# Photosynthesis and foliar vascular adjustments to growth light intensity in summer annual species with symplastic and apoplastic phloem loading

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## Highlights

- Summer annuals exhibited variation in multiple features of minor foliar veins
- Photosynthetic capacity and leaf-level volume of all vascular cell types were correlated
- Relationship of loading-cell size and number varied by phloem-loading mechanism
- The proportion of vascular cell number to size was greater in apoplastic loaders

## Abstract

Concomitant adjustments in photosynthetic capacity and size, composition, and/or density of minor foliar veins in response to growth environment were previously described primarily for winter annuals that load sugars into foliar phloem apoplastically. Here, common trends, differences associated with phloem-loading mechanism, and species-dependent differences are identified for summer annuals (loading sugars either symplastically [cucumber, pumpkin, and basil] or apoplastically [tomato and sunflower]) that were grown in low and high light. Photosynthetic capacity per leaf area was significantly positively correlated with leaf-level volume of phloem-loading cells (LCs), sugar-export conduits (sieve elements), and water conduits (tracheary elements) irrespective of phloem-loading mechanism. The relative contribution to leaf-level volume of LC numbers versus individual LC size was greater in apoplastic loaders than in symplastic loaders. Species-dependent differences included different vein density within each loading group and either greater or lower numbers of cells per minor vein (especially of tracheary elements in the symplastic loaders basil versus cucumber, respectively), which may be due to genetic adaptation to different environmental conditions. These results indicate considerable plasticity in foliar vascular features in summer annuals as well as some loading-mechanism-dependent trends.

Keywords: light response, loading cells, phloem, photosynthesis, summer annuals, xylem

Abbreviations: Loading cell (LC), photon flux density (PFD), sieve elements (SE), tracheary element (TE)

## Introduction

Minor foliar veins can exhibit a strong response to growth environment (Sack et al., 2012). During leaf development, the architecture of the foliar vasculature can acclimate in concert with photosynthetic capacity in response to environmental conditions, including adjustment in vein density, vascular cell size and numbers, and transfer cell wall/membrane ultrastructure (Cohu et al., 2013a; Adams et al., 2014, 2016; Stewart et al., 2016, 2017b; for a review, see Adams et al., 2018). This latter evidence was derived mainly from winter annual species that actively load sugars into the phloem of minor foliar veins via an apoplastic mechanism (for details on loading mechanisms, see below). Much less information of this kind is available for summer annuals and species that load sugar symplastically. Available studies either compared several summer annual species using different active phloem-loading mechanisms in plants grown under a single environmental condition (Muller et al., 2014a) or made comparisons that involved only a single species each of two active phloem-loading mechanisms tested under different growth environments - different temperature and/or light intensity (Cohu et al., 2014; Muller et al., 2014b). Moreover, Cohu et al. (2014) reported little difference in certain vascular parameters assessed for two summer annual species using different active phloem-loading mechanisms, whereas Muller et al. (2014b) did report differences in other vascular parameters in response to growth temperature for a comparison of two biennial species (one an apoplastic loader and one a symplastic loader).

The current paper addresses the effect of both phloem-loading mechanism and growth environment (low versus high light intensity) on the foliar vascular features of five species of summer annuals that actively load sugars into the phloem via two different mechanisms. Two of the species (sunflower and tomato) were previously characterized as apoplastic phloem loaders (Gamalei, 1989; Lalonde et al., 1999; Turgeon et al., 2001; Turgeon and Ayre, 2005) that transport sugars through a $H^+$-sucrose symporter embedded in the cell membranes of the loading cells (LCs) (Ramsperger-Gleixner et al., 2004; Sondergaard et al., 2004; Klepek et al., 2005). Three species (cucumber, pumpkin, and basil) were previously characterized as symplastic phloem loaders (Volk et al., 1996; Büchi et al., 1998; Kang et al., 2007; Turgeon, 2016) that use polymer trapping (converting sucrose/galactose into larger raffinose-family oligosaccharides) by enzymes in the cytosol of the loading cells that prevents the larger sugars from flowing back into the leaf (Rennie and Turgeon, 2009; Slewinski et al., 2013).

The choice to include the specific apoplastic loaders tomato and sunflower and the two symplastic loading cucurbits is based on the previous work from Muller et al. (2014a) who reported a significant positive correlation between photosynthetic capacity and either the number or the individual size (cross-sectional area) of phloem-loading cells in apoplastic and symplastic loaders, respectively, when grown under a single growth condition of high light. Larger loading cells can presumably accommodate more of the enzymes that produce oligosaccharides in the symplastic loaders. On the other hand, positive correlations between photosynthetic capacity and loading-cell number in apoplastic loaders were suggested to provide benefit via enhanced membrane area available for placement of membrane-bound transport proteins for protons and sugars (Adams et al., 2013, 2018). Both the larger loading cells in symplastic loaders and the more numerous cells in apoplastic loaders presumably increase export capacity for photosynthate from the leaf. Use of a single growth condition (high light) likely limited the range of variation in the numeric value of vascular parameters in the study by Muller et al. (2014a).

We selected those species from each phloem-loading type used by Muller et al. (2014a) that exhibited the greatest differences in photosynthetic capacity and in foliar vein density for additional testing for any robust differences that depend on loading mechanisms independent of such other features (e.g., vein density differences). Use of species with very different vein densities also facilitated assessment of scale-dependent differences that may reside at the single vein level and/or the whole leaf level. Furthermore, to ascertain whether the relationships reported by Muller et al. (2014a) apply beyond the four species from the cucurbit family that represented symplastic loaders in the latter study, basil was added as another symplastic loader

(Büchi et al., 1998; Kang et al., 2007) from the well-characterized symplastic phloem-loading family Lamiaceae (Davidson et al., 2011).

The present study involved growth under low versus high light that typically lead to pronounced differences in photosynthetic capacity on a leaf area basis. This approach allows further testing of which foliar vascular features correlate best with photosynthetic capacity, and addresses whether the constitutive differences in features correlated with photosynthetic capacity (loading-cell size in symplastic loaders and loading-cell numbers in apoplastic loaders) reported by Muller et al. (2014a) extend to differences in foliar vascular features that may be adjusted in response to growth light environment in concert with adjustments in photosynthetic capacity.

The present study tested the hypotheses that (i) either cross-sectional area or number of phloem-loading cells in minor foliar veins will be adjusted in symplastic and apoplastic loaders, respectively, in response to growth light intensity and (ii) additional adjustments in foliar vascular anatomy may occur in response to growth light environment irrespective of phloem-loading type that correlate with photosynthetic capacity in both groups.

## Materials and Methods

### *Plant material and growth conditions*

Symplastic phloem loaders included the cucurbits (Cucurbitaceae) pumpkin (*Cucurbita pepo* L. cv. Autumn Gold) and cucumber (*Cucumis sativus* L. cv. Straight Eight) as well as basil (*Ocimum basilicum* L. cv. large leaf Italian), a member of the Lamiaceae. Apoplastic phloem loaders included sunflower (*Helianthus annuus* L. ANN 2199; Asteraceae) and tomato (S*olanum lycopersicum* L. cv. Brandywine; Solanaceae). Sunflower seeds were obtained from USDA-ARS Germplasm Resources Information Network, and all other seeds were obtained from Park Seed Co. (Hodges, SC, USA).

Plants were grown in growth chambers (E15 and PGR15, Conviron, Winnipeg, Canada) controlled for temperature and light environment including photoperiod (12 hour) and light intensity of low light (100 µmol photons $m^{-2}$ $s^{-1}$) or high light (750 µmol photons $m^{-2}$ $s^{-1}$). Air temperatures during the light period were set to 28°C and 27°C in low and high light, respectively, which resulted in similar leaf temperatures in both light environments for each species (see Supplemental Table 1). Leaf temperature was measured with an infrared thermometer (Fisher Scientific Traceable Infrared Thermometer, Fisher Scientific, Pittsburgh, PA). During the dark period, air and leaf temperature was kept at 22°C. Plants were grown from seed in soil (Fafard Canadian Growing Mix 2, Conrad Fafard Inc., Agawam, MA, USA) in 6.3 L pots, receiving water daily with added nutrients every other day. Characterized leaf samples from non-flowering plants were collected from portions of expanded leaves not subject to self-shading. For plant age at harvest and leaf temperature, see Supplemental Table 1.

*Foliar vascular anatomy and photosynthesis*

Light- and $CO_2$-saturated capacity of photosynthetic oxygen evolution was determined with leaf-disk oxygen electrode systems (Hansatech Instruments, King's Lynn, Norfolk, United Kingdom) using leaf samples maintained at 25°C with circulating water baths (Fisher Scientific, Pittsburgh, PA, United States). Light saturation of photosynthesis for plants grown under low and high light conditions was reached at 1500 and 2000 µmol photons $m^{-2}$ $s^{-1}$, respectively.

Vein density (vein length per leaf area) was measured from leaves that were cleared of pigments by soaking in 70% (v/v) ethanol followed by 5% (w/v) NaOH. Cross-sections for characterization and measurements of minor foliar veins were prepared with an Ultracut E microtome (Reichert-Jung, Wien, Austria) from leaf sections that had been fixed in a glutaraldehyde and paraformaldehyde solution before they were dehydrated and embedded in Spurr resin (Spurr, 1969).

Sieve elements (SEs) are the conduits through which sugars can be moved out of the leaf to the rest of the plant. Loading cells (LCs) facilitate the movement of sugar into the SEs and comprise the rest of the phloem tissue. The xylem is composed of water conduits (tracheary elements, TEs) and xylem parenchyma cells that likely support water movement and facilitate embolism reversal (Secchi et al., 2017; Brodersen et al., 2018; Cardoso et al., 2018; Klein et al., 2018; Trifilò et al., 2019). For schematic representations of each cell type, see Supplemental Figure 1.

Images of leaf sections used for vein anatomy and vein density were captured using a light microscope (Axioskop 20, Carl Zeiss AG, Oberkochen, Germany) with a digital camera (OptixCam Summit Series, Microscope.com, Roanoke, VA, USA). Vascular features were quantified using the computer program ImageJ (Rasband WS, National Institute of Health, Bethesda, MD, USA). Visual identification of cell type was conducted from light microscopic images. For more information on how leaf segments for foliar vascular measurements (vein density, cell cross-sectional area, and conduit volume) were prepared and quantified see Stewart et al. (2019).

### *Measured and derived vascular parameters*

Individual cross-sectional area was quantified for each vascular cell type (the average of all individual cells from minor vein images) as was the cross-sectional area occupied by all cells of that type per minor vein. In addition, the two-dimensional cross-sectional area per vein for each cell type was multiplied by a third dimension of vein length. Multiplication by minor vein density (length of minor veins per unit leaf area) provides an estimation of the total volume of a given vascular cell type per unit leaf area (Stewart et al., 2019). Additionally, the product of cell number per vein × vein density provided a normalization across species to account for the differing minor vein densities among species. These latter two calculations scale the vasculature measurements to the level of a leaf from the level of an individual minor vein.

### *Statistical analysis*

Mean values and standard errors were calculated from seven to ten cross sections from each of three plants per species under each growth light regime. Differences in minor-vein vascular features were assessed via one-way analysis of variance with post-hoc Tukey–Kramer HSD test for comparison of means across species. Linear regressions and density ellipses ($P = 0.950$) were used for comparisons in scatter plots. All statistical analysis was carried out using JMP Pro 15.0.0 (SAS Institute Inc., Cary, NC, USA).

## **Results**

### *General features of apoplastic and symplastic loaders*

Schematic images (based on light microscopy) of representative cross-sections of minor foliar veins (Fig. S1) illustrate that basil exhibited intermediate features between those of the apoplastic loaders and the two cucurbit symplastic loaders with respect to number of cells per vein. Total cell number per minor vein showed no consistent effect of either loading mode or growth photon flux density (PFD), except for a somewhat higher cell number in tomato grown under high versus low light (Fig. 1A). Total cross-sectional area per minor vein was not significantly different among the five species in plants grown in low light, was greater in all species except basil when grown in high light (Fig. 1B), but again with no clear discernable effect of loading mode. Maximal photosynthetic capacity per leaf area was considerably and significantly greater in plants grown under high versus low PFD in all species (Fig. 1C). Basil had a significantly lower photosynthetic capacity than any of the other species especially at high light intensity (Fig. 1C). Vein density was either similar (tomato) or modestly greater (sunflower, basil, cucumber, pumpkin) in plants grown under high versus low PFD, again with no clear difference by loading type (Fig. 1D).

### *Relationship between photosynthetic capacity and foliar vascular infrastructure in summer annual apoplastic and symplastic phloem loaders*

Leaf photosynthetic capacity (Fig. 1C) was significantly and positively correlated with leaf-level volume of phloem-loading cells (LCs; Fig. 2A), sugar-export conduits (SEs; Fig. 2B), and water-transport conduits (TEs; Fig. 2C) for both symplastic and apoplastic loaders. Both sugar and water conduit volume of basil fell considerably below the general relationship between sieve and tracheary volume and photosynthetic capacity, whereas both sugar and water conduit volume of pumpkin fell considerably above this relationship (Fig. 2B,C).

### *Analysis of loading cell-associated features in apoplastic and symplastic phloem loaders*

There was a positive relationship between LC volume and sugar conduit (SE) volume on a leaf area basis regardless of phloem-loading mechanism (Fig. 3). Most data points for the symplastic loaders from the cucurbit family fell to the left of that relationship for the apoplastic loaders, with relatively greater LC versus SE volumes. However, the data points for LC versus SE volume for the symplastic loader basil fell on the same relationship as those of the apoplastic loaders. There was thus no consistent difference in this relationship between phloem-loading mode with the inclusion of basil.

Further analysis showed that the relationship between LC number at the leaf level (LC number per vein × vein density) and individual LC size (average cross-sectional area per LC) between the different phloem loading mechanisms reduced the overlap between the apoplastic and symplastic phloem loaders (Fig. 4). There were fewer, larger LCs for the symplastic loaders compared to smaller more numerous LCs in apoplastic loaders (Fig. 4)

This separation of apoplastic and symplastic loaders was driven predominantly by the greater number of LCs on a leaf area basis in the apoplastic loaders (Fig. 5A). In addition, LC number was significantly greater in high versus low growth PFD in all species except cucumber (Fig. 5A). Individual LC cross-sectional area did not differ consistently between apoplastic and symplastic loaders, with similarly small LCs in basil as in the two apoplastic loaders but larger LCs in the cucurbits (Fig. 5B). Individual LC cross-sectional area was significantly greater in plants grown in high versus low light only among the two cucurbit symplastic loaders (Fig. 5B). Total LC cross-sectional area per vein (as the product of individual LC size and LC number per vein) was also relatively similar across species (Fig. 5C), i.e., not significantly different in plants grown in low light across all five species, but significantly greater in high light in tomato compared to sunflower, basil, and pumpkin (but not compared to cucumber). Although LC cross-sectional areas per minor vein were higher in high versus low PFD among all species (Fig. 5C), this difference was thus not dependent on loading mechanism.

The relationship between the ratio of LCs to SEs number and the ratio of cross-sectional LC to SE area in minor veins also led to separation of the data points for the two apoplastic loaders versus all three symplastic loaders (Fig. 6). Apoplastic loaders fell above the symplastic loaders with relatively more numerous LCs per SE in a minor vein. Basil exhibited a similarly low ratio of LC to SE cross-sectional area as the apoplastic loaders, which contrasted with the higher ratio of LC to SE cross-sectional area in the two cucurbits (Figs. 6 and 7A). While the ratio of LC to SE number in minor veins was consistently higher in leaves that developed under high versus low light (albeit not significantly so; Fig. 7A), the ratio of LC to SE cross-sectional area in minor veins exhibited no consistent nor significant response to light intensity (Fig. 7B).

### *Analysis of sugar and water conduit features in apoplastic and symplastic phloem loaders*

There was a significant and positive correlation between the leaf-level volume of sugar (SEs) and water (TEs) conduits for all species as well as for each loading group when considered independently (Fig. 8). In contrast to the differences between apoplastic and symplastic loaders with respect to some features involving LCs, the relationships between TE number and TE size (Fig. 9A) or SE number and SE size (Fig. 9B) overlapped among the apoplastic and symplastic loaders. Likewise, none of the individual parameters for SEs or TEs exhibited significant differences between apoplastic and symplastic loaders (Fig. 10).

In particular, basil exhibited similarly high TE and SE numbers per vein as the two apoplastic loaders whereas the two cucurbits had significantly lower TE and SE numbers per vein (Fig. 10A,B). Basil's high sugar and water conduit numbers per minor vein were associated with a low vein density (Fig. 1D), which resulted in the overall cell volume per leaf area being low (Fig. 2B,C). TE number per minor vein was significantly higher in high compared to low growth PFD in only the apoplastic loaders (Fig. 10A) whereas SE number per minor vein did not vary significantly in any species when compared between growth light intensities (Fig. 10B). Cross-sectional areas of both TEs and SEs were larger in leaves that developed under high compared to low light intensity, but only significantly so for the TEs of pumpkin (Fig. 10C) and the SEs of both cucurbits (Fig. 10D).

## Discussion

Leaf vasculature offers structural support to the leaf as a platform for photosynthesis, with larger leaf veins scaling in size with leaf dimension (Sack et al., 2012). The minor foliar veins, on the other hand, are adjustable in number per unit leaf area, composition, and size to meet the leaf's needs for sugar loading/export and water transport in response to plant growth environment (Sack et al., 2012; Adams et al., 2013; Cohu et al., 2013a,b; Stewart et al., 2017b, 2016; Polutchko et al., 2018). Close positive correlations between foliar minor vein density, size, composition, and/or ultrastructure of veins and maximal photosynthetic capacity (Cohu et al., 2013b, 2014; Adams et al., 2014, 2016, 2018), transpiration rate (Cohu et al., 2013b; Muller et al., 2014a; Adams et al., 2016, 2018), or foliar hydraulic conductance (Brodribb et al., 2005; 2007; 2010; Franks, 2006; McKown et al., 2010; Scoffoni et al., 2016) have been demonstrated.

Much of the work on links between foliar vasculature and photosynthetic capacity has focused on apoplastically loading winter annuals (Cohu et al., 2013b; Adams et al., 2016; Stewart et al., 2016, 2017a,b), with some evidence for differences between winter and summer annuals (Cohu et al., 2014) as well as between symplastic and apoplastic loaders (Adams et al., 2013; Muller et al., 2014a,b; Polutchko et al., 2018). For example, Muller and colleagues reported constitutive differences in numbers and sizes of the sugar-loading cells in minor foliar veins in summer annual apoplastic compared to symplastic phloem loaders (Muller et al., 2014a) as well as between a biennial apoplastic loader and a biennial symplastic loader (Muller et al., 2014b; see also Adams et al., 2013, 2018; Polutchko et al., 2018). Specifically, it was proposed that numerous loading cells in foliar minor veins of apoplastic loaders may provide a large membrane area for transport proteins (Adams et al., 2013, 2016, 2018; Cohu et al., 2013a) and that large loading cells in symplastic loaders may provide ample volume for enzymes that convert sucrose to raffinose-family oligosaccharides (Adams et al., 2013; Muller et al., 2014a). However, Stewart et al. (2017a,b) also showed that the winter annual apoplastic loader *Arabidopsis thaliana* responded to light intensity by featuring small phloem cells when grown under low light and much larger cells when grown under high light.

These previous characterizations were extended in the present study by including the additional symplastic loading species (basil) from an additional family (Lamiaceae) beyond species from the Cucurbitaceae used by Muller et al. (2014a) and plant growth in low light in addition to high light (summer annuals were only grown in high light in the latter study). Growth under two very different light intensities in the present study broadened the range of variation in some of the characterized parameters' numerical values. Furthermore, basil exhibited a particularly high number of cells in minor veins (which differs from the low cell numbers in the cucurbits) and a low photosynthetic capacity (which differed from all other species). The other four species – beyond basil – included in the present comparison represented the two previously characterized extremes in vascular features among the four apoplastically-loading species and four symplastically-loading species examined by Muller et al. (2014a). Together, this variation in multiple features enhanced the testing rigor of what vascular features, if any, vary consistently with phloem-loading mechanism.

The resulting findings show that cell number per minor vein did not vary much (in tomato) or at all (all other species) with acclimation to low versus high light intensity nor was loading-cell number constitutively and consistently higher in apoplastic compared to symplastic loaders. Furthermore, total cross-sectional area per minor vein was significantly greater irrespective of loading mechanism in four of the five species (all except basil) despite no significant differences in average vascular cell number per minor vein in four of the five species (all except tomato) in high versus low light. Larger veins and larger individual vascular cell sizes (cross-sectional areas) in high versus low light were observed, as had also been seen in *A. thaliana* (Stewart et al., 2017a,b; Adams et al., 2018; Polutchko et al., 2018). These findings on adjustments in individual vascular cell size are consistent with those of Muller et al. (2014b) who reported on a comparison of a single apoplastically loading biennial (*Malva neglecta*) and a single symplastically loading biennial (*Verbascum phoniceum*) grown under two temperatures, where both species exhibited growth-environment-dependent differences in individual vascular cell size as well as total cross-sectional area per vein. The latter two-species comparison also showed a significant, albeit minor, difference in cell number per vein of sieve elements, loading cells, and tracheary elements depending on growth temperature in the apoplastic loader but not in the symplastic loader.

The absence of consistent loading-mechanism-dependent differences in vascular cell number is consistent with the finding of Cohu et al. (2014) that a symplastically loading (squash) and an apoplastically loading (sunflower) summer annual grown under two temperatures in either moderate or high light intensity exhibited “little evidence for a different response” as dependent on loading mechanism in total cross-sectional area of either sieve elements or loading cells per vein. A dichotomy between individual cell size versus cell number as exclusive targets of adjustment in symplastic versus apoplastic of adjustments is thus not consistent with the evidence. Furthermore, larger cells can be expected to not only accommodate more enzymes for the sucrose trapping mechanism of symplastic loaders but also contribute to greater membrane area for placing of transporters (ATPases and sucrose-proton symporters in apoplastic loaders; Vaughn et al., 2002; Amiard et al., 2005, 2007) as well as greater cell wall area for plasmodesmata (between bundle sheath and intermediary cells of symplastic loaders and between companion cells and sieve elements of species of either loading mechanism; Giaquinta 1983; Schulz 2015).

However, the above considerations do not exclude the possibility that the relative contribution of modulation in vascular cell number versus individual size may differ dependent on loading mechanism. A trend for different clusters of proportionally greater individual loading cell size and lower loading cell number for the symplastic versus apoplastic summer annuals used by Muller at al. (2014a) was suggested by principal component analysis (Polutchko et al., 2018). Likewise, the species examined here, that varied profoundly in vein density and photosynthetic capacity within loading mechanism, exhibited distinct clusters by loading mechanism for the relationship between individual loading cell size and either (i) loading cell number at the scale of the whole leaf (features at the vein scale × total vein length per leaf area, which considers all cells per unit of leaf) or (ii) the ratio of loading cell to sieve element number as a measure of loading capacity per sugar-conducting conduit (see Adams et al., 2007). Thus, relative differences are important, and it is necessary to consider features at multiple scales beyond a single vein, including at both the individual cell and whole leaf levels.

On the other hand, the relationship between photosynthetic capacity and the volume of each type of vascular cell type at the scale of the whole leaf (total volume of cells per minor vein × total length of veins per leaf area) for all plants grown under low and high light intensity was similar among all summer annual species examined in the current study, and largely independent of phloem-loading mechanism. Such a relationship had only been apparent among symplastic loaders, but not among apoplastic loaders, in previous analyses that utilized plants grown solely under high light (Muller et al., 2014a,b; Polutchko et al., 2018), where loading cell volume did not vary widely. The significant correlation was particularly strong for sugar-loading cells but also present (albeit less strong) for sieve elements and tracheary elements. The assessment of cell volume per leaf area (as the product of cell number per minor vein × vein density) allows for an estimation of the capacity of these different cell types per unit of leaf rather than for an individual vein, which provides for an appropriate comparison with foliar photosynthetic capacity that is also expressed on a leaf area basis. A similar relationship between photosynthetic capacity (on a leaf area basis) and the capacity of vascular infrastructure at the scale of the whole leaf is intuitive since both symplastic and apoplastic loaders presumably need to enhance vascular capacity to support enhanced photosynthetic capacity – with the secondary effect that the vascular capacity (volume of vascular cells at the leaf level) is adjusted with a greater emphasis on loading-cell number (apoplastic loaders) versus loading-cell size (symplastic loaders).

However, there were also notable deviations from the general relationships for photosynthetic capacity versus vascular cell volume within the same loading-mode group, e.g., between basil and cucumber (both symplastic loaders). Data points fell above (cucumber) or below (basil) the general relationships between maximal photosynthetic capacity and especially tracheary element volume. The greater (in basil) or lesser (in cucumber) sieve element or tracheary element volume can be traced to the high (basil) or low (cucumber) cell numbers per vein and are evidently not related to loading mode but to some other driver. One may speculate that these species-dependent differences could be driven by differences in evaporative demand in an evolutionary context. For example, ecotypes of *Arabidopsis thaliana* from sites of origin with different precipitation levels exhibited different ratios of tracheary to sieve elements as well as different transpiration rates under common conditions (Adams et al., 2016, 2018). Beyond any species-dependent differences, all species in the present study are summer annuals and likely have some adaptations that support high rates of transpiration under high temperature and large water potential gradient (Boyce et al., 2009). High vein density and relatively large volumes of water-transport infrastructure for supporting photosynthesis under these conditions could concomitantly aid in cooling the plant on a hot summer day (Nardini et al., 2005; Pagano and Storchi, 2015; Lin et al., 2017).The results presented here support the need for further research into constitutive differences in foliar vascular architecture of summer annuals, winter annuals, and biennials with different loading mechanisms (see also Cohu et al., 2014; Polutchko et al., 2018) as well as their acclimation of the foliar vasculature at different scales (subcellular, cellular, vein, and leaf) to various environments. Such insight may aid in development of crops for improved leaf vascular features and superior photosynthetic performance in specific climates.

## Author Contributions

SKP: Investigation, Formal analysis, Visualization, Supervision, Writing - Original Draft; JJS: Investigation, Formal analysis, Visualization, Supervision, Funding acquisition, Writing - Review & Editing; BD-A: Conceptualization, Formal analysis, Visualization, Funding acquisition, Resources, Supervision, Project administration, Writing - Original Draft. WWA: Conceptualization, Methodology, Formal analysis, Visualization, Funding acquisition, Resources, Supervision, Project administration, Writing - Original Draft.

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## Conflict of Interest

We declare no conflict of interest.

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## Figure Legends

**Figure 1.** (A) Total number of cells per foliar minor vein, (B) average cross-sectional area of foliar minor veins, (C) photosynthetic capacity (light- and $CO_2$-saturated rate of oxygen evolution at 25°C), and (D) foliar minor vein density of two apoplastic and three symplastic loaders grown under low light (light columns) and high light (dark columns). Mean values ± standard errors. Mean values that differed significantly from one another, based on ANOVA across all species and both growth light intensities, are indicated by different lower-case letters.

**Figure 2.** Relationship between photosynthetic capacity and volume (per unit leaf area) of (A) loading cells, (B) sieve elements, and (C) tracheary elements of apoplastic (purple line and symbols) and symplastic (blue line and symbols) loaders. Species are represented by different symbols: tomato (circles), sunflower (triangles), basil (inverted triangles), pumpkin (squares), and cucumber (diamonds). All linear regressions were statistically significant ($P < 0.05$). The $R^2$ values for LCs (Fig 4A) are $R^2_{\text{Apoplastic}} = 0.84$, $R^2_{\text{Symplastic}} = 0.79$, and $R^2_{\text{AllSpecies}} = 0.81$. The $R^2$ values for SEs (Fig 4B) are $R^2_{\text{Apoplastic}} = 0.71$, $R^2_{\text{Symplastic}} = 0.34$, and $R^2_{\text{AllSpecies}} = 0.52$. The $R^2$ values for TEs (Fig 4C) are $R^2_{\text{Apoplastic}} = 0.79$, $R^2_{\text{Symplastic}} = 0.32$, and $R^2_{\text{AllSpecies}} = 0.56$.

**Figure 3.** Relationship between loading-cell and sieve-element volume on a leaf area basis with density ellipses ($P = 0.950$) among apoplastic loaders (purple ellipse and symbols) and symplastic loaders (blue ellipse and symbols). Species are represented by different symbols: tomato (circles), sunflower (triangles), basil (inverted triangles), pumpkin (squares), and cucumber (diamonds).

**Figure 4.** Relationship between loading cell cross-sectional area and loading volume with density ellipses ($P = 0.950$) of the apoplastic phloem loaders (purple ellipse and symbols) and the symplastic phloem loaders (blue ellipse and symbols). Species are represented by different symbols: tomato (circles), sunflower (triangles), basil (inverted triangles), pumpkin (squares), and cucumber (diamonds).

**Figure 5.** (A) Number of loading cells (LC) per foliar minor vein normalized by vein density, (B) average cross-sectional area of individual loading cells, and (C) average cross-sectional area of foliar minor veins occupied by loading cells in two apoplastic and three symplastic loaders grown under low light (light columns) and high light (dark columns). Mean values ± standard errors. Mean values that differed significantly from one another, based on ANOVA across all species and both growth light intensities, are indicated by different lower-case letters.

**Figure 6.** Relationship between the ratio of loading cell (LC) to sieve element (SE) cross-sectional area and the ratio of loading cell to sieve element number in minor veins, shown with density ellipses ($P = 0.950$) for apoplastic loaders (purple ellipse and symbols) and symplastic loaders (blue ellipse and symbols). Species are represented by different shapes: tomato (circles), sunflower (triangles), basil (inverted triangles), pumpkin (squares), and cucumber (diamonds).

**Figure 7.** (A) Ratio of minor vein loading cell (LC) cross-sectional area to sieve element (SE) cross-sectional area and (B) ratio of loading cell number to sieve elements per foliar minor vein normalized by vein density of the two apoplastic and three symplastic species grown under low light (light columns) and high light (dark columns). Mean values ± standard errors. Mean values that differed significantly from one and other, based on ANOVA across all species and both growth light intensities, are indicated by different lower-case letters.

**Figure 8.** Relationship between minor vein tracheary element volume and sieve element volume on a leaf area basis for the apoplastic (purple line and symbols; $R^2_{\text{Apoplastic}} = 0.82$) and symplastic (blue line and symbols; $R^2_{\text{Symplastic}} = 0.86$) loaders. Species are represented by different symbols: tomato (circles), sunflower (triangles), basil (inverted triangles), pumpkin (squares), and cucumber (diamonds). All linear regressions were significant at $P < 0.001$; $R^2_{\text{AllSpecies}} = 0.82$.

**Figure 9.** Relationship between (A) average cross-sectional area of individual tracheary elements and tracheary element number per minor vein and (B) average cross-sectional area of individual sieve elements and sieve element number per minor vein, shown with density ellipses ($P = 0.950$) for apoplastic loaders (purple ellipse and symbols) and symplastic loaders (blue ellipse

and symbols). Species are represented by different symbols: tomato (circles), sunflower (triangles), basil (inverted triangles), pumpkin (squares), and cucumber (diamonds).

**Figure 10.** Average number of (A) tracheary elements and (B) sieve elements per minor vein and average cross-sectional area of (C) individual tracheary elements and (D) individual sieve elements in two apoplastic and three symplastic loaders grown under low light (light columns) and high light (dark columns). Mean values ± standard errors. Mean values that differed significantly from one another, based on ANOVA across all species and both growth light intensities, are indicated by different lower-case letters.

## Supplemental Tables

**Supplemental Table 1.** Leaf age at characterization and leaf temperature during growth. Plants grown in high light (HL) grew more quickly compared to plants grown under low light (LL). Leaves were characterized when fully expanded and of a similar size between light treatments. Leaf temperature was assessed for each species and treatment, except for basil. Air temperatures during the light period were set to 28°C and 27°C in low and high light, respectively, which resulted in similar leaf temperatures in LL and HL for each species**.**

| | Age at characterization (days) | | Leaf temperature (°C) | |
|---|---|---|---|---|
| **Species** | LL | HL | LL | HL |
| Pumpkin | 22 | 19 | 25.8 ± 1.1 | 26.6 ± 1.7 |
| Cucumber | 21 | 21 | 25.4 ± 0.7 | 25.8 ± 0.8 |
| Basil | 37 | 26 | | |
| Sunflower | 43 | 27 | 27.0 ± 0.8 | 27.8 ± 0.3 |
| Tomato | 29 | 29 | 26.9 ± 0.8 | 27.9 ± 0.6 |

## Supplemental Figure Legends

**Supplemental Figure 1.** Schematic depictions of representative cross-sectional images of foliar minor veins from each species with different cell types given in blue (xylem tissue; tracheary elements in dark blue and xylem parenchyma cells in light blue) or green (phloem tissue; sieve elements in dark green and sugar-loading cells in light green). Species in Cucurbitaceae have bicollateral phloem with some additional, adaxial phloem cells (shown in white since these cells apparently do not transport photosynthate; Schmitz et al., 1987; Sui et al., 2021) above the xylem cells.

Figure 1

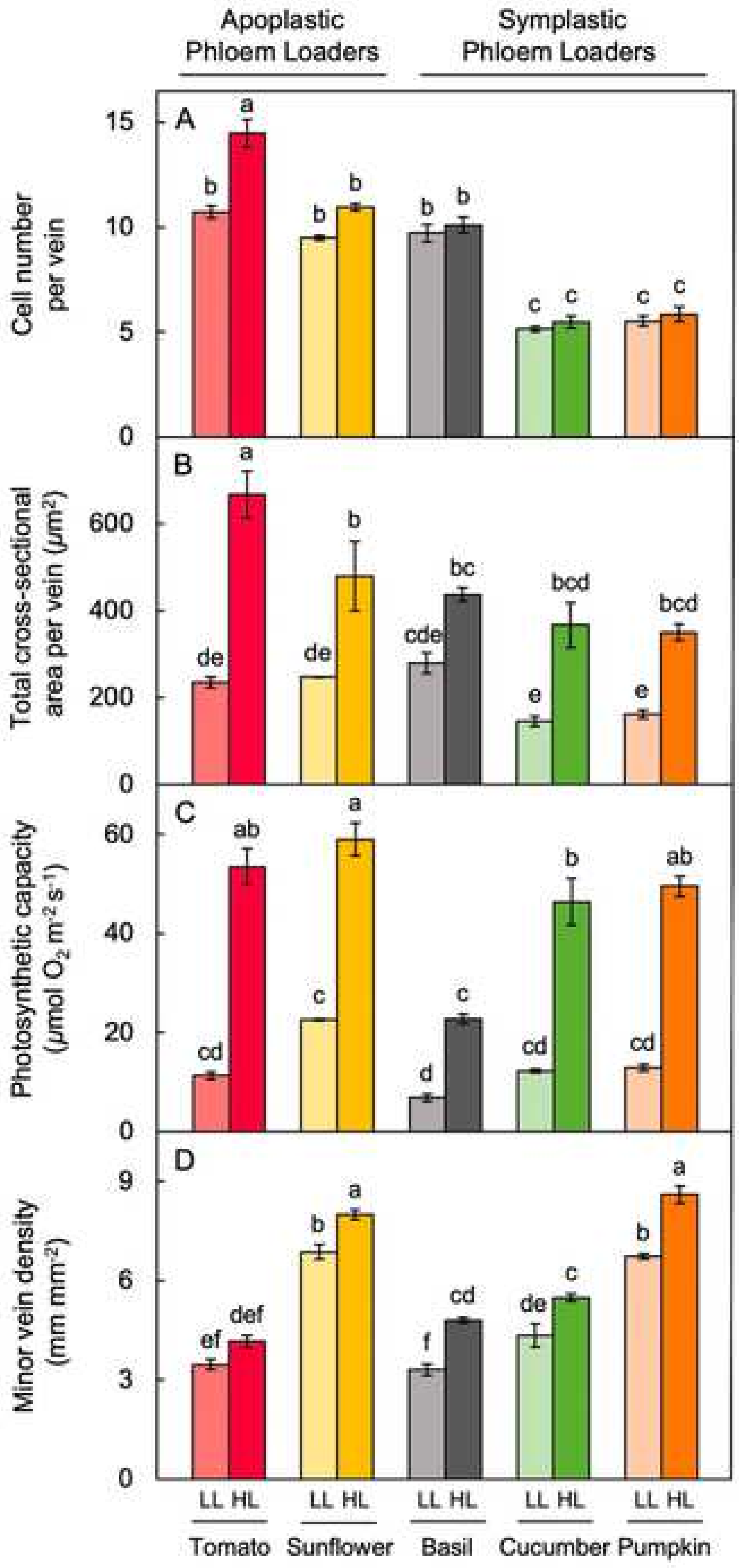
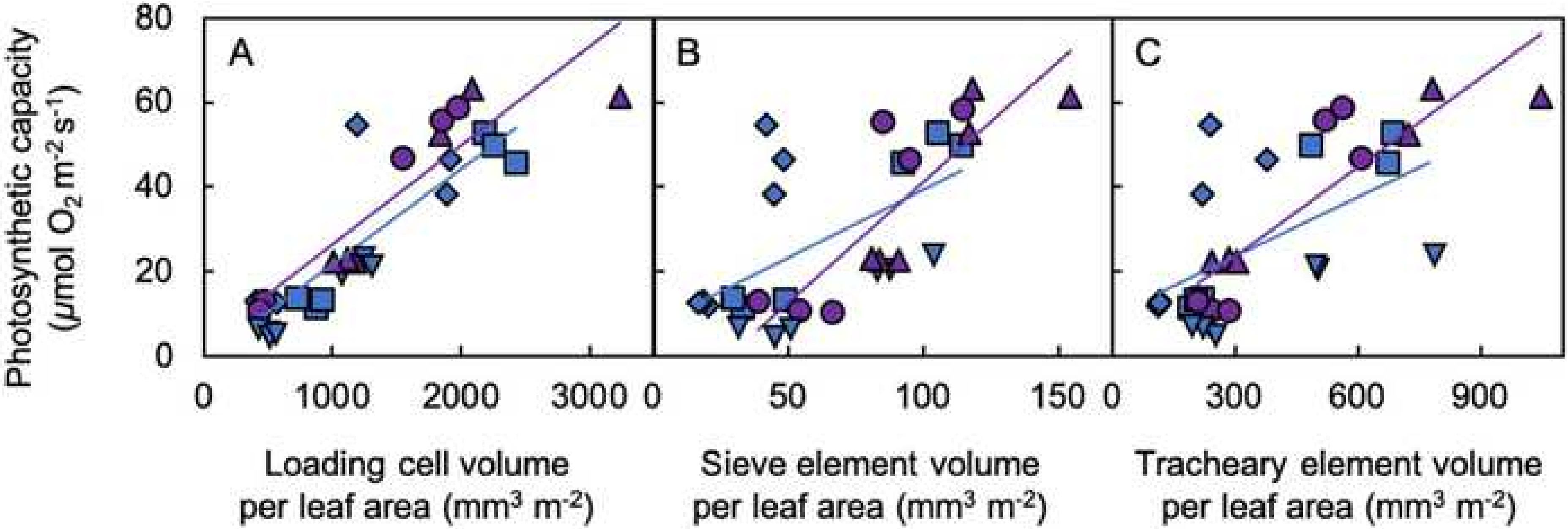

Apoplastic Phloem Loaders
Symplastic Phloem Loaders
A
Cell number per vein
0
5
10
15
b
a
b
b
b
b
c
c
c
c
B
Total cross-sectional area per vein ($\mu m^2$)
0
200
400
600
de
a
de
b
cde
bc
e
bcd
e
bcd
C
Photosynthetic capacity ($\mu$mol $O_2$ $m^{-2}$ $s^{-1}$)
0
20
40
60
cd
ab
c
a
d
c
cd
b
cd
ab
D
Minor vein density (mm $mm^{-2}$)
0
3
6
9
ef
def
b
a
f
cd
de
c
b
a
LL HL
LL HL
LL HL
LL HL
LL HL
Tomato
Sunflower
Basil
Cucumber
Pumpkin

Figure 2



Photosynthetic capacity ($\mu$mol $O_2$ $m^{-2}$ $s^{-1}$)
0
20
40
60
80
A
B
C
0
1000
2000
3000
0
50
100
150
0
300
600
900
Loading cell volume per leaf area ($mm^3$ $m^{-2}$)
Sieve element volume per leaf area ($mm^3$ $m^{-2}$)
Tracheary element volume per leaf area ($mm^3$ $m^{-2}$)

Figure 3

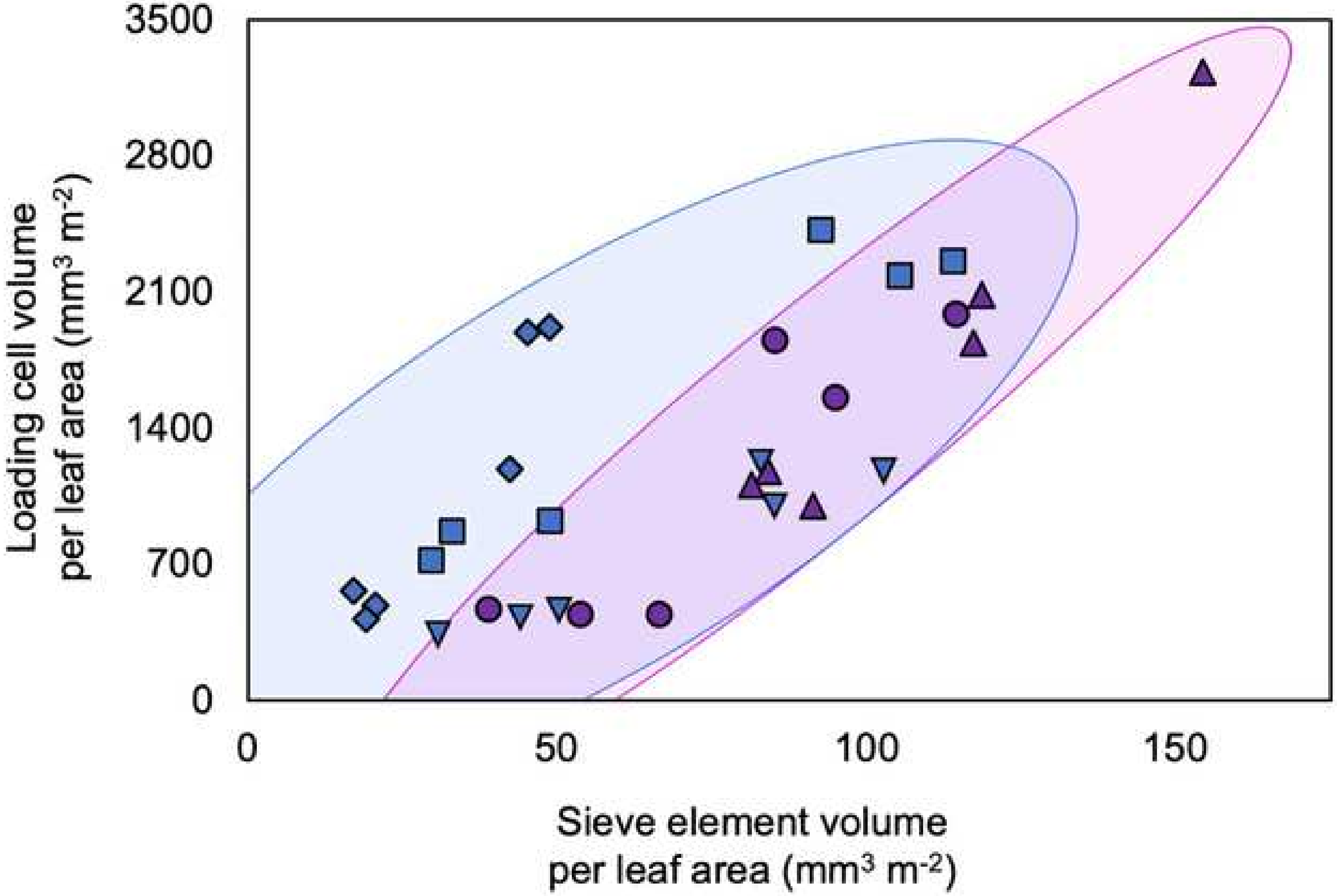
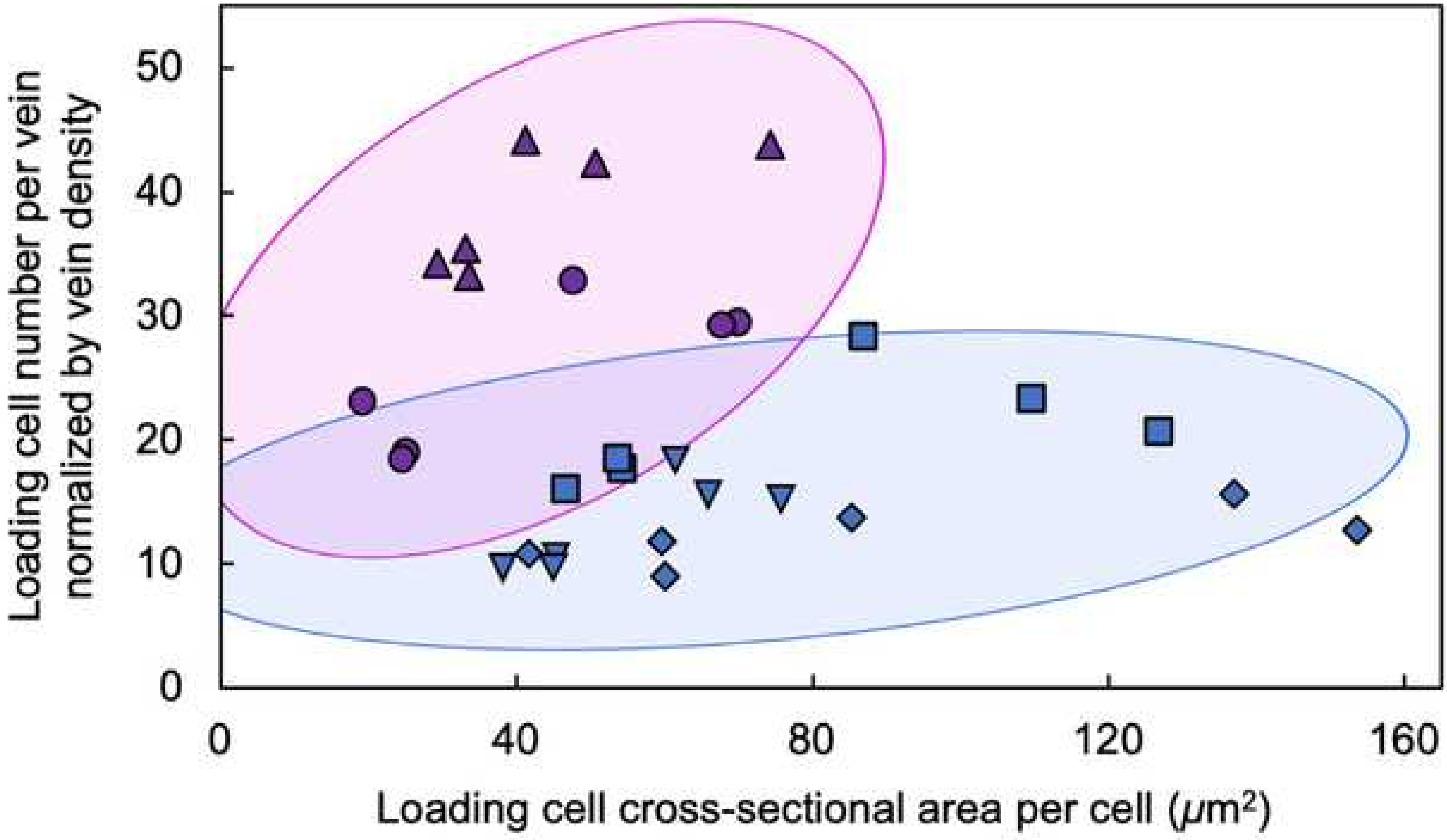
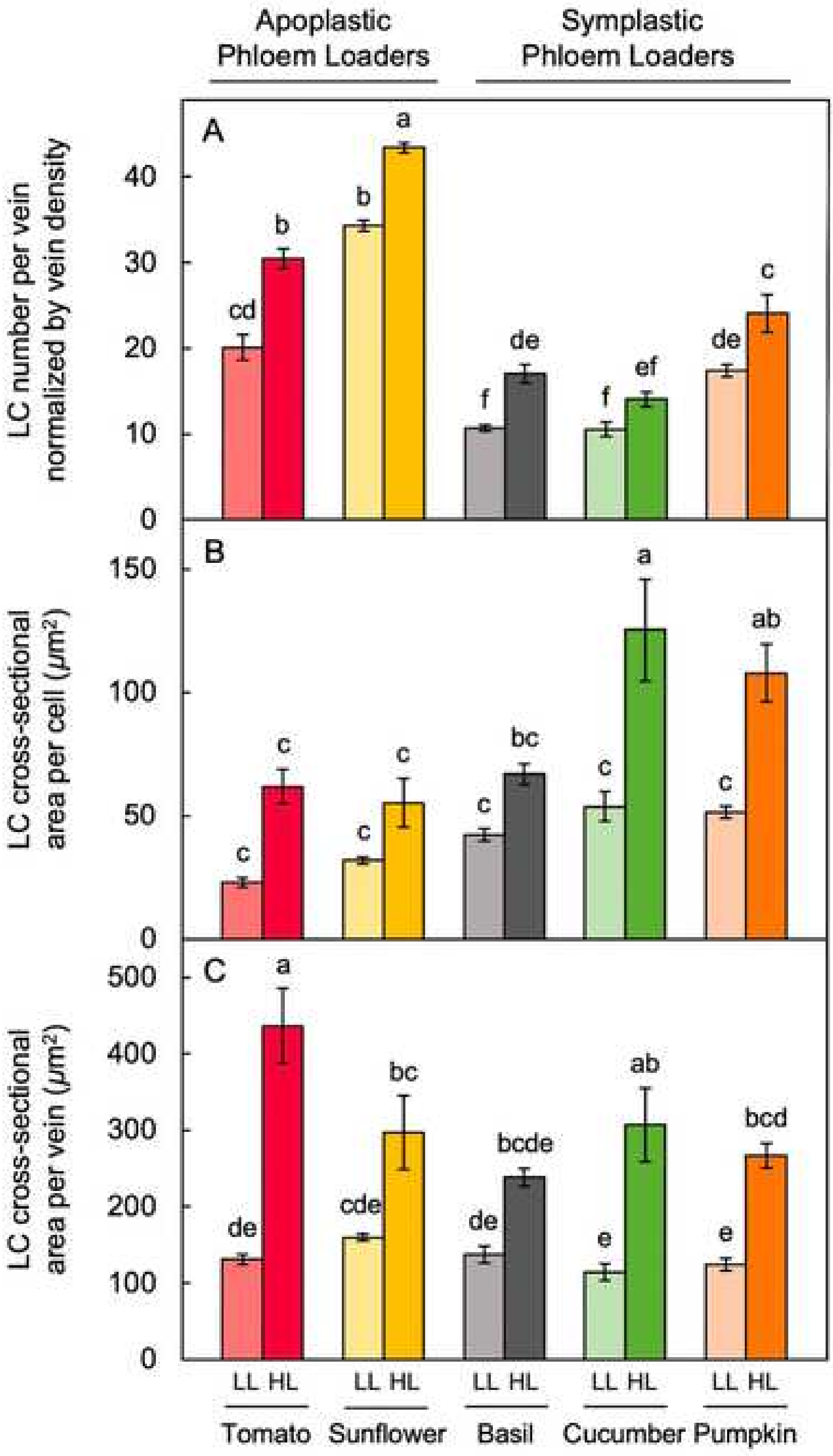

3500
2800
2100
1400
700
0
0
50
100
150
Loading cell volume
per leaf area (mm³ m⁻²)
Sieve element volume
per leaf area (mm³ m⁻²)

Figure 4



Loading cell number per vein normalized by vein density
0
10
20
30
40
50
0
40
80
120
160
Loading cell cross-sectional area per cell ($\mu m^2$)

Figure 5



Apoplastic Phloem Loaders
Symplastic Phloem Loaders
A
LC number per vein normalized by vein density
0
10
20
30
40
cd
b
b
a
f
de
f
ef
de
c
B
LC cross-sectional area per cell ($\mu m^2$)
0
50
100
150
c
c
c
c
c
bc
c
a
c
ab
C
LC cross-sectional area per vein ($\mu m^2$)
0
100
200
300
400
500
de
a
cde
bc
de
bcde
e
ab
e
bcd
LL HL
LL HL
LL HL
LL HL
LL HL
Tomato
Sunflower
Basil
Cucumber
Pumpkin

Figure 6

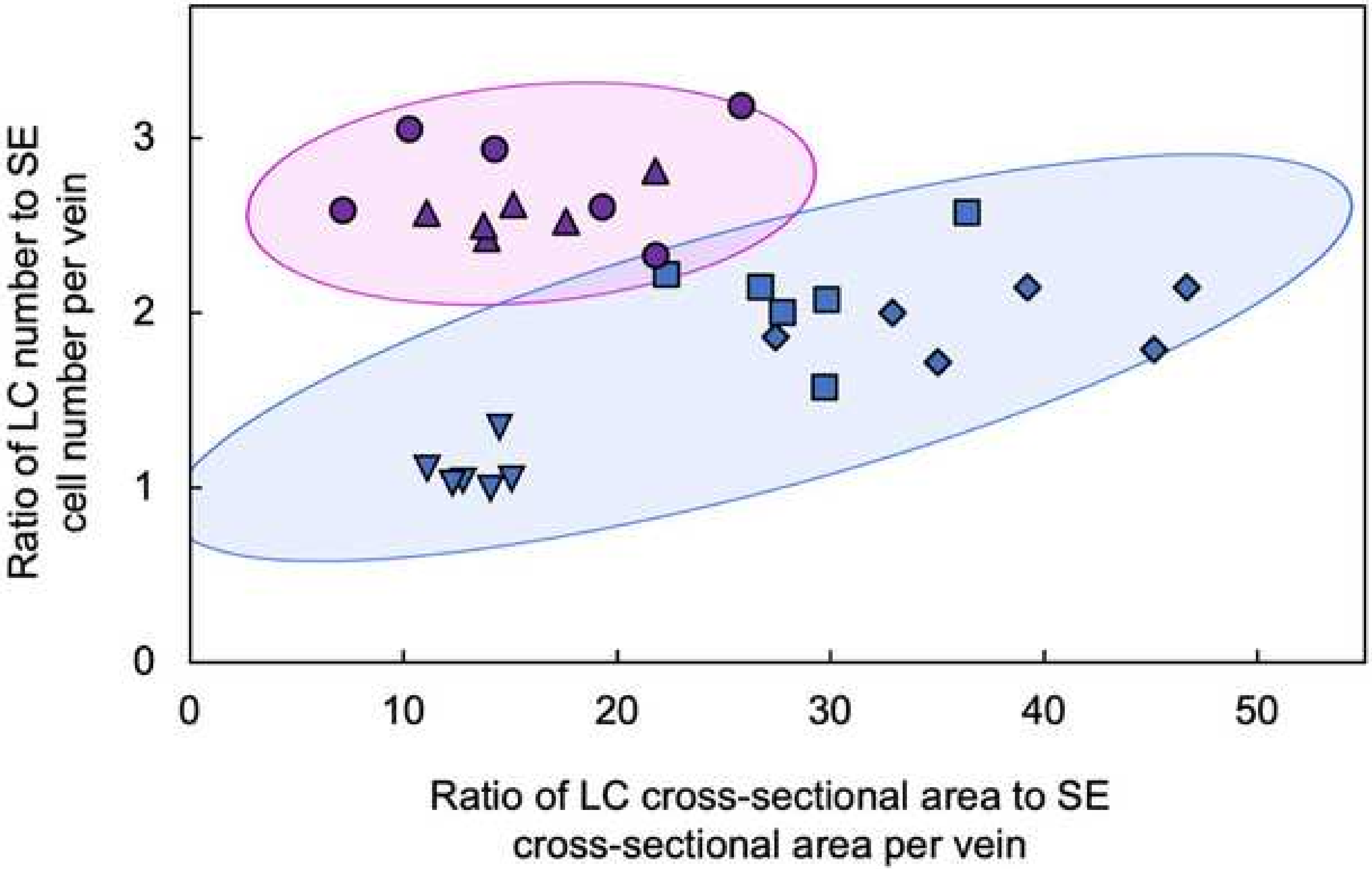
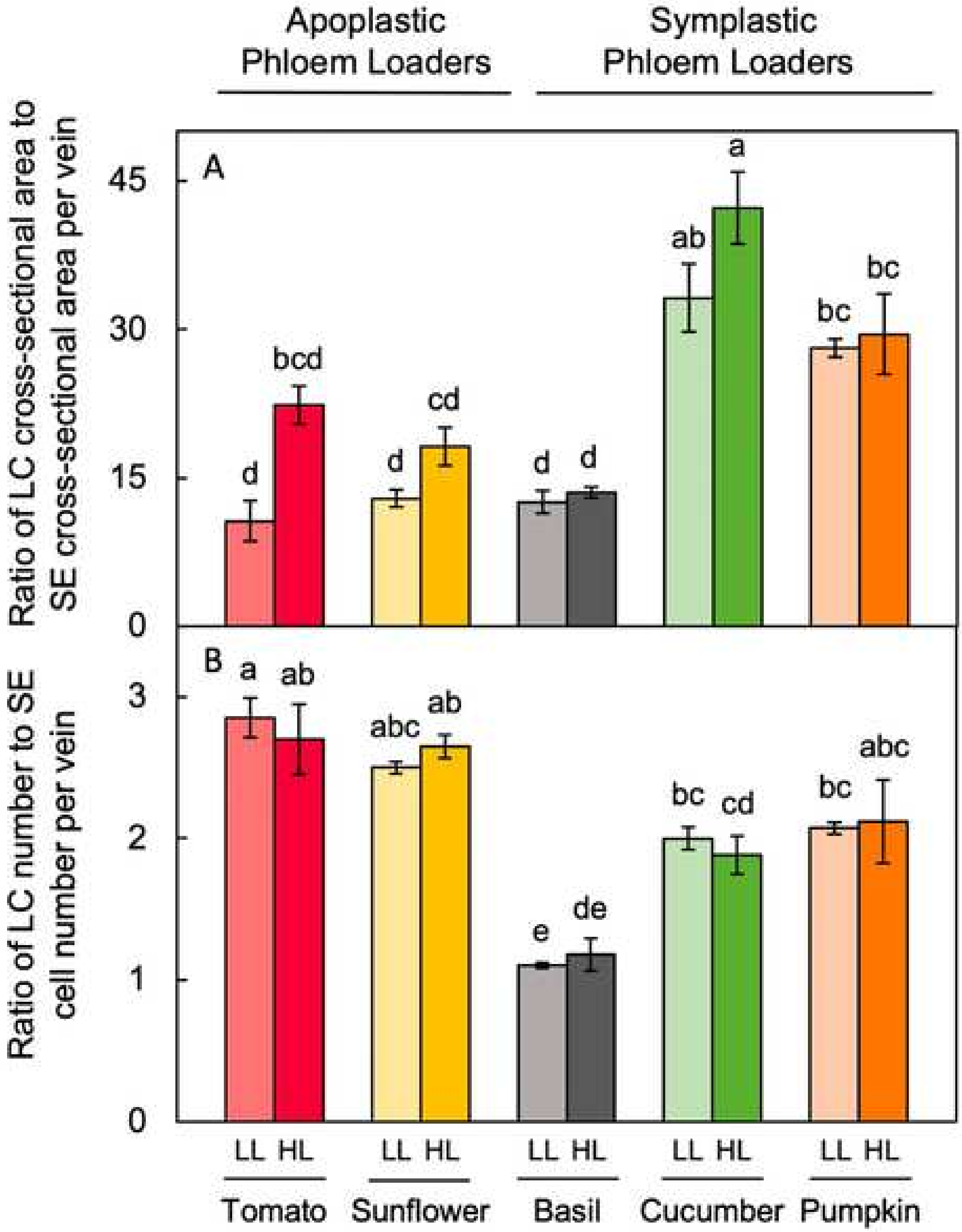

Ratio of LC number to SE cell number per vein
0
1
2
3
0
10
20
30
40
50
Ratio of LC cross-sectional area to SE cross-sectional area per vein

Figure 7



Apoplastic Phloem Loaders
Symplastic Phloem Loaders
Ratio of LC cross-sectional area to SE cross-sectional area per vein
A
45
30
15
0
d
bcd
d
cd
d
d
ab
a
bc
bc
Ratio of LC number to SE cell number per vein
B
3
2
1
0
a
ab
abc
ab
e
de
bc
cd
bc
abc
LL HL
LL HL
LL HL
LL HL
LL HL
Tomato
Sunflower
Basil
Cucumber
Pumpkin

Figure 8

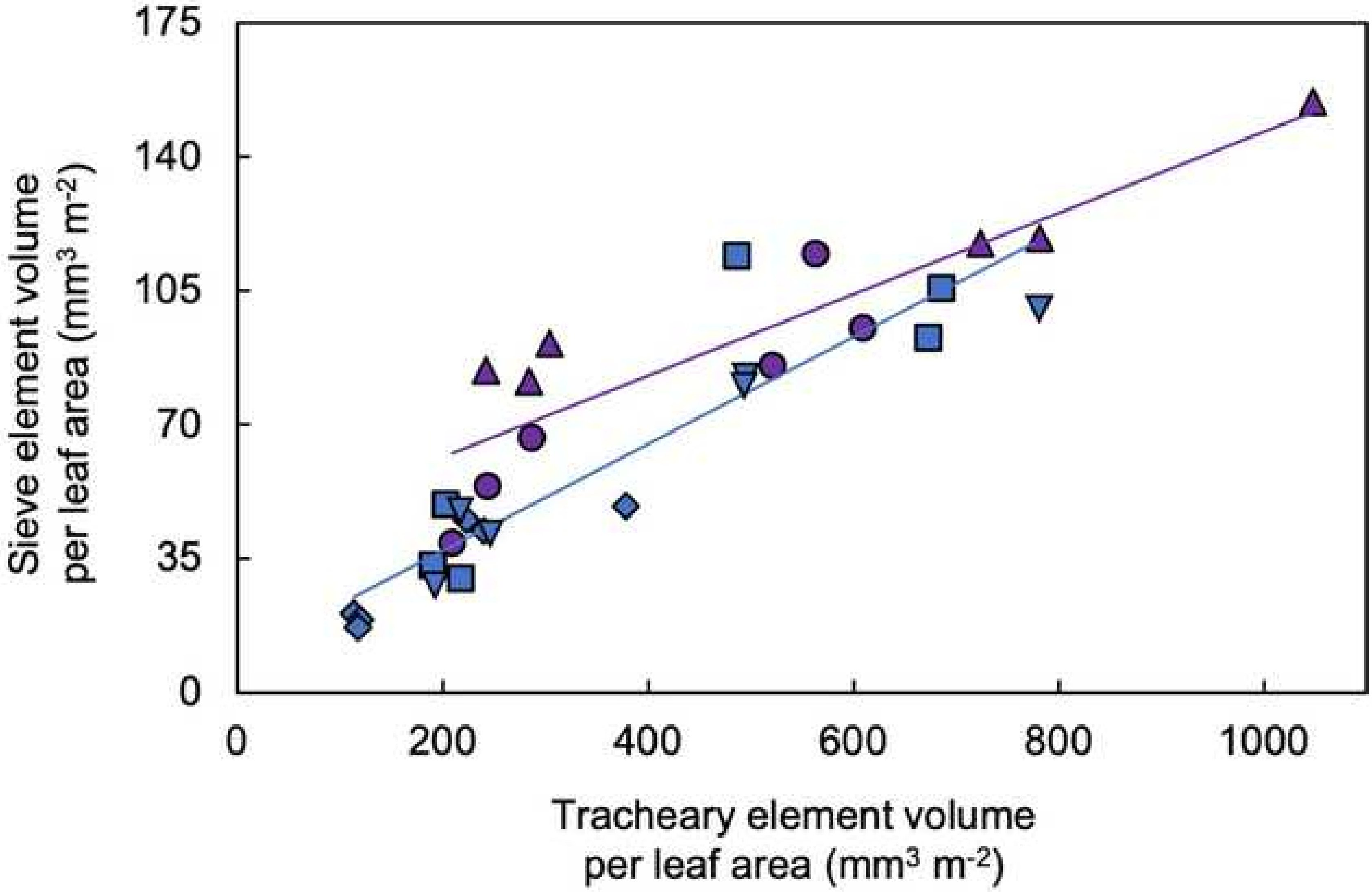
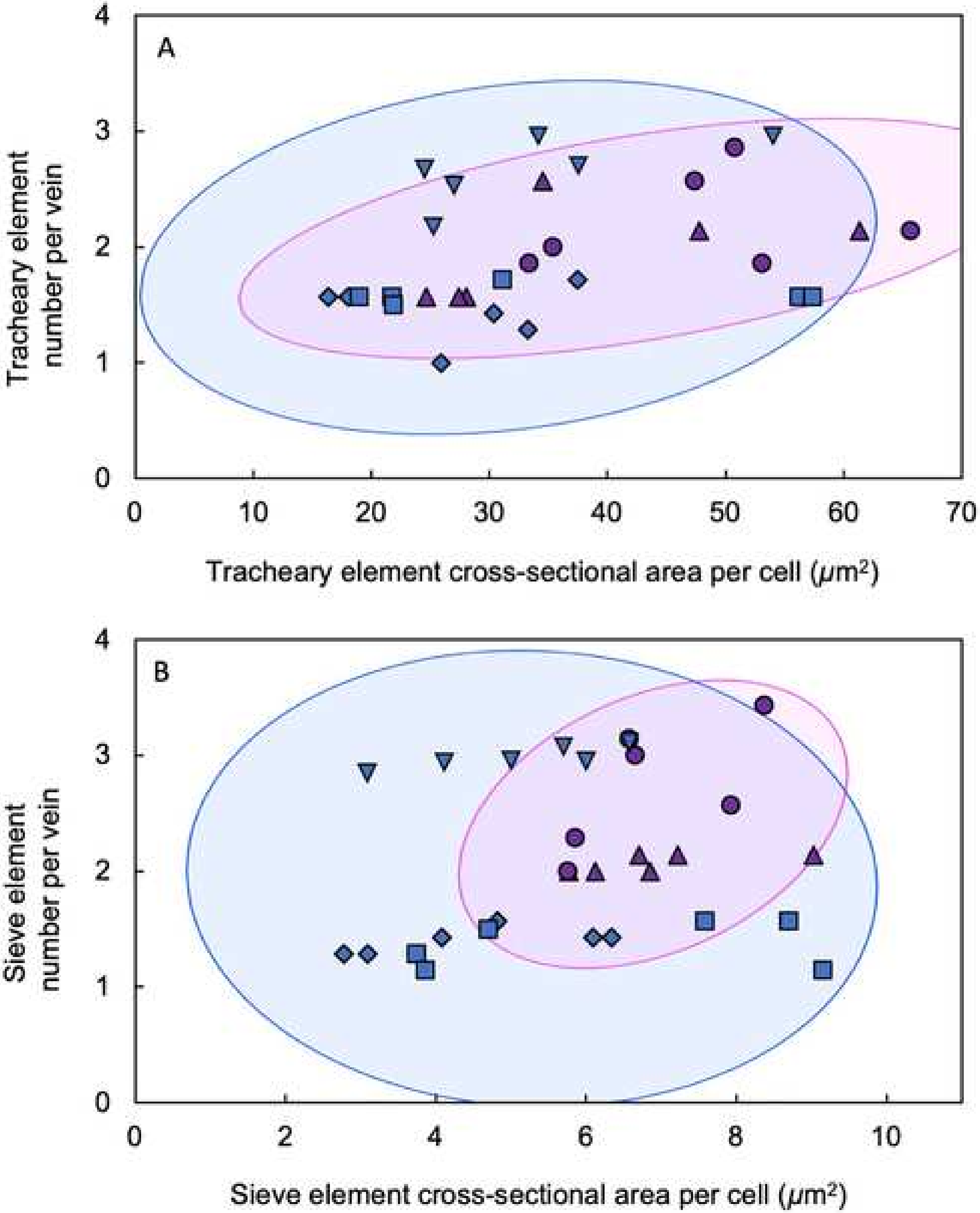
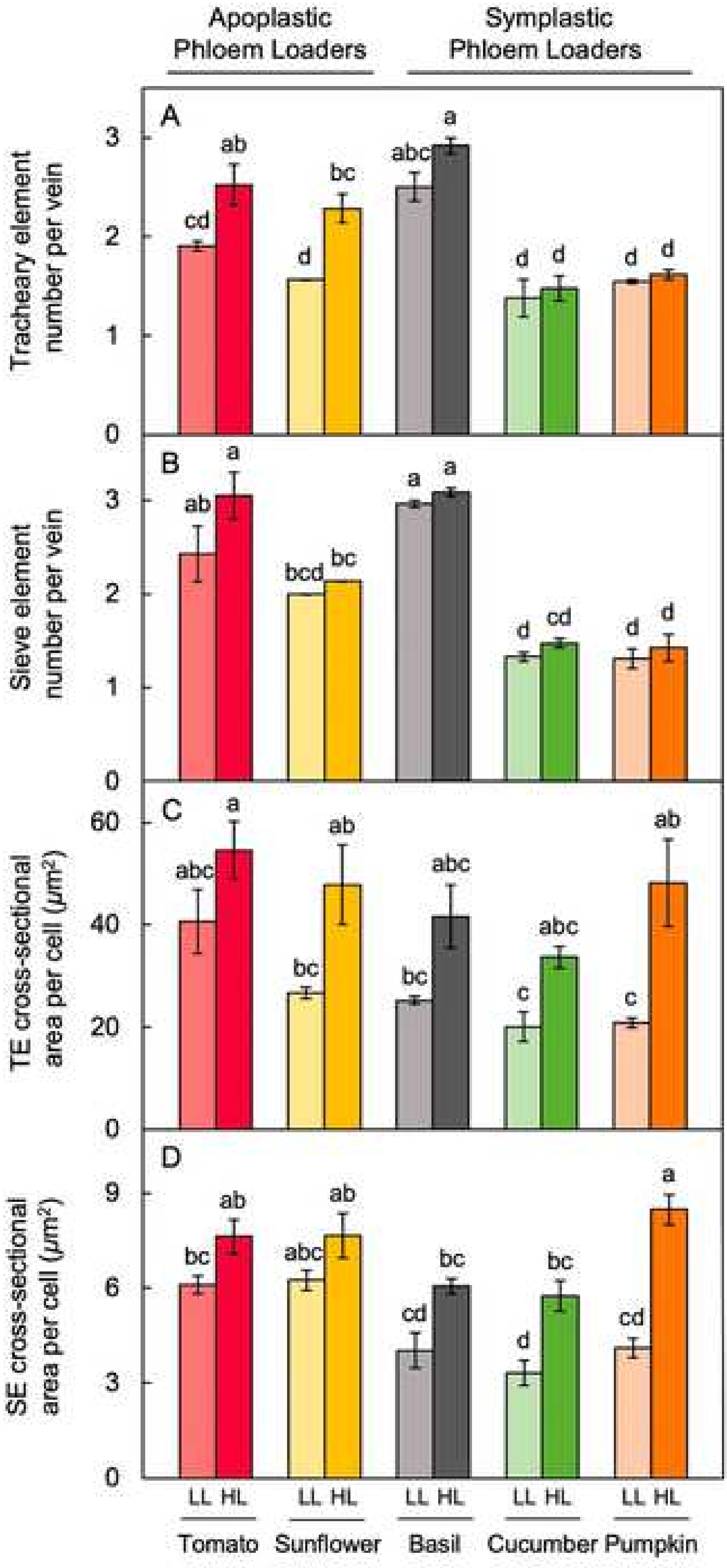

175
140
105
70
35
0
0
200
400
600
800
1000
Sieve element volume per leaf area (mm$^3$ m$^{-2}$)
Tracheary element volume per leaf area (mm$^3$ m$^{-2}$)

Figure 9

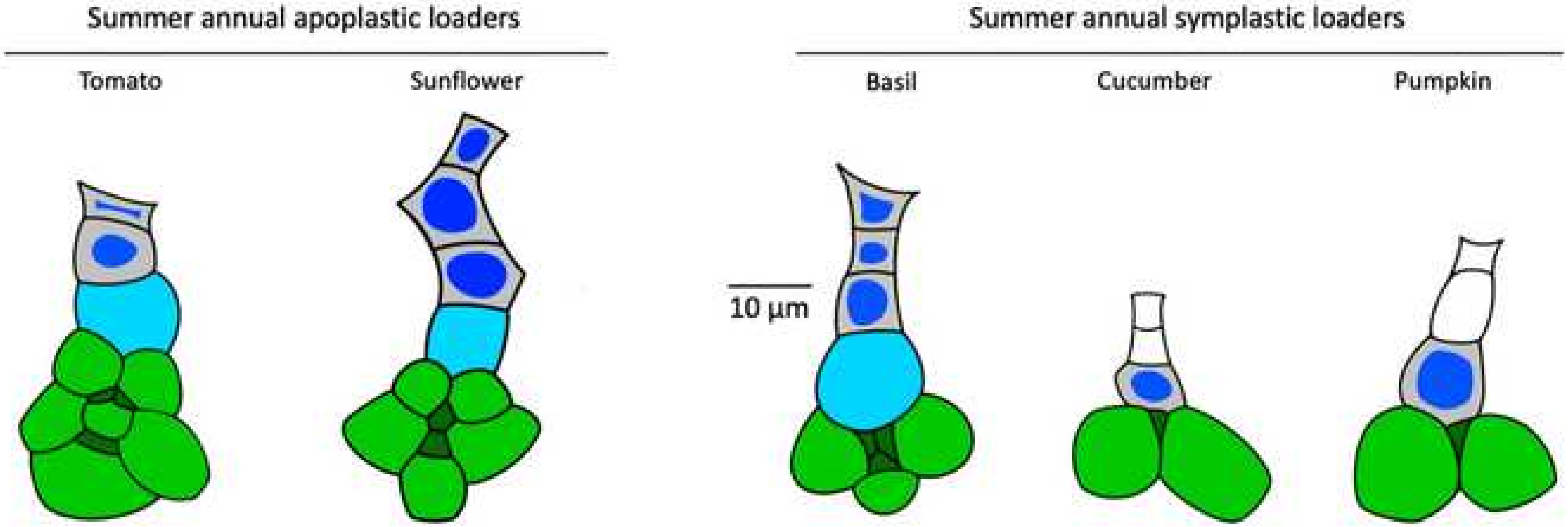


A
Tracheary element number per vein
0
1
2
3
4
0
10
20
30
40
50
60
70
Tracheary element cross-sectional area per cell ($\mu m^2$)
B
Sieve element number per vein
0
1
2
3
4
0
2
4
6
8
10
Sieve element cross-sectional area per cell ($\mu m^2$)

Figure 10


Apoplastic Phloem Loaders
Symplastic Phloem Loaders
A
Tracheary element number per vein
0
1
2
3
cd
ab
d
bc
abc
a
d
d
d
d
B
Sieve element number per vein
0
1
2
3
ab
a
bcd
bc
a
a
d
cd
d
d
C
TE cross-sectional area per cell ($\mu m^2$)
0
20
40
60
abc
a
bc
ab
bc
abc
c
abc
c
ab
D
SE cross-sectional area per cell ($\mu m^2$)
0
3
6
9
bc
ab
abc
ab
cd
bc
d
bc
cd
a
LL HL
LL HL
LL HL
LL HL
LL HL
Tomato
Sunflower
Basil
Cucumber
Pumpkin

Supplemental Figure 1



## Highlights

- Summer annuals exhibited variation in multiple features of minor foliar veins
- Photosynthetic capacity and leaf-level volume of all vascular cell types were correlated
- Relationship of loading-cell size and number varied by phloem-loading mechanism
- The proportion of vascular cell number to size was greater in apoplastic loaders

# Conclusion

Photosynthetic capacity per leaf area was significantly positively correlated with leaf-level volume of phloem-loading cells, sugar-export conduits (sieve elements), and water conduits (tracheary elements) irrespective of phloem-loading mechanism. The relative contribution to leaf-level volume of loading cell numbers versus individual loading cell size was greater in apoplastic loaders than in symplastic loaders. Species-dependent differences included different vein density within each loading group and either greater or lower numbers of cells per minor vein (especially of tracheary elements in the symplastic loaders basil versus cucumber, respectively), which may be due to genetic adaptation to different environmental conditions. These results indicate considerable plasticity in foliar vascular features in summer annuals as well as some loading-mechanism-dependent trends. The results presented here support the need for further research into constitutive differences in foliar vascular architecture of summer annuals, winter annuals, and biennials with different loading mechanisms as well as their acclimation of the foliar vasculature at different scales (subcellular, cellular, vein, and leaf) to various environments. Such insight may aid in development of crops for improved leaf vascular features and superior photosynthetic performance in specific climates.