Tocopherols modulate leaf vein arrangement and composition without impacting photosynthesis

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Abstract

Growth of the tocopherol-deficient *vte1* mutant and Col-0 wild type of *Arabidopsis thaliana* in a sunlit glasshouse revealed both similarities and differences between genotypes. Photosynthetic capacity and leaf mesophyll features did not differ between mutant and wild type. Likewise, the total volume of water conduits (tracheary elements, TEs), sugar conduits (sieve elements, SEs), and sugar-loading cells (companion and phloem parenchyma cells) on a leaf area basis were unaffected by tocopherol deficiency. However, tocopherol deficiency yielded smaller and more numerous minor veins with fewer phloem cells and smaller TEs, resulting in greater ratios of TEs to SEs. The smaller TEs in the *vte1* mutant may present a decreased risk for cavitation under high evaporative demand or in response to freezing. In turn, compensation for fewer phloem cells and smaller TEs by more numerous veins may bolster resistance to cavitation at no cost to photosynthetic capacity.

Additional key words: foliar vasculature; leaf venation; vein density; vitamin E deficiency; xylem.

Introduction

Photosynthesis provides energy and building materials for plant growth and development in natural communities and agricultural settings. Sugars produced in photosynthesis serve as energy carriers and building blocks, and also as inputs for sugar-based signaling networks that maintain a balance between sugar production in the plant's source leaves and the demand for sugars from the plant's sink tissues (Paul and Driscoll 1997, Paul and Foyer 2001, Körner 2013, Fatichi *et al.* 2014).

The leaf vascular network collects sugars produced in photosynthesis for export to the rest of the plant, imports water and nutrients, and also exchanges various signals with other parts of the plant (Sack and Scoffoni 2013). Close correlations exist between photosynthetic capacity and various phloem features involved in sugar loading and sugar export from leaves (Adams *et al.* 2013, 2014, 2016; Cohu *et al.* 2013a, 2014; Muller *et al.* 2014a,b; Stewart *et al.* 2016, 2017). In addition, sufficient water import into leaves is a prerequisite not only for stomatal opening (Sack and Holbrook 2006, Sack *et al.* 2015) but also to facilitate

sugar movement out of the leaf (Hölttä *et al.* 2006, Hölttä and Nikinmaa 2013, Nikinmaa *et al.* 2013).

Many components of plant growth and development are regulated by the cellular balance of oxidants and antioxidants (Foyer and Noctor 2009, Considine and Foyer 2014). This balance, the cellular redox state, has been shown to modulate growth regulators like the phytohormone class of auxins (Foyer and Noctor 2013) that serve in vascular differentiation (Mattsson *et al.* 2003, Scarpella and Meijer 2004). Several *Arabidopsis thaliana* mutants with shifts in cellular redox balance exhibited alteration in the ultrastructure of sugar-loading phloem cells (Maeda *et al.* 2006, 2014, Demmig-Adams *et al.* 2013). *Arabidopsis thaliana* lines deficient in tocopherols, in particular, developed more pronounced wall ingrowths in phloem cells than wild type after transfer to cold temperatures (Maeda *et al.* 2006, 2014). The present study utilized the same tocopherol-deficient *vte1* mutant as a previous study (Stewart *et al.* 2017) to further address the impact of tocopherol status on the foliar vasculature.

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Abbreviations: CC – companion cell; Chl – chlorophyll; DM – dry mass; PC – phloem parenchyma cell; SE – sieve element; TE – tracheary element; VD – vein density.

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The *vte1* mutant is deficient in tocopherols, but, unlike the *vte2* mutant, contains redox-active tocopherol precursors (Maeda *et al.* 2006, Sattler *et al.* 2006). Both mutants have been used to illustrate functions of tocopherols in photo-protection, synergistic interaction with other antioxidants, and signaling events presumably involving modulation of the production of oxidation-based messengers (e.g., Maeda *et al.* 2006, Ksas *et al.* 2015, Stewart *et al.* 2017; see also below).

While current efforts to limit crop losses under extreme environmental conditions (e.g., drought and unfavorable temperatures) include overexpression of antioxidant systems (for a review of efforts, see Logan *et al.* 2006), it has been cautioned that plant response to the environment relies on oxidative signaling (Foyer and Noctor 2009; see also Demmig-Adams *et al.* 2013, 2014a,b, Kurepin *et al.* 2013). For example, Allu *et al.* (2017) recently reported differences in hormonal and transcriptional responses to soil phosphate availability between the *vte1* mutant and wild-type *A. thaliana*. Additional information is, therefore, needed to understand how altered leaf antioxidant contents affect plant development and to identify possible trade-offs in adjustments to contrasting environments. Previous studies had shown that growth under natural conditions led to enhanced foliar α -tocopherol contents compared to growth under controlled laboratory conditions (see, e.g., Semchuk *et al.* 2009; for a recent review, see Havaux and García-Plazaola 2014), which is similar to trends seen for other antioxidant systems, such as zeaxanthin and photo-protective thermal energy dissipation (Demmig-Adams *et al.* 2012, 2014c, Mishra *et al.* 2012; see also Külheim *et al.* 2002). In the present study, we characterized foliar features of the *vte1* mutant vs. wild type in plants grown in a sunlit glasshouse with intermittently high irradiance levels and temperatures at midday, and compared genotypic differences identified here with those seen in controlled growth-chamber conditions under which plants were exposed to high light intensities or high temperatures during the photoperiod (Stewart *et al.* 2017).

We previously used two ecotypes of *A. thaliana* from habitats in Sweden and Italy with contrasting temperature and day-length profiles (Ågren and Schemske 2012, Adams *et al.* 2016) to study acclimation of photosynthesis and the leaf's vasculature to growth conditions varying in light and/or temperature (Adams *et al.* 2014, 2016, Cohu

et al. 2013a,b, Stewart *et al.* 2016). The Swedish and Italian ecotypes differed in their acclimation of both photosynthetic capacity and vascular features associated with the leaf's capacities for sugar and water transport (Adams *et al.* 2014, 2016, Cohu *et al.* 2013a, Stewart *et al.* 2016). While the Swedish ecotype exhibited superior acclimation to cold temperatures and high light intensities, the Italian ecotype performed better under hot temperatures and lower light intensities (Adams *et al.* 2014, 2016, Cohu *et al.* 2013a,b, Stewart *et al.* 2015, 2016). In addition, the Italian ecotype accumulated less tocopherol (vitamin E) in its leaves when grown under hot temperatures (Stewart *et al.* 2016). To assess possible connections between tocopherol status and the foliar vasculature, we compared the Col-0 wild type of *A. thaliana* with the tocopherol-deficient *vte1* mutant in the Col-0 background under four different conditions in environmentally controlled growth chambers. These conditions included very low and very high light intensities under an intermediate temperature regime and cool and hot temperature regimes under an intermediate light intensity. Relative to Col-0, the *vte1* mutant exhibited a different ratio of water conduits to sugar conduits only in plants grown under the hot temperature of 35°C (i.e., no differences between Col-0 and *vte1* under the other environmental conditions used). Furthermore, under this very hot growth temperature, *vte1* and Col-0 both showed unusually high vein densities (for this winter annual), but exhibited no significant differences in vein density between the genotypes. The present study addresses three follow-up research questions:

(1) What, if any, differences in foliar vascular anatomy exist between *vte1* and Col-0 when both are grown under more natural conditions in a sunlit glasshouse with light and temperature variation over the course of the day?

(2) Are any differences in foliar vascular features associated with differences in photosynthetic capacities and leaf morphological features?

(3) Is vein density in leaves grown in a naturally-lit greenhouse lower in both genotypes than growth under either high light or high growth temperature, and may this less extreme condition reveal a significant difference in foliar vein density between *vte1* and Col-0 that may have been obscured by the extreme conditions used in the previous study?

Materials and methods

Plant material: Two lines of *Arabidopsis thaliana* (L.) Heynhold were compared, wild type Columbia (Col-0) and the *vte1-1* mutant from the same genetic background (Sattler *et al.* 2003), but with a disruption in the pathway of tocopherol synthesis. The *vte1* mutant is missing tocopherol cyclase and is therefore deficient in all (α -, β -, γ -, and δ -) tocopherols (for additional details on *vte1-1*, see Mène-Saffrané and DellaPenna 2010). *Arabidopsis thaliana* seeds were vernalized at 4°C for 4 d and

germinated in six-plug trays (50-mL volume). Emerging seedlings were transplanted individually into large (2.9-L) pots containing *Canadian Growing Mix 2* (Conrad Fafard Inc., Agawam, MA, USA). Seeds were germinated in a partially shaded portion of the glasshouse with a peak PPFD of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ before transfer to their final growth location with a peak PPFD of 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Col-0 and *vte1* were grown in a location of the sunlit glasshouse with mean daytime and nighttime air

temperatures of 22°C and 17°C, respectively, as described in Stewart *et al.* (2015). In the sunlit glasshouse, air temperatures during exposure to peak PPFDs reaching or exceeding 26°C were recorded on six separate days, two of which reached or exceeded 27°C. Several fully expanded leaves, which developed under the growth conditions of a naturally lit glasshouse, were used for the parameters assessed. For a given parameter, one leaf each from five plants were used (resulting in five biological replicates per genotype).

Photosynthesis, transpiration, leaf morphology, and minor vein anatomy: Measurements of photosynthetic capacity (light- and CO₂-saturated rate of oxygen evolution at 25°C), transpiration rate, leaf dry mass per unit of leaf area, leaf and palisade mesophyll thickness, number of palisade mesophyll cell layers, minor vein density [mm(minor vein length) mm⁻²(leaf area)], minor vein cell numbers and cross-sectional areas, and sample preparation for microscopy were conducted as described previously (Amiard *et al.* 2005, Cohu *et al.* 2013b, Dumlao *et al.* 2012, Muller *et al.* 2014b, Stewart *et al.* 2015). Further details of leaf clearing for vein-density determinations are described in Stewart *et al.* (2017). Minor-vein density and minor-vein architecture remain constant in

A. thaliana as leaves expand (see Kang and Dengler 2004, Sack *et al.* 2012, Stewart *et al.* 2017). Photosynthetic oxygen evolution was measured in a temperature-controlled leaf-disc oxygen electrode chamber (LD2/2, Hansatech Instruments, King's Lynn, Norfolk, UK) in water-saturated air with 5% CO₂ (Delieu and Walker 1981). Transpiration measurements were conducted as described in Stewart *et al.* (2015) at a PPFD of 1,500 µmol m⁻² s⁻¹, a leaf temperature of 27.6 ± 0.7°C, a vapor-pressure difference of 3.31 ± 0.18 kPa, and a CO₂ concentration of 428 ± 7 ppm.

Leaf chlorophyll (Chl) and tocopherol content: Chl content was determined, and the absence of tocopherols in *vtel* confirmed, *via* high-performance liquid chromatography as described in Stewart *et al.* (2015) on leaf discs of 0.30 cm² collected before sunrise from one leaf from each of the five different plants for each genotype.

Statistical analyses: All statistical analyses were conducted using JMP software (Pro 13.0.0, SAS Institute Inc., Cary, NC, USA). Student's *t*-test was used for comparison of two means, and linear regression analysis was used for comparison of two variables.

Results

Minor vein density and size: Fig. 1 shows that the tocopherol-deficient *vtel* mutant exhibited more numerous and smaller foliar minor veins than the Col-0 wild type when both genotypes were grown in a naturally lit glasshouse. Foliar minor vein density, expressed as vein length per leaf area, was significantly greater (Fig. 1A) while the cross-sectional area per minor vein was significantly smaller (Fig. 1B) in *vtel* compared to Col-0. The product of vein density and cross-sectional area per vein (Fig. 1C), representing the total vein volume on a leaf area basis, did not differ significantly between *vtel* and Col-0. When total cross-sectional areas per vein as well as vein volumes per leaf area were broken down into the veins' water-moving xylem component and sugar-moving phloem component, a similar picture as for the whole vein emerged for the individual xylem and phloem portions of veins (Fig. 2). The combined cross-sectional area of all xylem cells per vein (Fig. 2A) and of all phloem cells per vein (Fig. 2B) was significantly smaller in the *vtel* mutant compared to Col-0. After normalizing for expression on a leaf area basis (by calculating the product of foliar vein density and the cross-sectional area of each vascular cell type per minor vein) to enable direct comparison with photosynthetic rates on a leaf area basis (see below), however, the total volumes for both xylem (Fig. 2C) and phloem (Fig. 2D) per leaf area were not significantly different between *vtel* and Col-0.

Photosynthesis and leaf morphology: A variety of leaf

metrics (Table 1) and the capacity for photosynthesis (assessed as the light- and CO₂-saturated rate of photosynthetic oxygen evolution) expressed on several bases (Fig. 3) also exhibited no significant differences between the *vtel* and Col-0 lines (Table 1). Chl content per leaf area, leaf mass per area, and leaf thickness were similar in both lines (Table 1), and photosynthetic capacities expressed on each of these bases [*i.e.*, per leaf area (Fig. 3A), per Chl (Fig. 3B), and per leaf dry mass (Fig. 3C)] were likewise indistinguishable between the two. There were, furthermore, no significant differences in thickness of the palisade or spongy mesophyll tissue, the ratio of palisade to spongy mesophyll tissue, the number of palisade cell layers, or numbers of palisade or spongy mesophyll cells per leaf cross-sectional area (Table 1). Lastly, overall appearance and plant size was similar for *vtel* mutant and Col-0 wild type plants (not shown).

Smaller water conduits and fewer sugar conduits and loading cells in *vtel* minor veins: Phloem and xylem each consist of several functional cell types. These different cell types were individually quantified for a better estimate of the flux capacities of conduits for water and sugar movement in the leaf. The cross-sectional area per minor vein for a given vascular cell type is determined by both numbers and individual area of these vascular cells. While individual cell cross-sectional area of each water-conducting TE was significantly smaller in *vtel* than in Col-0 (Fig. 4A), individual cross-sectional area of sugar-

Fig. 1. (A) Foliar minor vein density [mm(vein length) mm⁻²(leaf area)], (B) cross-sectional area of all vascular cells per minor vein, and (C) vein volume per leaf area (as the product of the cross-sectional area of all vascular cells per minor vein and minor vein density) in leaves of *Arabidopsis thaliana* Col-0 wild type (light gray columns) and *vte1* mutant (dark gray columns). Mean values $\pm$ standard deviation for (A) and $\pm$ standard error for (B,C); statistically significant differences between genotypes are indicated with asterisks (* – $P < 0.05$; *** – $P < 0.001$; n.s. – not significantly different).

exporting SEs (Fig. 4B) or sugar-loading CCs + PCs (Fig. 4C) did not differ between the genotypes. Conversely, while TE numbers per minor vein were similar in

vte1 and Col-0 (Fig. 4D), the number of SEs (Fig. 4E) and CCs + PCs (Fig. 4F) per minor vein were both significantly lower in the *vte1* mutant. The smaller total cross-sectional area of *vte1* minor veins compared to Col-0 minor veins (Fig. 1B) thus resulted from a combination of fewer similarly sized phloem cells and a similar number of narrower tracheary elements. This differential trend in cell numbers between water and sugar conduits resulted in a significantly greater ratio of the number of water conduits (TEs) to sugar conduits (SEs) in *vte1* vs. Col-0 (Fig. 5A). In contrast, the ratio of major loading cells (CCs + PCs) to sugar-exporting SEs in minor veins was the same in the two genotypes (Fig. 5B).

Proxies for total foliar water and sugar transport capacities per leaf area were unaffected by tocopherol status: Normalization for the differences in vein density between the two *A. thaliana* lines revealed that the product of cell number per vein and vein density was greater in *vte1* than in Col-0 for TEs (Fig. 6A), but not for SEs (Fig. 6B) or CCs + PCs (Fig. 6C). However, the most relevant parameters for estimating flux capacity for water distribution throughout the leaf, sugar loading, and sugar export are the volumes of TEs, CCs + PCs, and SEs, respectively, per leaf area (calculated as the product of vein density and cross-sectional areas per minor vein for each type of vascular cell). There was no significant difference between *vte1* and Col-0 in these respective total volumes per leaf area of water-conducting tracheary elements of the xylem (Fig. 6D), sugar-exporting sieve elements of the phloem (Fig. 6E), or major sugar-loading cells (CCs + PCs) of the phloem (Fig. 6F). From the finding that the total volume of water conduits (TEs) per leaf area is not significantly different between *vte1* and Col-0, one would predict that transpiration rate per leaf area should also not be significantly different. Transpiration measurements indeed support this notion. Transpiration rates per leaf area were 7.5 ± 0.5 and 8.8 ± 1.7 mmol(H₂O) m⁻² s⁻¹ ($P = 0.260$) in Col-0 and *vte1*, respectively, i.e., slightly, albeit not significantly, higher in *vte1*. This result mirrors the result shown in Fig. 6D for total TE volume per leaf area of 196 ± 11 and 225 ± 28 mm³ m⁻² ($P = 0.185$) for Col-0 and *vte1*, respectively, i.e., slightly, albeit not significantly, higher for *vte1* (see also Fig. 7).

Discussion

Importance of growth conditions: We here describe a phenotype for the tocopherol-deficient *vte1* mutant of *Arabidopsis thaliana* featuring more numerous, smaller veins with a greater emphasis on water-transporting tracheary elements vs. to sugar-transporting sieve elements compared to the wild type. These features of the *vte1* mutant in comparison to wild type plants grown under exposure to natural sunlight are reminiscent of the features of hot- compared to cool-grown wild type Col-0 plants (Stewart *et al.* 2017). Wild-type plants that had developed

in a growth chamber with high leaf temperatures of 36°C during the photoperiod exhibited a higher vein density, more numerous and smaller TEs, and a higher ratio of TEs to SEs than cool-grown plants (see also Adams *et al.* 2016). Tocopherol deficiency thus produces a vascular phenotype under moderate temperature and natural light fluctuations that is similar to that of wild-type plants acclimated to hot temperature conditions (Adams *et al.* 2016, Stewart *et al.* 2016) with a greater evaporative demand than that at cool conditions.

Fig. 2. Cross-sectional area of (A) all xylem cells per minor vein and (B) all phloem cells per minor vein as well as vein volumes on a leaf area basis from the product of minor vein density, and (C) cross-sectional area of all xylem cells per vein or (D) all phloem cells per vein in leaves of *Arabidopsis thaliana* Col-0 wild type (light gray columns) and *vte1* mutant (dark gray columns). Mean values $\pm$ standard error; statistically significant differences between genotypes are indicated with asterisks (* – $P < 0.05$; n.s. – not significantly different).

Table 1. Chlorophyll content per leaf area and leaf morphological features of wild-type Col-0 and the *vte1* mutant of *Arabidopsis thaliana* grown in a sunlit glasshouse. Mean values $\pm$ standard error (or $\pm$ standard deviation for chlorophyll and leaf mass per area). There were no significant differences between Col-0 and *vte1*. DM – dry mass.

Parameter	Col-0	<i>vte1</i>
Chlorophyll <i>a</i> + <i>b</i> [$\mu\text{mol m}^{-2}$]	848 $\pm$ 71	873 $\pm$ 47
Leaf mass per area [g(DM) m^{-2}]	18.2 $\pm$ 2.0	18.0 $\pm$ 1.6
Leaf thickness [μm]	237 $\pm$ 9	240 $\pm$ 2
Palisade mesophyll thickness [μm]	131 $\pm$ 7	132 $\pm$ 6
Spongy mesophyll thickness [μm]	72.0 $\pm$ 4.8	76.7 $\pm$ 4.9
Ratio of palisade to spongy mesophyll thickness	1.91 $\pm$ 0.14	1.82 $\pm$ 0.20
Number of palisade cell layers	2.80 $\pm$ 0.13	2.70 $\pm$ 0.20
Palisade mesophyll cells per cross-sectional area [cells mm^{-2}]	552 $\pm$ 25	637 $\pm$ 43
Spongy mesophyll cells per cross-sectional area [cells mm^{-2}]	811 $\pm$ 41	742 $\pm$ 36

A previous study (Stewart *et al.* 2017) compared the features of *vte1* and Col-0 under those same growth chamber conditions of high leaf temperatures and found some but not all of the differences in the leaf vascular network identified in the present study for glasshouse-grown plants. The previous study reported TEs that were even smaller, more numerous TEs, and with even greater ratios of TEs to SEs in the foliar minor veins of the *vte1* mutant compared to wild type, but found no significant difference in vein density between the two lines. Instead, the high leaf temperature of 36°C induced similarly high vein densities in both genotypes (for more detail on vein density, *see below*). The previous study also assessed the effect of high growth light intensity on leaf vasculature in the *vte1* mutant. Unlike high temperature, growth chamber conditions of constant high light during the photoperiod did not induce any differences in leaf vascular organization between *vte1* and Col-0 with respect to either vein density or TE sizes and numbers (Stewart *et al.* 2017). There was thus no difference in vein density between *vte1* and wild type in either hot-grown plants or plants grown under high

light intensities, both of which exhibited vein densities that were comparatively high for *A. thaliana* (Stewart *et al.* 2017). In contrast, the difference in vein density between *vte1* and Col-0 seen in the present study was revealed under growth conditions where vein density was not already high in Col-0. While vein density in Col-0 was 2.4 mm mm⁻² in glasshouse-grown plants, vein densities were 2.7 and 3.1 mm mm⁻² in Col-0 plants grown in growth chambers under constant high light intensity or constant hot temperature, respectively (Stewart *et al.* 2017). A significant difference in vein density was thus only detectable between *vte1* and Col-0 when both plant lines were grown under moderate conditions with natural variation in light and temperature over the course of the day.

Peak light intensities of 1,500 $\mu\text{mol(photon) m}^{-2} \text{ s}^{-1}$ were experienced in the sunlit glasshouse for only 1 to 2 h per day, with light intensities otherwise following a typical natural bell-shaped curve (in the absence of clouds) and lower light intensities for most of the day. Air temperatures during peak irradiance in the glasshouse reached 26–27°C, whereas plants grown in growth chambers in the

Fig. 3. Photosynthetic capacity (light- and CO₂-saturated rate of oxygen evolution) on a (A) leaf area basis [$\mu\text{mol}(\text{O}_2) \text{m}^{-2} \text{s}^{-1}$], (B) chlorophyll basis [$\text{mmol}(\text{O}_2) \text{mol}^{-1}(\text{Chl}) \text{s}^{-1}$], and (C) dry mass [$\mu\text{mol}(\text{O}_2) \text{g}^{-1}(\text{DM}) \text{s}^{-1}$] basis in leaves of *Arabidopsis thaliana* Col-0 wild type (light gray columns) and *vte1* mutant (dark gray columns). Mean values $\pm$ standard deviation; n.s. – not significantly different.

previous study (Stewart *et al.* 2017) experienced a constant air temperature of 35°C throughout the entire photoperiod. Since it has been suggested that cellular redox state may serve as the plant's sensor of changes in temperatures (Hüner *et al.* 1996; see also Hüner *et al.* 2012, 2016), the transiently moderately elevated leaf temperature in the glasshouse may have been sufficient to trigger the heat-acclimated vascular phenotype in *vte1* plants.

Why organize leaf vasculature differently to support the same photosynthetic capacity? Despite significantly different minor vein densities, cross-sectional areas of vascular tissue per vein, number of phloem cells per vein, and ratios of TEs to SEs per vein, the total volumes of water and sugar conduits per leaf area were unaffected by tocopherol status as were photosynthetic capacity and a host of leaf morphological features. Smaller individual cross-sectional area (as a proxy for cell diameter) of the TE

water conduits in the tocopherol-deficient *vte1* mutant can thus be thought of as having been compensated for by a greater vein density. Mathematically, total cross-sectional area per vein of a vascular cell type (such as TEs) is the product of numbers and individual cell sizes of this cell type. Furthermore, the product of total cross-sectional TE area per vein and vein length per leaf area (vein density) reflects the total conduit volume available for water movement through TEs on a leaf area basis. Due to the compensation for smaller TE size by maintaining TE numbers per vein and featuring more numerous veins, the smaller diameters of individual water conduits did not translate into a reduced total volume of water conduits on a leaf area basis.

The smaller tracheary element diameter seen in *vte1* vs. wild type under either hot temperatures or in the sunlit greenhouse are reminiscent of the smaller TE sizes in hot-grown vs. cool-grown wild type and may offer the advantage of lowering the risk of xylem cavitation (air-bubble formation that can block water flow in the xylem) under conditions of high evaporative demand (Hargrave *et al.* 1994, Hacke and Sperry 2001, Hacke *et al.* 2009, Sterck *et al.* 2012). Evaporative demand is typically increased under elevated temperature. A high vein density is thought to enhance maximal leaf hydraulic conductance and gas exchange (Sack and Scoffoni 2013), and may thereby facilitate leaf cooling under hot temperatures. In addition, narrower xylem conduits may confer greater resistance against cavitation due to freeze-thaw events (Davis *et al.* 1999) and may, therefore, be advantageous for both extreme hot and cold temperatures.

Compensation for smaller water conduits by greater vein density may allow the leaf to maintain overall hydraulic conductivity for moving similar volumes of water to a given leaf area. Such compensatory adjustments may thus afford a reduced likelihood for cavitation with no penalty to overall water supply to the leaf — in other words, a decrease in cavitation risk at no cost to photosynthetic performance. Likewise, there is no apparent cost to transpiration rate per leaf area; similar water-transport capacities and similar transpiration rates on a leaf area basis in *vte1* with its narrower TEs compared to Col-0 are attained in *vte1* by virtue of its greater TE numbers and greater TE/SE ratios. This finding, that transpiration rate correlates with total TE volume, is further supported by data from our previous study (Stewart *et al.* 2017) on Col-0 and *vte1* under contrasting light intensities and temperatures in environmentally controlled growth chambers (Fig. 7). Such adjustment of the leaf vascular network's organization may serve to co-optimize plant productivity and heat stress tolerance. These findings further illustrate the high degree of flexibility of developmental adjustment in multiple individual components of the foliar vascular network, resulting in differential adjustment of vascular features in leaves with similar overall photosynthetic activity and similar apparent capacities for sugar and water flux.

Fig. 4. (A,B,C) Cross-sectional area per cell and (D,E,F) cell number per minor vein for (A,D) tracheary elements, (B,E) sieve elements, and (C,F) companion cells and phloem parenchyma cells (CCs + PCs) in leaves of *Arabidopsis thaliana* Col-0 wild type (light gray columns) and *vte1* mutant (dark gray columns). Mean values $\pm$ standard error; statistically significant differences between genotypes are indicated with asterisks (* – $P < 0.05$; n.s. – not significantly different).

Fig. 5. The ratio of (A) tracheary to sieve elements, TE/SE ratio, and (B) companion cells + phloem parenchyma cells to sieve elements, (CC + PC)/SE ratio, in foliar minor veins of *Arabidopsis thaliana* Col-0 wild type (light gray columns) and *vte1* mutant (dark gray columns). Mean values $\pm$ standard error; statistically significant differences between genotypes are indicated with asterisks (* – $P < 0.05$; n.s. – not significantly different).

While recognizing that the *vte1* *A. thaliana* line is a mutant system, one may speculate that these findings could nevertheless be relevant for understanding the implications of differences in leaf vascular organization among species and ecotypes. Differences in leaf vascular organization combined with similar maximal photosynthesis rates were seen between summer and winter annual species, with more numerous, smaller veins with higher minor vein xylem-to-phloem ratios in summer vs. winter annuals (Cohu *et al.* 2014, Demmig-Adams *et al.* 2014a, Stewart *et al.* 2016). Differences in leaf vascular organization were also seen between the Italian and Swedish ecotypes of *A. thaliana*, with smaller foliar veins in the Italian ecotype under some growth conditions (Adams *et al.* 2016, Stewart *et al.* 2016).

Fig. 6. The product of minor vein density (VD) and (A,B,C) cell number per minor vein and (D,E,F) cell cross-sectional area per minor vein (which provides the estimated volume of each vascular cell type servicing a given leaf area) in order to normalize for differences in minor vein density. (A,D) tracheary elements, (B,E) all sieve elements, and (C,F) all companion cells and phloem parenchyma cells (CCs + PCs) in leaves of *Arabidopsis thaliana* Col-0 wild type (light gray columns) and *vte1* mutant (dark gray columns). Mean values $\pm$ standard error; statistically significant differences between genotypes are indicated with asterisks (* – $P < 0.05$; n.s. – not significantly different).

Fig. 7. Relationship between transpiration rate per leaf area and the total volume of water conduits (tracheary elements, TE) per leaf area for Col-0 (triangles) and *vte1* (diamonds) grown in a sunlit glasshouse (GH; light gray symbol for Col-0 and dark gray symbol for *vte1*) in comparison with plants grown under low light intensity (LL; PPFD of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$; leaf temperature of 20°C), high light intensity (HL; PPFD of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$; leaf temperature of 20°C), cool temperature (CT; PPFD of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$; leaf temperature of 14°C) and hot temperature (HT; PPFD of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$; leaf temperature of 36°C) in environmentally controlled growth chambers (data from Stewart *et al.* 2017). Mean values $\pm$ standard deviation for transpiration and $\pm$ standard error for TE volume.

Tocopherol signals, plant development, and further implications:

The majority of foliar tocopherols is α -tocopherol localized in the chloroplast (Mène-Saffrané and DellaPenna 2010), and the chloroplast provides significant input into cellular redox signaling networks (Foyer and Noctor 2013). Tocopherols might directly modulate regulators such as plant hormones involved in vascular differentiation or, alternatively, interact with transcription factors that control regulators of leaf development (see Allu *et al.* 2017). Cellular redox state regulates the activity of phytohormones such as auxins (Foyer and Noctor 2013) that are involved in vascular differentiation (Mattsson *et al.* 2003, Scarpella and Meijer 2004). Differential temperature acclimation in the Swedish vs. Italian ecotypes involves many genes (Oakley *et al.* 2014), and includes a difference in one of the members of the C-repeat binding factor (CBF) transcription factor family implicated in temperature adaptation (Gehan *et al.* 2015, Kang *et al.* 2013). The greater cold-temperature tolerance of the Swedish ecotype is associated with a functional CBF2, while the Italian ecotype that features superior acclimation to hot temperature (Adams *et al.* 2016, Stewart *et al.* 2016), encodes a dysfunctional CBF2. The CBF family of transcription factors has also been linked to cellular redox state (Hüner *et al.* 2012, 2016; Kurepin *et al.* 2013). Moreover, we previously reported

lower concentrations of foliar tocopherols in the Italian ecotype under several conditions, with the most pronounced differences between the ecotypes seen in plants grown with short daily exposures to high light (Stewart *et al.* 2015) or under hot temperatures (Stewart *et al.* 2016). The available evidence is thus consistent with a direct or indirect role of tocopherols in leaf vascular differentiation.

Since tocopherol deficiency resulted in smaller apparent TE diameters, one may postulate that high tocopherol contents will promote development of TEs with larger diameters. High tocopherol contents may thus increase the risk for cavitation. Future research should address a possible involvement of the lower tocopherol contents in hot-grown plants in the superior heat tolerance of the

Italian ecotype compared to the Swedish ecotype (Adams *et al.* 2016, Stewart *et al.* 2016, 2017). Moreover, it should be recognized that overexpression of tocopherols or other antioxidants could potentially negatively affect plant performance at hot temperature. Due to the vital role of reactive oxygen and other redox-active species and states in signaling cascades (Foyer and Noctor 2009), modulation of oxidative-signaling events by antioxidant overexpression has the potential to interfere with the beneficial effects of oxidative signaling. The present findings provide incentive to explore novel trade-offs between adjustments to high vs. low growth temperature and the use of tocopherol contents, and possibly other antioxidants, to screen for plant lines with differential temperature tolerance.

References

- Adams W.W. III, Muller O., Cohu C.M., Demmig-Adams B.: Foliar phloem infrastructure in support of photosynthesis. – *Front. Plant Sci.* **4**: 194, 2013.
- Adams W.W. III, Cohu C.M., Amiard V., Demmig-Adams B.: Associations between phloem-cell wall ingrowths in minor veins and maximal photosynthesis rate. – *Front. Plant Sci.* **5**: 24, 2014.
- Adams W.W. III, Stewart J.J., Cohu C.M. *et al.*: Habitat temperature and precipitation of *Arabidopsis thaliana* ecotypes determine the response of foliar vasculature, photosynthesis, and transpiration to growth temperature. – *Front. Plant Sci.* **7**: 1026, 2016.
- Ågren J., Schemske D.W.: Reciprocal transplants demonstrate strong adaptive differentiation of the model organism *Arabidopsis thaliana* in its native range. – *New Phytol.* **194**: 1112–1122, 2012.
- Allu A.D., Simancas B., Balazadeh S., Munné-Bosch S.: Defense-related transcriptional reprogramming in vitamin E-deficient *Arabidopsis* mutants exposed to contrasting phosphate availability. – *Front. Plant Sci.* **8**: 1396, 2017.
- Amiard V., Mueh K.E., Demmig-Adams B. *et al.*: Anatomical and photosynthetic acclimation to the light environment in species with differing mechanisms of phloem loading. – *P. Natl. Acad. Sci. USA* **102**: 12968–12973, 2005.
- Cohu C.M., Muller O., Stewart J.J. *et al.*: Association between minor loading vein architecture and light- and CO₂-saturated oxygen evolution among *Arabidopsis thaliana* ecotypes from different latitudes. – *Front. Plant Sci.* **4**: 264, 2013a.
- Cohu C.M., Muller O., Demmig-Adams B., Adams W.W. III: Minor loading vein acclimation for three *Arabidopsis thaliana* ecotypes in response to growth under different temperature and light regimes. – *Front. Plant Sci.* **4**: 240, 2013b.
- Cohu C.M., Muller O., Adams W.W. III, Demmig-Adams B.: Leaf anatomical and photosynthetic acclimation to cool temperature and high light in two winter versus summer annuals. – *Physiol. Plantarum* **152**: 164–173, 2014.
- Considine M.J., Foyer C.H.: Redox regulation of plant development. – *Antioxid. Redox Sign.* **21**: 1305–1326, 2014.
- Davis S.D., Sperry J.S., Hacke U.G.: The relationship between xylem conduit diameter and cavitation caused by freezing. – *Am. J. Bot.* **86**: 1367–1372, 1999.
- Demmig-Adams B., Cohu C.M., Muller O., Adams W.W. III: Modulation of photosynthetic energy conversion efficiency in nature: from seconds to seasons. – *Photosynth. Res.* **113**: 75–88, 2012.
- Demmig-Adams B., Cohu C.M., Amiard V. *et al.*: Emerging trade-offs – impact of photoprotectants (PsbS, xanthophylls, and vitamin E) on oxylipins as regulators of development and defense. – *New Phytol.* **197**: 720–729, 2013.
- Demmig-Adams B., Stewart J.J., Adams W.W. III: Multiple feedbacks between chloroplast and whole plant in the context of plant adaptation and acclimation to the environment. – *Philos. T. R. Soc. B* **269**: 20130244, 2014a.
- Demmig-Adams B., Stewart J.J., Adams W.W. III: Photoprotection and the trade-off between abiotic and biotic defense. – In: Demmig-Adams B., Garab G., Adams W.W. III, Govindjee (ed.): *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*. Pp. 631–643. Springer, Dordrecht 2014b.
- Demmig-Adams B., Stewart J.J., Burch T.A., Adams W.W. III: Insights from placing photosynthetic light harvesting into context. – *J. Phys. Chem. Lett.* **5**: 2880–2889, 2014c.
- Delieu T., Walker D.A.: Polarographic measurements of photosynthetic oxygen evolution by leaf discs. – *New Phytol.* **89**: 165–178, 1981.
- Dumlao M.R., Darehshouri A., Cohu C.M. *et al.*: Low temperature acclimation of photosynthetic capacity and leaf morphology in the context of phloem loading type. – *Photosynth. Res.* **113**: 181–189, 2012.
- Fatichi S., Leuzinger S., Körner C.: Moving beyond photosynthesis: from carbon source to sink-driven vegetation modeling. – *New Phytol.* **201**: 1086–1095, 2014.
- Foyer C.H., Noctor G.: Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. – *Antioxid. Redox Sign.* **11**: 861–905, 2009.
- Foyer C.H., Noctor G.: Redox signaling in plants. – *Antioxid. Redox Sign.* **18**: 2087–2090, 2013.
- Gehan M.A., Park S., Gilmour S.J. *et al.*: Natural variation in the C-repeat binding factor cold response pathway correlates with local adaptation of *Arabidopsis* ecotypes. – *Plant J.* **84**: 682–693, 2015.
- Hacke U.G., Sperry J.S.: Functional and ecological xylem anatomy. – *Perspect. Plant Ecol.* **4**: 97–115, 2001.
- Hacke U.G., Jacobsen A.L., Pratt R.B.: Xylem function of arid-land shrubs from California, USA: an ecological and evolutionary analysis. – *Plant Cell Environ.* **32**: 1324–1333, 2009.

- Hargrave K.R., Kolb K.J., Ewers F.W., Davis S.D.: Conduit diameter and drought-induced embolism in *Salvia mellifera* Greene (Labiatae). – *New Phytol.* **126**: 695-705, 1994.
- Havaux M., García-Plazaola J.I.: Beyond non-photochemical fluorescence quenching: the overlapping antioxidant functions of zeaxanthin and tocopherols. – In: Demmig-Adams B., Garab G., Adams W.W. III, Govindjee (ed.) *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*. Pp. 583-603. Springer, Dordrecht 2014.
- Hölttä T., Nikinmaa E.: Modelling the effect of xylem and phloem transport on leaf gas exchange. – *Acta Hort.* **991**: 351-358, 2013.
- Hölttä T., Vesala T., Sevanto S. *et al.*: Modeling xylem and phloem water flows in trees according to cohesion theory and Münch hypothesis. – *Trees* **20**: 67-78, 2006.
- Hüner N.P.A., Maxwell D.P., Gray G.R. *et al.*: Sensing environmental temperature change through imbalances between energy supply and energy consumption: redox state of photosystem II. – *Physiol. Plantarum* **98**: 358-364, 1996.
- Hüner N.P.A., Bode R., Dahal K. *et al.*: Chloroplast redox imbalance governs phenotypic plasticity: the “grand design of photosynthesis” revisited. – *Front. Plant Sci.* **3**: 255, 2012.
- Hüner N.P.A., Dahal K., Bode R. *et al.*: Photosynthetic acclimation, vernalization, crop productivity and the ‘grand design of photosynthesis’. – *J. Plant Physiol.* **203**: 29-43, 2016.
- Kang J., Dengler N.: Vein pattern development in adult leaves of *Arabidopsis thaliana*. – *Int. J. Plant Sci.* **165**: 231-242, 2004.
- Kang J., Zhang H., Sun T. *et al.*: Natural variation of *C-repeat-binding factor* (CBFs) genes is a major cause of divergence in freezing tolerance among a group of *Arabidopsis thaliana* populations along the Yangtze River in China. – *New Phytol.* **199**: 1069-1080, 2013.
- Körner C.: Growth controls photosynthesis – mostly. – *Nova Act. Lc.* **114**: 273-283, 2013.
- Ksas B., Becuwe N., Chevalier A., Havaux M.: Plant tolerance to excess light energy and photooxidative damage relies on plastoquinone biosynthesis. – *Sci. Rep.* **5**: 10919, 2015.
- Külheim C., Ågren J., Jansson S.: Rapid regulation of light harvesting and plant fitness in the field. – *Science* **297**: 91-93, 2002.
- Kurepin L.V., Dahal K.P., Savitch L.V. *et al.*: Role of CBFs as integrators of chloroplast redox, phytochrome and plant hormone signaling during cold acclimation. – *Int. J. Mol. Sci.* **14**: 12729-12763, 2013.
- Logan B.A., Korniyev D., Hardison J., Holaday A.S.: The role of antioxidant enzymes in photoprotection. – *Photosynth. Res.* **88**: 119-132, 2006.
- Maeda H., Song W., Sage T.L., DellaPenna D.: Tocopherols play a crucial role in low-temperature adaptation and phloem loading in *Arabidopsis*. – *Plant Cell* **18**: 2710-2732, 2006.
- Maeda H., Song W., Sage T., DellaPenna D.: Role of callose synthases in transfer cell wall development in tocopherol deficient *Arabidopsis* mutants. – *Front. Plant Sci.* **5**: 46, 2014.
- Mattsson J., Ckurshumova W., Berleth T.: Auxin signaling in *Arabidopsis* leaf vascular development. – *Plant Physiol.* **131**: 1327-1339, 2003.
- Mène-Saffrané L., DellaPenna D.: Biosynthesis, regulation and function of tocopherols in plants. – *Plant Physiol. Bioch.* **48**: 301-309, 2010.
- Mishra Y., Jänkänpää H.J., Kiss A.Z. *et al.*: *Arabidopsis* plants grown in the field and climate chambers significantly differ in leaf morphology and photosystem components. – *BMC Plant Biol.* **12**: 6, 2012.
- Muller O., Cohu C.M., Stewart J.J. *et al.*: Association between photosynthesis and contrasting features of minor veins in leaves of summer annuals loading phloem *via* symplastic versus apoplastic routes. – *Physiol. Plantarum* **152**: 174-183, 2014a.
- Muller O., Stewart J.J., Cohu C.M. *et al.*: Leaf architectural, vascular, and photosynthetic acclimation to temperature in two biennials. – *Physiol. Plantarum* **152**: 763-772, 2014b.
- Nikinmaa E., Hölttä T., Hari P. *et al.*: Assimilate transport in phloem sets conditions for leaf gas exchange. – *Plant Cell Environ.* **36**: 655-669, 2013.
- Oakley C.G., Ågren J., Atchison R.A., Schemske D.W.: QTL mapping of freezing tolerance: links to fitness and adaptive trade-offs. – *Mol. Ecol.* **23**: 4304-4315, 2014.
- Paul M.J., Driscoll S.P.: Sugar repression of photosynthesis: The role of carbohydrates on signaling nitrogen deficiency through source:sink imbalance. – *Plant Cell Environ.* **20**: 110-116, 1997.
- Paul M.J., Foyer C.H.: Sink regulation of photosynthesis. – *J. Exp. Bot.* **52**: 1383-1400, 2001.
- Sack L., Holbrook N.M.: Leaf hydraulics. – *Annu. Rev. Plant Biol.* **57**: 361-381, 2006.
- Sack L., Scoffoni C.: Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. – *New Phytol.* **198**: 983-1000, 2013.
- Sack L., Scoffoni C., McKown A.D. *et al.*: Developmentally based scaling of leaf venation architecture explains global ecological patterns. – *Nat. Commun.* **3**: 837, 2012.
- Sack L., Scoffoni C., Johnson D.M. *et al.*: The anatomical determinants of leaf hydraulic function. – In: Hacke U. (ed.): *Functional and Ecological Xylem Anatomy*. Pp. 255-271. Springer, Dordrecht 2015.
- Sattler S.E., Cahoon E.B., Coughlan S.J., DellaPenna D.: Characterization of tocopherol cyclases from higher plants and cyanobacteria. Evolutionary implications for tocopherol synthesis and function. – *Plant Physiol.* **132**: 2184-2195, 2003.
- Sattler S.E., Mène-Saffrané L., Farmer E.E. *et al.*: Nonenzymatic lipid peroxidation reprograms gene expression and activates defense markers in *Arabidopsis* tocopherol-deficient mutants. – *Plant Cell* **18**: 3706-3720, 2006.
- Scarpella E., Meijer A.H.: Pattern formation in the vascular system of monocot and dicot plant species. – *New Phytol.* **164**: 209-242, 2004.
- Semchuk N.M., Lushak O.V., Falk J. *et al.*: Inactivation of genes, encoding tocopherol biosynthesis pathway enzymes, results in oxidative stress in outdoor grown *Arabidopsis thaliana*. – *Plant Physiol. Bioch.* **47**: 384-390, 2009.
- Sterck F.J., Martínez-Vilalta J., Mencuccini M. *et al.*: Understanding trait interactions and their impacts on growth in Scots pine branches across Europe. – *Funct. Ecol.* **26**: 541-549, 2012.
- Stewart J.J., Adams W.W. III, Cohu C.M. *et al.*: Differences in light-harvesting, acclimation to growth-light environment, and leaf structural development between Swedish and Italian ecotypes of *Arabidopsis thaliana*. – *Planta* **242**: 1277-1290, 2015.
- Stewart J.J., Demmig-Adams B., Cohu C.M. *et al.*: Growth temperature impact on leaf form and function in *Arabidopsis thaliana* ecotypes from northern and southern Europe. – *Plant Cell Environ.* **39**: 1549-1558, 2016.
- Stewart J.J., Polutcho S.K., Adams W.W. III *et al.*: Light, temperature and tocopherol status influence foliar vascular anatomy and leaf function *Arabidopsis thaliana*. – *Physiol. Plantarum* **160**: 98-110, 2017.