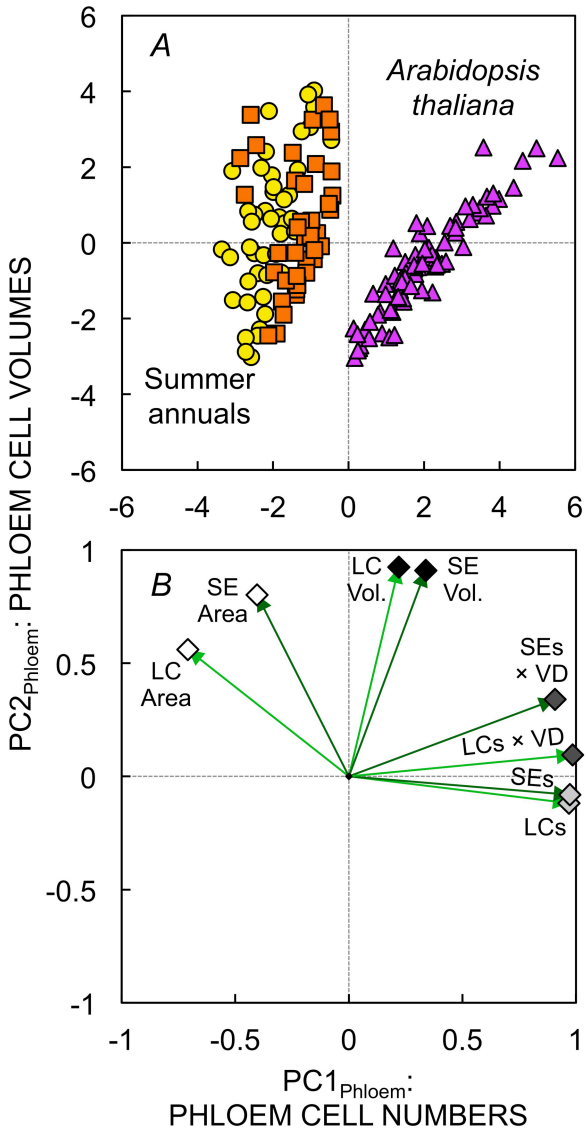

Figure 2. The (*A*) score and (*B*) loading plots for the first ($PC1_{Phloem}$) and second ($PC2_{Phloem}$) principal components, which explain the majority of the variation in the data ($PC1_{Phloem}$ = 56.3%; $PC2_{Phloem}$ = 34.8%), from a PCA of phloem cell cross-sectional areas (*open diamonds*), cell numbers per minor vein (*light gray diamonds*), cell numbers per minor vein normalized per leaf area (*dark gray diamonds*), and volumes per leaf area (*black diamonds*) of sieve elements (SE Area, SEs, SEs × VD, and SE Vol., respectively) and companion and phloem parenchyma cells (LC Area, LCs, LCs × VD, and LC Vol., respectively) in the leaves of several summer annual symplastic (*circles*; n = 39) and apoplastic (*squares*; n = 45) loaders, as well as the winter annual apoplastic loader *A. thaliana* (*triangles*; n = 72).

Table 1. Partial contributions [%] of the foliar phloem anatomical parameters for the first two components ($PC1_{Phloem}$, $PC2_{Phloem}$) from a PCA of several summer annual symplastic (n = 39) and apoplastic (n = 45) loaders, as well as the winter annual apoplastic loader *A. thaliana* (n = 72), as well as total partial contributions [%] of the types of parameters and types of cells. CC – companion cell; PPC – phloem parenchyma cell.

		$PC1_{Phloem}$	$PC2_{Phloem}$
Parameter	CCs + PPCs per vein	20.8	0.5
	Sieve elements per vein	21.0	0.2
	CCs + PPCs per vein × vein density	21.5	0.3
	Sieve elements per vein × vein density	18.3	4.2
	CC & PPC cross-sectional area	11.1	11.3
	Sieve element cross-sectional area	3.6	23.1
	CC + PPC volume per leaf area	1.1	30.7
	Sieve element volume per leaf area	2.5	29.7
Parameter type	Cell number per vein	41.8	0.7
	Cell number per vein × vein density	39.8	4.5
	Cell cross-sectional area	14.8	34.4
	Cell volume per leaf area	3.6	60.4
Cell type	CC & PPC	54.6	42.8
	Sieve elements	45.4	57.2

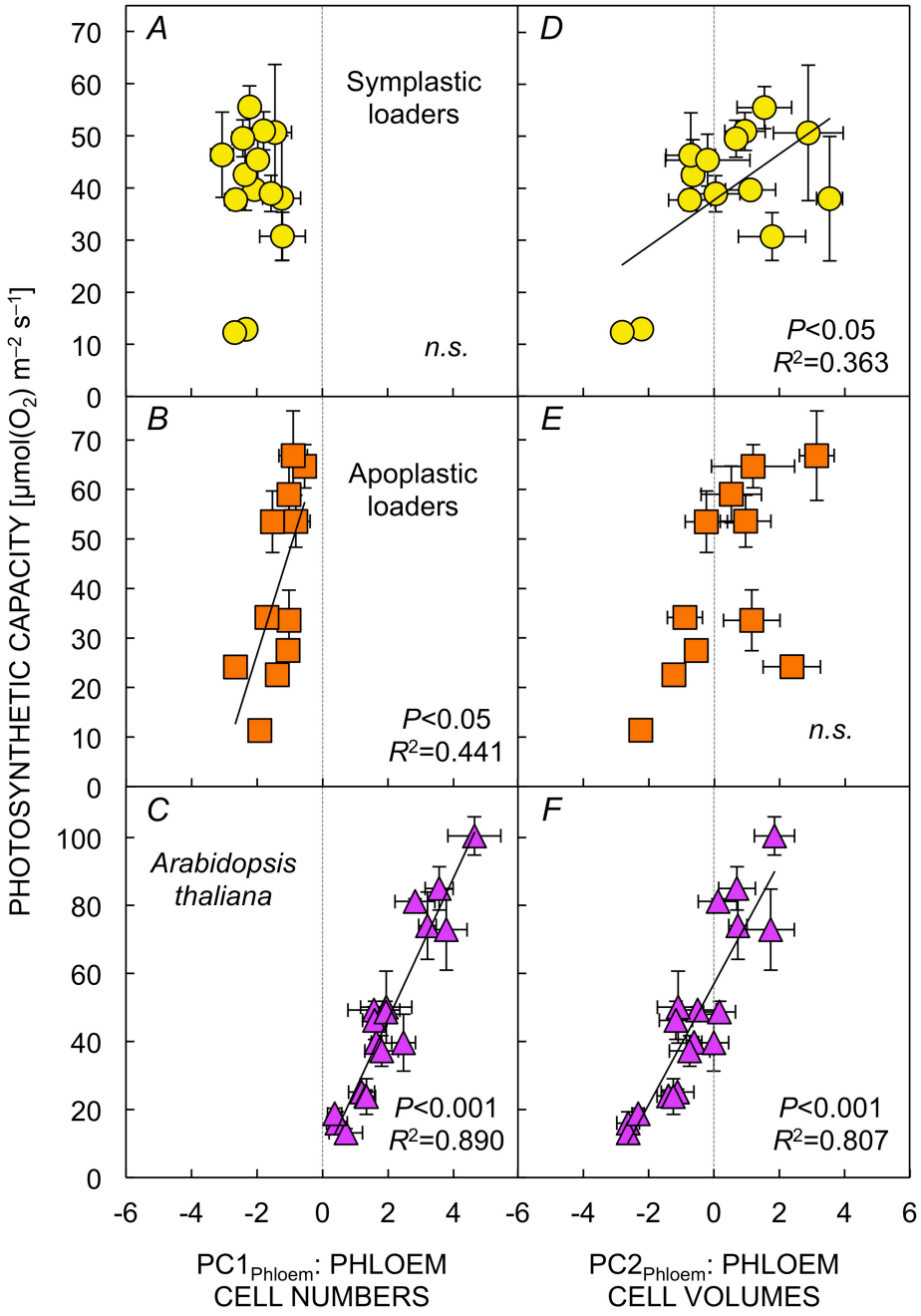

Figure 3. Relationships between photosynthetic capacity and the (*A*,*B*,*C*) $PC1_{Phloem}$ and (*D*,*E*,*F*) $PC2_{Phloem}$ scores of several summer annual (*A*,*D*) symplastic (*circles*) and (*B*,*E*) apoplastic (*squares*) loaders, as well as (*C*,*F*) the winter annual apoplastic loader *A. thaliana* (triangles). Mean values ± standard deviations; significant linear relationships are indicated with *P*-values and determination coefficients (*n.s.* – relationship not significant).

The first two principal components from the PCA of leaf minor vein xylem (tracheary element) features, $PC1_{TE}$ and $PC2_{TE}$ (Fig. 4), accounted for 94.9% of variation in these data. $PC1_{TE}$ was negatively loaded by tracheary element number per vein and positively loaded by the volume of tracheary elements per leaf area, the tracheary element cross-sectional area, and the product of vein density and tracheary element number per vein (Fig. 4B, Table 2). $PC2_{TE}$ was positively loaded by tracheary element number per vein (and the product of the latter × vein density) and negatively loaded by tracheary element cross-sectional area (Fig. 4*B*, Table 2). Both $PC1_{TE}$ and $PC2_{TE}$ showed considerable and significant ($P<0.0001$) segregation between summer annuals and the winter annual *A. thaliana* (Fig. 4*A*). This almost complete separation was driven by larger tracheary element cross-sectional areas in summer annuals, greater numbers of tracheary elements per vein of *A. thaliana*, and higher vein density in summer annuals (*see* also Fig. 1). Across the entire range of growth conditions represented by these data, photosynthetic capacity in the summer annuals (both symplastic and apoplastic loaders) and the winter annual *A. thaliana* was significantly and positively correlated with the xylem cell features that constituted $PC1_{TE}$ (Fig. 5*A,B,C*; particularly tracheary element cross-sectional area and, due to higher vein density, the volume of tracheary elements per leaf area; Fig. 4*B* and Table 2). On the other hand, the xylem cell features that constituted $PC2_{TE}$ (number of tracheary elements per minor vein and the product of the latter × vein density; Fig. 4*B*, Table 2) exhibited no relationship with photosynthetic capacity among summer annual symplastic loaders (Fig. 5*D*), but positive and significant relationships with photosynthetic capacity among apoplastic loaders (both summer annuals and the winter annual *A. thaliana*; Fig. 5*E,F*).

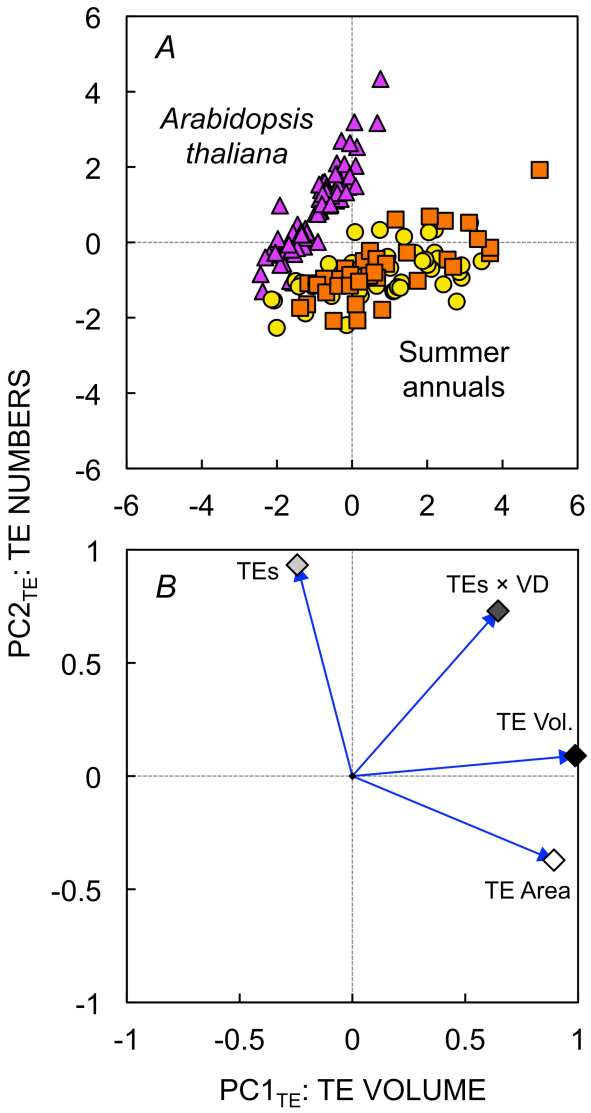

Figure 4. The (*A*) score and (*B*) loading plots for the first ($PC1_{TE}$) and second ($PC2_{TE}$) principal components, which explain the majority of the variation in the data ($PC1_{TE}$ = 56.2%; $PC2_{TE}$ = 38.7%), from a PCA of tracheary element cross-sectional areas (TE Area; *open diamond*), number per minor vein (TEs; *light gray diamond*), number per minor vein normalized per leaf area (TEs × VD; *dark gray diamond*), and volume per leaf area (TE Vol.; *black diamond*) in the leaves of several summer annual symplastic (*circles*; n = 39) and apoplastic (*squares*; n = 45) loaders, as well as the winter annual apoplastic loader *A. thaliana* (*triangles*; n = 72).

Table 2. Partial contributions [%] of the foliar xylem anatomical parameters for the first two components ($PC1_{TE}$, $PC2_{TE}$) from a PCA of several summer annual symplastic (n = 39) and apoplastic (n = 45) loaders, as well as the winter annual apoplastic loader *A. thaliana* (n = 72).

	$PC1_{TE}$	$PC2_{TE}$
Tracheary element cross-sectional area	35.5	8.9
Tracheary element volume per leaf area	43.4	0.5
Tracheary elements per vein	2.7	56.2
Tracheary elements per vein × vein density	18.5	34.4

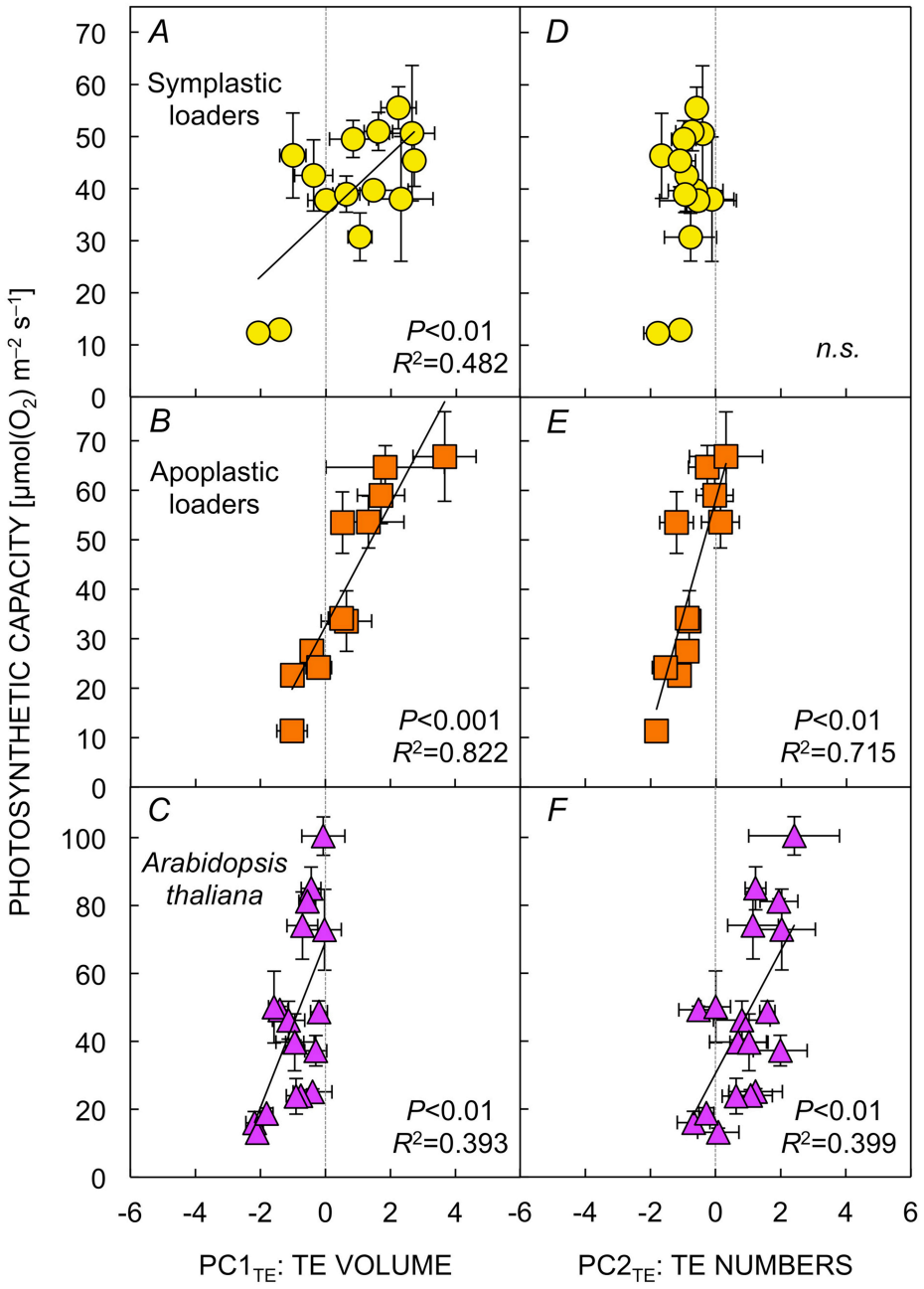

Figure 5. Relationships between photosynthetic capacity and the (*A*,*B*,*C*) $PC1_{TE}$ and (*D*,*E*,*F*) $PC2_{TE}$ scores of several summer annual (*A*,*D*) symplastic (*circles*) and (*B*,*E*) apoplastic (*squares*) loaders, as well as (*C*,*F*) the winter annual apoplastic loader *A. thaliana* (*triangles*). Mean values ± standard deviations; significant relationships are indicated with *P*-values and determination coefficients (*n.s.* – relationship not significant).

DISCUSSION

Inclusion of multiple species and growth light and temperature conditions resulted in a wide range of photosynthetic capacities, foliar minor vein densities, and minor-vein cellular features for evaluation. This data set thus encompasses both genetic differences and acclimatory adjustments of leaf anatomy and function in response to a wide range of different growth conditions.

Photosynthesis, vein density, and water conduits in summer annuals and the winter annual *Arabidopsis thaliana*: Photosynthesis depends on water supply to the leaf via the xylem to support stomatal opening for CO_2 uptake and replenishment of water lost through the stomates at varying levels determined by evaporative demand (Bond and Kavanagh 1999, Brodribb 2009, Brodribb and Jordan 2008, Hubbard *et al*. 2001, Zweifel *et al*. 2007). A greater supply of water to mesophyll tissue during the longer days and warmer temperatures of summer months may be facilitated by constitutively higher vein density (thus contributing to a higher volume of tracheary elements per unit leaf area) in the summer annuals compared to the winter annual *A. thaliana*. Higher vein density has been associated with higher leaf hydraulic conductance and can also contribute to a more even distribution of water across the leaf (*see* Sack and Scoffoni 2013, Prado and Maurel 2013). Summer annuals typically feature relatively high vein densities, especially when grown under high light intensities (Adams *et al*. 2005, Amiard *et al*. 2005). While vein densities in *A. thaliana* are relatively low, leaves acclimated to either high (versus low) light intensity or hot (versus cool) temperature exhibited somewhat greater vein densities (Adams *et al*. 2016, Stewart *et al*. 2016, 2017a,b), presumably to support greater evaporative demand under these conditions.

The larger individual cross-sectional areas of water-transporting TEs in summer annuals (compared to *A. thaliana*) may also be adaptive in the context of water flux. A greater radius of water-transporting conduits results in less frictional resistance to water flow (Zimmerman 1983; for other factors, *see* Sperry *et al.* 2006). The constitutively large TEs in minor veins of the summer annuals, the individual cross-sectional areas of which were almost twice as large as those of *A. thaliana*, may support the high demand for water during elevated summer temperatures. It should be noted that these conclusions apply to mesophytic species that tend to move large volumes of water under conditions of high evaporative demand and are typically found in habitats with sufficient access to soil water (*see* discussion in Adams *et al.* 2016). Woody species can grow in sites and during times of the year when water availability can be more limiting, and such species often employ different strategies that may involve a reduced volume of water movement under conditions of high evaporative demand in conjunction with mechanisms that reduce leaf heat load through means other than evaporative cooling (*see* discussion in Adams *et al.* 2016). Furthermore, the water-transporting conduits of the winter annual *A. thaliana* with smaller radii may be less susceptible to cavitation (Hacke and Sperry 2001, Pratt and Jacobsen 2017). Cold temperatures during the winter months pose a high risk of TE cavitation due to freeze-thaw events and low availability of liquid water in frozen soils (Langan *et al.* 1997, Davis *et al.* 1999, Repo *et al.* 2005, 2008; Beikircher *et al.* 2016). In fact, Cavender-Bares *et al.* (2005) suggested that narrower TEs confer greater freezing tolerance and thus a selective advantage in colder environments.

Many studies have documented the importance of foliar vein density and hydraulic conductance for photosynthetic CO_2 fixation (Beerling and Franks 2010, Blonder *et al.* 2011, Boyce *et al.* 2009, Brodribb and Feild 2010, Brodribb *et al.* 2005, 2007, 2010; Franks 2006, Hubbard *et al.* 2001, Maherali *et al.* 2008, McKown *et al.*

2010, Nardini *et al*. 2005, Sack and Holbrook 2006, Santiago *et al*. 2004, Walls 2011, Zhu *et al*. 2013), and TE number per minor vein (normalized for vein density) did indeed exhibit a significant and positive linear relationship with the capacity for photosynthetic oxygen evolution among eight summer annual symplastic and apoplastic loaders (Muller *et al*. 2014a). In *A. thaliana*, similar correlations between TE features and photosynthetic capacity were documented in response to experimental growth under high compared to low light intensity (Stewart *et al*. 2017a), but not in response to experimental growth at hot versus cold temperatures (Adams *et al*. 2016, Stewart *et al*. 2016). For a winter annual like *A. thaliana*, experimental growth at low temperature results in thicker leaves with higher capacities for photosynthetic oxygen evolution and minor veins with more numerous phloem cells but fewer xylem cells, whereas experimental growth at moderate and higher temperature conversely results in progressively thinner leaves with lower photosynthetic capacities and minor veins with fewer phloem but more numerous xylem cells that support higher transpiration rates (Adams *et al*. 2013, 2014, 2016; Cohu *et al*. 2013a,b, 2014; Stewart *et al*. 2016, 2017a). Furthermore, ecotypes of *A. thaliana* originating from progressively colder habitats exhibited greater acclimatory upregulation of both photosynthesis and phloem features in response to experimental growth at low temperature, whereas ecotypes of *A. thaliana* from progressively drier sites exhibited greater acclimatory upregulation of vein density, xylem features, and transpiration in response to experimental growth at high temperature (Adams *et al*. 2016). Despite these divergent responses between phloem (coupled to photosynthetic capacity under all growth conditions) and xylem (not always coupled to photosynthetic capacity), the current evaluation of *A. thaliana* integrating multiple experimental growth conditions was able to reveal a significant positive linear relationship between photosynthetic capacity and both the first (primarily driven by tracheary element volume per leaf area) and second

(primarily driven by tracheary element number per minor vein) principal components of the xylem features across all growth conditions. It is notable that the number of tracheary elements per minor vein was also significantly associated with photosynthetic capacity among summer annual apoplastic loaders. Given the association between phloem cell number per minor vein and photosynthetic capacity previously documented among apoplastic loaders (Adams *et al.* 2013, 2016; Cohu *et al.* 2013a, 2014; Muller *et al.* 2014a,b; Stewart *et al.* 2016, 2017a,b; *see* also below), it seems probable that altering the number of cells per minor vein is the primary means of adjusting the architecture of foliar vasculature among apoplastic loaders (at the genetic level among species and through phenotypic plasticity within a species acclimating to different growth conditions). The number of vascular cells in the minor veins of summer annual symplastic loaders exhibits very little variation, with phenotypic plasticity among symplastic loaders instead apparent in the cross-sectional area of vascular cells as well as in foliar vein density (Adams *et al.* 2013, Amiard *et al.* 2005, Dumlao *et al.* 2012, Muller *et al.* 2014a,b).

Photosynthesis and phloem features in summer annuals and the winter annual *Arabidopsis thaliana*: In contrast to the different volumes of water-transporting TEs per unit leaf area between the summer annuals and *A. thaliana*, the total volumes of both phloem-loading cells and sugar-transporting SEs per unit leaf area were comparable in the summer annuals and the *A. thaliana* ecotypes. The volume of SEs per unit leaf area is a proxy for the capacity to export the products of photosynthesis out of the leaf and would thus be expected to correlate with photosynthetic capacity. The finding of a similar range of SE volumes in the summer annuals and *A. thaliana* is consistent with the assumption that, while photosynthetic capacities vary widely with growth environment in all species, there is no systematic difference in maximal photosynthetic capacity between summer

and winter annuals (*see* also Demmig-Adams *et al*. 2014). The optimization of leaf function under a given set of environmental conditions involves developmental coordination of multiple features, including palisade and spongy mesophyll tissue (cell numbers and sizes contributing to leaf thickness, mesophyll cell surface area to volume ratio, chloroplast density per unit leaf area, *etc*.), stomata (density, aperture, responsiveness to environmental cues), as well as xylem and phloem features of the minor veins and vein density (Adams *et al*. 2005, 2013, 2014, 2016; Amiard *et al*. 2005, 2007; Brodribb and Feild 2010, Brodribb *et al*. 2005, 2007; Cohu *et al*. 2013a, 2014; Demmig-Adams *et al*. 2017, Dumlao *et al*. 2012, Evans *et al*. 2009, Hubbard *et al*. 2001, Jumrani *et al*. 2017, Kundu and Tigerstedt 1999, Maherali *et al*. 2008, Muller *et al*. 2014a,b; Oguchi *et al*. 2005, 2006, 2008; Stewart *et al*. 2015, 2016, 2017a,b; Strand *et al*. 1999, Tanaka *et al*. 2013, Taylor *et al*. 2012, Terashima *et al*. 2001, 2006, 2011; Wang *et al*. 2016, Wu *et al*. 2014, Zweifel *et al*. 2007).

Photosynthesis and phloem features in symplastic and apoplastic phloem loaders: We have argued previously (Adams *et al*. 2013, Muller *et al*. 2014a,b) that the cross-sectional area of sugar-loading cells might serve as a proxy for the capacity to synthesize the larger raffinose-family sugars as an essential feature of symplastic phloem loading, with the rationale that a larger cell would accommodate more of the enzymes responsible for catalyzing the attachment of galactose molecules to sucrose. Under the assumption that a greater capacity to load the phloem with the products of photosynthesis could support higher rates of photosynthesis, the significant linear relationships found for symplastic loaders in the present study between photosynthetic capacity and a principal component loaded primarily by phloem cell cross-sectional area (multiplied by vein density to yield the volume of phloem cells per leaf area) supports such an interpretation.

Sieve elements with a larger cross-sectional area contributing to this component would also provide a greater flux capacity for moving sugars from the leaves to the rest of the plant.

On the other hand, studies of apoplastic loaders had identified the number of phloem cells per minor vein as a feature associated with the photosynthetic capacity of leaves using this phloem loading mechanism (Adams *et al*. 2013, 2016; Cohu *et al*. 2013a, 2014; Muller *et al*. 2014a,b; Stewart *et al*. 2016, 2017a,b). It was therefore argued that variation in the number of phloem loading cells (genetically, among different species and ecotypes, as well as by acclimation to different growth conditions) was a way to modulate phloem-cell membrane area available for placement of membrane transporters utilized in active apoplastic sugar loading. For both the summer annual apoplastic loaders and the winter annual apoplastic loader *A. thaliana*, the significant positive linear relationships between photosynthetic capacity and a principal component driven largely by phloem cell numbers per minor vein is consistent with this interpretation. However, it should be noted that this principal component also included a contribution from vein density and phloem cell cross-sectional area . In the present study, summer annuals and *A. thaliana* grown in low light were included for the first time, and acclimation between low and high light involved a wider range of cell sizes than had been observed before, with very small vascular cells in low light for all species examined. The much larger minor vein phloem cells of leaves acclimated to high compared to low light have a larger membrane surface area available for placement of cell membrane-spanning transport proteins that facilitate phloem loading. In addition, the previously identified positive linear relationship between the number of phloem cells per minor vein and photosynthetic capacity among summer annual apoplastic loaders was only revealed when normalized for different vein densities in the different species (*i.e*., phloem cell number per minor vein × vein density) (Muller *et al*. 2014a).

Therefore, a direct assessment of cell surface area is needed, not only for individual cells, but also scaled to leaf area by multiplying cell perimeter by vein length per leaf area to obtain total cell surface area per leaf area for comparison with photosynthetic capacity per leaf area (for more detail, *see* below).

Towards a mechanistic framework: Whereas numerous experiments involving transport between source and sink tissues suggested that long-distance flux capacity of the phloem was unlikely to be limiting (for reviews, *see* Gifford and Evans 1981, Gifford *et al.* 1984, Wardlaw 1990), subsequent studies provided evidence that sugar loading into the phloem of foliar minor veins and sugar export from leaves can be limiting. Low-light-grown leaves (with low vein density and low capacity for loading and exporting sugar) were unable to upregulate photosynthetic capacity to that exhibited by high-light-grown leaves (with higher vein densities) of the same species upon sudden transfer from low to high light, suggesting a structural and functional limitation to carbon export and photosynthetic upregulation (Adams *et al.* 2007, Amiard *et al.* 2005). A similar outcome was observed for warm-grown leaves of *A. thaliana* following transfer to cool temperature (unpublished data). Moreover, the significant relationships between various minor vein phloem parameters and photosynthetic capacity in multiple species grown under various environmental conditions provide strong evidence of co-regulation between leaf structure and function during leaf development as well as potential limitations to photosynthetic capacity by the infrastructure of the foliar phloem (Adams *et al.* 2013, 2014, 2016; Cohu *et al.* 2013a,b, 2014; Muller *et al.* 2014a,b; Stewart *et al.* 2016, 2017a,b).

The current analyses provide new insight into the features of leaf minor-vein phloem with the potential to support photosynthetic capacity universally across species with differing vascular organizations, growth habits, and phloem

loading mechanisms, as well as those acclimated to different growth conditions. We have thus far focused on the quantification of vascular cell cross-sectional areas and vascular cell numbers per minor vein, individually and as products with minor vein density, as proxies for the flux capacity of vascular conduits (TEs or SEs) and the phloem-loading capacity of companion cells. While this approach allowed the identification of strong associations between photosynthetic capacity and leaf vascular features, future work could benefit from an approach based on features with a stronger mechanistic basis. A combination of three primary parameters may be able to provide such a mechanistic framework:

1) The total volume of sugar-exporting SEs per leaf area, which can be estimated from the product of total SE cross-sectional area per minor vein and minor vein length per unit leaf area. This parameter should be a common feature important to sugar-export capacity for all leaves regardless of phloem loading strategy or plant growth habit.
2) The total volume of sugar-loading cells per unit leaf area, which can be estimated from the product of loading cell cross-sectional area per minor vein and minor vein length per unit leaf area. This may be a feature that is only important for symplastic loaders, given their reliance on enzymes within such cells for the active loading step.
3) The surface area of phloem-loading cells, which is needed as a proxy for sugar flux into sugar-loading cells in both symplastic and apoplastic loaders, as well as a proxy for the capacity of active apoplastic loading (for estimates of surface areas and sugar flux rates, *see* Fondy and Geiger 1977, Giaquinta 1983).

To scale the perimeter of individual cells to a leaf area basis, the combined perimeter per vein of phloem cells involved in loading can be multiplied by vein length per unit leaf area (vein density) to obtain total cell surface area per leaf area

for comparison with photosynthetic capacity per leaf area. For apoplastic loaders, this cell surface area represents the area available for placement of transport proteins. These transporters include sugar efflux proteins and ATPases in PPCs, ATPases and H^+-sucrose (or H^+–sugar alcohol) symporters in CCs, and, in some species such as *A. thaliana*, H^+-sucrose symporters in SEs (Geiger 2011, Rennie and Turgeon 2009, Slewinski *et al.* 2013). Special care will need to be taken to quantify such areas in species possessing PPC and/or CC transfer cells with surface-area expanding cell wall ingrowths (Adams *et al.* 2014, 2016; Amiard *et al.* 2005, 2007). For symplastic loaders, as well as those species that do not employ any active phloem loading mechanism, the surface area of cells adjacent to sugar-exporting SEs approximates the area available for placement of plasmodesmata through which sugars flow into SEs. The interface between CCs and SEs is another region that might be useful to quantify, since plasmodesmata between the two provide the conduit for sugar passage from the former to the latter in all species regardless of phloem loading mechanism employed. The conductivity of plasmodesmata should also be assessed (*see*, *e.g.*, Giaquinta 1983, Liesche and Schulz 2013, Schulz 2015).

Direct determination of cell perimeters and their use to derive total cell surface areas per leaf area for various phloem cells will also allow testing of the assumption that the number per cell surface area of membrane transporters in apoplastic loaders, and of plasmodesmata in symplastic loaders and nonloaders, is constant. The numbers of plasmodesmata per unit cell length were found to be unaffected by growth conditions in pumpkin (Amiard *et al.* 2005). Furthermore, the close correlations that have already been demonstrated between photosynthetic capacity and phloem features associated with phloem-cell surface area would not be expected if the number of membrane transporters or plasmodesmata per cell surface area were variable.

Strategic selection of additional species for characterization: PCAs, such as those employed in the present study, should prove useful in probing and understanding relationships between foliar vasculature and photosynthesis in additional species. For instance, all four symplastic loaders in the present study are closely related members of the Cucurbitaceae. While the biennial symplastic loader *Verbascum phoeniceum* was not included in the present analysis, PCAs comparing *V. phoeniceum* with the biennial apoplastic loader *Malva neglecta* yielded similar differences to those between summer annual symplastic and apoplastic phloem loaders shown above (data not shown; *see* Muller *et al*. 2014b). It should be fruitful to explore the relationships between photosynthetic capacity and minor vein vascular features in species with different growth habits (evergreen species, winter or drought deciduous species, perennial species that die back each year, aquatic species, *etc*.), different venation patterns (*e.g*., bifurcating, parallel, as opposed to the reticulate pattern present in all of the species examined in the present study), additional loading mechanisms (especially the many species that rely solely on diffusion, as opposed to active loading mechanisms used by all species in the present study), and other photosynthetic pathways (C_4, CAM). Moreover, many major crop species are grasses (some C_3 and some C_4) with parallel venation, for which possible correlations between specific features and photosynthetic capacity have yet to be assessed.

Leaf vascular organization in the context of crop productivity and plant stress tolerance: There are two perspectives for evaluating implications of differences in leaf vascular organization for crop productivity. One focus is on correlations between leaf vascular features and photosynthetic capacity as the engine of plant productivity. While photosynthetic capacity is not always aligned with plant vegetative growth, there is always a close association between

photosynthetic capacity and the sum total of all demands for photosynthate, including those by growth, reproduction, and storage (*see* discussion in Demmig-Adams *et al*. 2017). The relationships evaluated here between photosynthetic capacity and leaf vascular features associated with the capacities for sugar loading and export as well as water transport suggest that efforts to identify, breed, or engineer crops with increased productivity should give attention to not only photosynthesis and plant sink strength, but also to the capacity of the vascular system that moves water and sugar in and out of the leaf. In addition to sugar-export capacity, the capacity of sugar loading is adjusted to match photosynthetic capacity, with the specific loading-cell features subject to adjustment differing by phloem-loading mode.

The other focus is on scenarios where a given photosynthetic capacity and a given capacity for sugar export from the leaf are paired with differences in the organization of the leaf's vascular network that allow for differential tolerance of environmental stresses like either hot or cold temperatures. One such scenario is the difference between summer annuals and a winter annual like *A. thaliana* with a different emphasis on water movement, which likely contributes to the superior performance of summer annuals under conditions of high evaporative demand. These species-dependent differences further extend to differences in foliar vascular organization that concern the size of individual minor veins versus their density as well as numbers and sizes of individual cells within these veins. As discussed above, more numerous, smaller water-transporting TEs may offer advantages in certain growth environments and, at the same time, support similar photosynthetic capacities. These insights promise to make contributions to the co-optimization of crop productivity and stress tolerance during a time of increasing demand for food against the backdrop of changing climate.

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