From fossils to physiology: testing the functional significance of leaf shape

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Abstract

From fossils to physiology: testing the functional significance of leaf shape

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Leaf morphological diversity has captured the attention of botanists for hundreds of years and while several correlative relationships between leaf form and climate have been confirmed through experimental testing, much of the adaptive significance is still an open question. We consider this link to be essential to the fields of plant physiology, cell and developmental biology, paleobotany, and abiotic stress physiology. Gaining a more complete understanding of how plants inherently acclimate and over time adapt to new environments can be approached by merging the science of two fields: 1) the paleobotanical approach of identifying morphological traits from fossils for climate reconstruction, and 2) the physiological approach for testing mechanistic hypothesis suggested from correlations. This merged approach will reveal how leaf shape contributes to overall plant performance and could yield considerable insight into species range-shifts and ecosystem flux due to future climate instability. It is also a novel way to confront inefficiencies and stress resiliencies within crops.

Leaf shape is largely determined by venation patterning, hardwired in many species, due to biomechanical limitations determining structure and physiological limitations determining resource movement. A subset of wild and domesticated tomato leaflets with a range of margin shapes (toothed, entire) was used to test the hypothesis that this morphological form provides a functional boost in the form of increased gas exchange and resilience during applied stress from water deficit. Here we show that leaf teeth in tomato do provide a disproportionately higher photosynthetic rate per area, and this pattern persists even as the water potential gradient across the leaf increases in magnitude suggesting margin shape, and not the species, underlies this pattern.

Lastly, we show that leaf hydraulic conductance and its coordinated response with gas exchange and vein structural traits give insight into plant adaptations to abiotic stress. The leaf shape, as determined by its vascular pattern and structural traits therein, is a critical point of resistance along the water pathway through the plant, and here we show that form matches functional potential as the venation traits, particularly vein surface area, is a strong predictor of maximum rates of gas exchange and hydraulic conductance.

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Chapter 1. INTRODUCTION

Leaves are an important structure for plants as they interface with the environment as the main site of gas exchange and light capture, which are fundamental for plant growth and survival. The diversity of leaf shapes speaks in part to their phylogenetic history, and also to their capacity to adapt morphologies to succeed in ecosystems all over the globe. For hundreds of years scientists have kept records of leaf shape, and using fossils have assembled a clear relationship of leaf shape with past-climate, which suggests that climate constrains leaf shapes to some degree and that leaf climate can impact leaf level functions.

Looking forward, rapidly changing climates will affect ecosystem structure and have palpable effects on how we grow food with the increasing costs and decreasing availability of fresh water for irrigation. There are environments with high temperatures, or with low water availability, or both, that have high incidence of particular leaf shapes according to the fossil record. While not 100% explanatory, these relationships could serve as the basis for exploring questions about the functional advantages of specific leaf shapes.

Some adaptations are well recognized for their benefits to specific climates (for example: succulence, spines, and vast root systems in deserts) and also some physiological responses (changing flowering time to avoid drought) associated with short term solutions to a changing climate. These inherent plant strategies for survival are far too often studied for the purpose of finding the genes to unlocking “drought tolerance”, or “drought resistance”, or “drought avoidance”. These key phrases can be misleading, as more often they refer to the behavior of a plant downstream from gene expression that combines so many genetic programs, hormone signals, and physiological responses of an entire plant. Responses to water limitation are remarkably complex, and worthy of research from an approach that that captures not only the gene expression and impact on yield, but the plant’s physiological response.

We chose an understated approach to researching plant responses to water deficit and the functional significance of leaf shapes by testing hypotheses raised in paleo-botanical literature with physiological approaches. We chose *Solanum* as the genus for this study because it is a model species for plant research with a wealth of genetic resources and also an economically important crop, making the results of this work broadly applicable. Previous work in tomato quantified contrasting leaf shapes that were selected for this study, and the compound leaf structure was well suited for experiments using the leaflet. Chapter 1 details the background for this study highlighted above, and includes relevant summaries from paleo, developmental, and physiological approaches to leaf shapes, leaf growth, and leaf functions. Chapter 2 focuses on the differences in leaf margin photosynthesis and responses to water deficit among the different leaf shapes. Chapter 3 further explores the functioning of leaf shapes by focusing on the coordination of hydraulic conductance and gas exchange, using vein traits to help explain patterns among the leaflets. Chapter 4 summarizes this work and points to future avenues of research.

Chapter 2. WHAT CAN LEAF SHAPE TELL US ABOUT CLIMATE, DEVELOPMENT, AND FUNCTION?

2.1 INTRODUCTION

Patterns in leaf form have piqued and inspired botanists' thinking for several centuries. The foundation laid by Carl Linnaeus' classification system which outlines the morphological diversity of leaf forms in 1735, and Goethe developed the philosophical notion that the inner workings, and notably polarity, determine leaf shape in his 1790 work 'Metamorphosis of Plants.' Over a century ago Bailey and Sinnott (1916) published their first work on the distribution of angiosperm leaves, drawing specific attention to the interaction between the leaf-margin and the environment. That paper is likely the first time in print that the question was asked: what is the physiological significance of leaf shape? Using both personal observation and rudimentary fossil data they concluded that with trees and shrubs, and to a lesser extent (due to a sparse dataset) herbs, a consistent pattern emerges with leaf margin and climate. Entire (smooth, non-toothed) margins occupy lowland tropics and toothed-margin (serrated) leaves dominate in the mesophytic cold temperate regions. This pattern can be observed today, and is evident in fossils spanning back over a hundred million years. This dichotomy in landscape and leaf shape has undoubtedly interested plant scientists of all sub disciplines, and its physiological significance is an open question that remains to this day.

That several fundamental questions about leaf form and function remain unanswered is a testament to the complexity of physiological adaptations, the design of meaningful experimentation, and data collection techniques. We consider this link to be essential to the fields of plant physiology, cell and developmental biology, paleobotany, and abiotic stress physiology. Understanding how leaf shape contributes to overall leaf and plant performance could give considerable insight into species range-shifts and ecosystem flux due to future climate instability, and is potentially a novel way to confront inefficiencies and stress resiliencies within crops. The following review is intended to highlight the existing hypotheses on the functionality of leaf shape, describe the fundamental elements of how leaves develop, and emphasize what we know so far about how leaf form is tied to function.

2.2 LEAF SHAPE HYPOTHESES

In the late 1960s paleobotanists began compiling more comprehensive surveys similar to those of Bailey and Sinnott (1916) where the leaf margin, either toothed or entire, was recorded as a proportion of the margin-type of all species found in a general region at a specific time in the fossil record. Time and again the conclusion, that the proportion of entire leaves is high in the tropics, and low in the temperate regions, is confirmed (Wolfe 1971; Royer et al. 2012; Wilf 1997; Little, Kembel, and Wilf 2010; Bailey and Sinnott 1916).

More recently, Givnish (1979) has emphasized that there must be physiological significance to the patterns of leaf shape (leaf margins, leaf size, and leaf complexity) with environment. Using maximum net photosynthesis as the metric of plant suitability to environment, Givnish demonstrates through various models that leaf size and shape determine the size of the leaf boundary layer. Specifically, two important distinctions come from this work about leaf shape: 1) Boundary layer thickness determines leaf temperature as a thick boundary layer leads to less convective heat loss, 2) A thick boundary layer adds resistance to the diffusion of gases, both water in the transpirational stream and CO_2 for fixation. In short, large leaves have correspondingly large boundary layers, leading to higher leaf temperatures and a larger resistance pathway to water loss. When other aspects of leaf shape like margin type (serrations, lobes) and leaf complexity (simple, compound) are considered, a spectrum of suitable temperature-gas exchange possibilities can be achieved. These models are key for understanding the potential significance of leaf shape, though are not yet empirically tested.

To understand how leaf morphology contributes biophysically to leaf temperature, Steven Vogel and others (Vogel 1969; Gottschlich and Smith 1982) detailed the convective cooling properties of leaves and identified several advantageous leaf shapes. In addition to carbon dioxide flux for photosynthesis, the convective cooling of transpiration maintains optimal leaf temperature for a myriad of biological processes driven by chemical reactions. Their simple and elegant set of experiments aimed empirically to test temperature differences between leaf surfaces and ambient air using simulated leaf shapes. The results of his study helped to explain heat dissipation in relation to leaf size and, to some extent, to lobing. Gottschlich and colleagues (1982) confirmed the connection between heat dissipation and margin shape, and thoughtfully noted that it may not invalidate other margin-shape hypotheses but instead be part of the leaf energy balance. The importance of temperature regulation through transpiration has been confirmed many times (Drake and Raschke 1970; D M Gates 1968; Hegazy and El Amry 1998), and so the external atmospheric conditions as well as the soil water availability influence the demand for evaporative cooling. These factors external to the leaf, together with its shape, may work in confluence to regulate temperature and meet functional demands. Added layers of complexity ensue when we aim to delineate at what level(s) in the system is the energy balance calibrated for: the leaf, a branch, a small sapling, or an entire tree.

For an ecological context of the importance of leaf shape, Baker-Broch and colleagues (1997) explored hypotheses that the area of teeth and lobes contribute to a photosynthetic boost, and for woody species in a deciduous forest, the fast maturing tissue in these regions help to achieve maximum plant growth during spring leaf-out. They showed this in experiments measuring photosynthesis spatially across lobes and sinuses as the leaf matured, and found that lobes outperform sinuses up to leaf maturity; at this size and developmental stage the two areas have equal rates (Baker-Brosh and Peet 1997). This type of experiment was repeated years later by Royer and Wilfe (2006) to show seasonality and temperature effect on tooth/lobe photosynthesis of woody angiosperms and to uncouple this observed phenomenon from leaf habit. They confirm that edges on lobed leaves have higher rates of photosynthesis than the interior space of lobed leaves before the leaves reached maturity, when the rates seemed to level out (Royer and Wilf 2006).

Alternatively, what if the leaf form has little to do with function during maturity, but instead is a consequence of function of the physical shape during the leaf initiation folding into the bud for woody plants? Since leaf development in many deciduous woody angiosperms initiates at the close of the prior season, the embryonic leaf for the following spring forms and is packed into a bud before dormancy. Perhaps the form of leaf teeth and lobes is due to an origami-esque technique to put as much tissue as possible into a small space (Couturier et al. 2012). Other hypotheses accounting for variation in leaf shape that are underexplored include the use of teeth to simulate herbivory thereby causing potential herbivores to pass over the eaten leaf (Brown and Lawton 1991). Studies on this topic (Givnish 1979; Rivero-Lynch 1996; Adam et al. 2011) are inconclusive for herbivory being a primary driver for the phenomenon of the leaf teeth – MAT (mean annual temperature) correlation observed countless times over several decades.

As we will review ahead, leaf shape is closely influenced by the vein architecture. This raises several hypotheses about the functional significance of leaf shape, and more specifically, the functional significance of vein patterning. Veins are responsible for shuttling photosynthate to and from active cells and also act as the water pipeline to irrigate all living cells. Angiosperm evolution offered gateways to new possible leaf shapes due to the unique leaf growth patterns including the presence of xylem vessels of increasingly small order (see next section), changing the hydraulic resistance of the leaf. Two clear patterns emerged that are worthy of further inspection to understand leaf shape functioning 1) hydraulic resistance increases along the pathway to evaporation leading to increasingly more negative water potentials along the path (Brodribb and Feild 2010) and 2) hydraulic resistance is negatively correlated with maximum photosynthetic rate (Sack and Tyree 2005). We will continue to explore these in later sections.

To consider herbs in this paradigm is to also consider the long-observed pattern of toothed herbaceous species along riparian habitats (Burgerstein 1904; Haberlandt 1914; Bailey and Sinnott 1916; Stocking 1956; Takeda, Wisniewski, and Glenn 2019). Riparian communities often facilitate conditions favoring guttation, where moisture-rich soils and high relative humidity cause a positive pressure to build inside plant tissue as water uptake from the roots exceeds transpiration rate at the stomata and the excess water exudes from leaf hydathodes. Hydathodes have a high incidence with leaf teeth and when in concert the tooth: 1) sheds guttation droplets due to less surface area and less surface tension for water to adhere and 2) can extend photosynthetic activity into this leaf area even when the conditions described above would normally cause water accumulation in the tissue resulting in high cell pressure, and could block CO_2 diffusion to mesophyll cells (Feild et al. 2005).

When considering the leaf lifespan and construction economies therein, teeth have been proposed as an economically practical way to increase leaf surface area with minimal carbon investment. The low order major veins (e.g., a secondary vein, one which branches from the primary) which run central through the tooth, provides significant structural support for the leaf lamina as well as being the pipeline for sugar and water transport. For deciduous species that invest in new leaf laminae every year, a miserly carbon expenditure makes sense since each leaf is not reusable. Teeth, also lobes, are a solution to this investment tradeoff as a way of keeping carbon cost low as the leaf can be thinner and still remain mechanically supported by vasculature (Niinemets 2001). For long-lived slow growing leaves, the carbon investment in the lamina is greater as it persists perennially and, as noted by botanists and naturalists for hundreds of years, the percent of toothed leaves in warm equatorial evergreen forests is low. The shorter growing season in temperate climates and the need for an early season ‘boost’ in photosynthetic activity also supports this minimalist leaf shape.

It would be unreasonable to conclude that there is a biophysical determination for leaf margin-shape consistent with the environment without incorporating the latent influence of phylogeny on global leaf-shape distribution. The idea that plant relatedness could account for the spatial patterning we see across the globe has been suggested and analyzed (Schmerler et al. 2012; Little, Kembel, and Wilf 2010) with varied results. In the study by Little et al (2010) the overall trend of mean annual temperature (MAT) and leaf shape correlation was weakened when phylogenetic signal was accounted for in their analysis. However, they also note that factors beyond presence/absence of teeth, such as the size and number of teeth, remained under strong influence of MAT. Schmerler and colleagues (2012) looked within the diversely shaped and globally stratified genus *Viburnum* and suggest that leaf margin shape is overall adaptive, as presence and absence of teeth have experienced multiple shifts with climate in several locations through time. Other studies on phylogeny and leaf shape point to the genetic predispositions of most plants to make simple (not complex) entire (not toothed) leaves (Geeta et al. 2012; Bharathan et al. 2002). The developmental model for leaves and leaf margins is discussed in the next section. Whether toothed leaves found themselves in climates that became cool-temperate or whether teeth evolved there in response to the climate they experienced, the point remains that this leaf shape, and not the others that are possible, thrives in these environments.

To conclude that the extra lamina produced by a leaf tooth or a lobe conforms to biomechanical properties allowing for maximum photosynthetic lamina per leaf, there must be evidence of increased photosynthesis in this leaf portion. Few studies have tested the photosynthetic functioning of leaf margins (Royer and Wilf 2006; Baker-Brosh and Peet 1997), and even fewer have explored this hypothesis in herbaceous species due to their inability to persist in the fossil record. In both of the mentioned studies, photosynthetic activity was quantified in the lobed/toothed leaf margins of over 20 temperate woody species and show increased rates as compared to the rest of the leaf. These findings are significant because they give credence to a rationale for the patterns that have motivated botanists' questions for several centuries. These results are of particular interest to this review as we aim to provide context for this frequently overlooked area of basic research: the functional importance of leaf form.

We have discussed how external environmental factors like temperature, light, water availability as well as genetically predetermined programs for phylogeny, lifespan, and mechanics all constrain the leaf shape. But how do leaves form and where are the potential feedback points from the environment?

2.3 LEAF PRIMORDIA & CAMBIUM DEVELOPMENT

Leaf morphogenesis is carefully summarized in several places (Kalve, De Vos, and Beemster 2014; N. Dengler and Kang 2001; Townsley and Sinha 2012; Sachs 1991). It is our goal here to give a synopsis of the current understanding and to point towards the parts of development critical to our discussion of leaf shape and the functioning of shapes specifically at the leaf margin.

For leaves to develop from the shoot apical meristem (SAM), a series of precisely timed events must occur. Using a simple (non-toothed, not compound) leaf as the basis for our discussion, we begin at the SAM where leaf precursor cells are produced. There is evidence that a mechanical "buckling" initiates a signal at the SAM (Green 1999) giving rise to the suppression of class 1 homeobox (KNOX) genes (Fleming et al. 1999) allowing the space (both spatial and temporal) for morphogenesis to begin. KNOX1 genes are known transcription factors (TF) for undifferentiated cell populations. It is only with suppression of KNOX1 that new primordia can arise and cell fates can be determined. Auxin produced at the SAM is directed through the dividing cell layers to accumulate in areas of high concentration (auxin maxima) distal to the center of the SAM, and with respect to each other, thus beginning the phyllotactic arrangement of leaves even as they exist only as precursors. The transporter protein PIN-FORMED1 (PIN1) directs the efflux of auxin establishing this auxin gradient (R. S. Smith and Bayer 2009). How the SAM is able to calculate precisely the rate of founder cell proliferation in tandem with the recruitment of cells for the budding primordia, and simultaneously maintain a founder cell population, is one example of the intricate feedback signaling and timing events involved in leaf morphogenesis and initiation.

Next, boundaries and polarity of the embryonic leaf are established. The CUP-SHAPED COTYLEDON2 (CUC2) genes trigger a suite of downstream gene activation regulating the boundary of the leaf primordium from the SAM and set up for the Homeodomain-leucine zipper (HD-ZIPIII) and auxin response factor 4 (ARF4) transcription factors in the adaxial and abaxial regions, respectively. Polarity signaling is complex and, as part of founding the dorsoventrality of the leaf, it also sets the space for vascular differentiation into xylem and phloem (Husbands et al. 2009; Fleming et al. 1999). With the establishment of polarity and the beginning of embryonic cell suppression and lateral growth out from the SAM, other complex gene regulatory networks (GRNs) come into play with the development of the leaf primordia (for a detailed summary of leaf initiation see Townsley and Sinha 2012). The outermost cells of the leaf primordia, termed the marginal blastozone, remains a blank slate of possible shapes that will be determined by the subsequent timing of the developmental sequence.

Just as KNOX suppression was necessary for cell differentiation at the SAM, for many species its suppression, or lack thereof, is again important for determining complexity and shape. In these cases when KNOX expression is extended within the cells of the marginal blastozone, they remain in a state of indeterminacy so that other developmental programs can take place. This temporal extension of indeterminacy is almost as if small meristems are intact along the edges of the leaf primordia. When KNOX expression is later downregulated, PIN activity and auxin accumulation at tips can then occur. Just as the pools of auxin accumulated at the leaf primordia tip cause more rapid cell differentiation and cell growth in that region, this program is repeated now along the margin of the leaf primordia, forming auxin maxima to delineate leaflets on a compound leaf. Now within a leaflet this fractal-like pattern can be repeated, if the window for differentiation is prolonged due to KNOX (or similar) suppression, and the leaf complexity increases. Evidence for this can be seen in examples of transgenic tomato where overexpression of the KNOX homeobox genes resulted in super-complexity, a layering of compound leaflets within the primary compound leaflets, repeated (Hareven et al. 1996; Champagne and Sinha 2004). If this regulation sequence at the leaf or leaflet margin is in play to a lesser degree (KNOX suppression at the marginal blastozone) the resultant shape can be serrations (teeth) instead of lobes. Recent evidence on margin-shape formation indicates that the epidermal patterning factor-like family of proteins, notably EPFL2, work along with and independent of KNOX and CUC2 to enforce the canalization of auxin at the leaf margin creating leaf teeth (Tameshige et al. 2016). This convergence of shape patterning mechanisms supports the significance of auxin accumulation for accelerated leaf growth resulting in teeth, lobes, or compound structures.

While PIN1 directs surface level auxin towards the leaf primordia tip, PIN1 also directs auxin from the surface maxima basipetally within internal cells initiating a midvein of procambium via canalization (N. G. Dengler 2001). Depending on whether the leaf is entire, lobed, or compound, this pattern is occurring at the leaf tip to initiate a midvein, or along the marginal blastozone at PIN-mediated auxin convergence zones. The procambium extends to the existing vascular tissue below the SAM, and from here protophloem is first to differentiate from the base of the leaf primordia to the tip. Interestingly, protoxylem differentiates in a tip to base orientation, eventually joining with the existing vascular bundle below. As the leaf primordia development continues, ground and mesophyll tissues differentiate, as well as epidermal characteristics in the outer layers of cells. It is important for vascular differentiation to occur at this stage in the developmental sequence because: 1) as the primordium matures and gets larger the cells become a sink for resources and therefore need pipelines to supply them, and 2) the major veins define the overall leaf shape and need to form with convergence points along the epidermis (Scarpella, Barkoulas, and Tsiantis 2010). During this first phase of leaf expansion when growth is predominantly accompanied by cell division, vein development is restricted to first and second order development. Depending on the hierarchy of the individual dicot, the next few orders fill in just before the leaf enters the rapid growth phase of cell expansion without mitosis. During this phase, it is the higher order veins that develop to fill in between and among the other vein tissues according to cell signaling and vascular spacing among the other cell types represented in the leaf (Sack et al. 2012). The process of differentiation of tissue types and maturation and expansion of cells occurs basipetally from the tip to the base and from the margins inward. Thus, the leaf tip and leaf edges are the first to mature.

Considering the developmental programming for leaves, it is interesting to consider how environmental conditions could potentially modulate the leaf in size or shape, and how the signals for acclimation could be incorporated into development for a functional leaf. Because cell expansion, the final stage of leaf growth, is complete within 20 days of primordia initiation in many dicots, if a leaf where to modulate size it would require signaling early in development so that the structural support and overall vein density could accomplish this. We have previously discussed the differences that arise from prolonged suppression of transcription factors at the SAM and that these temporal changes in expression pattern can lead to vastly different spatial landscape for subsequent gene patterning and in particular auxin concentrations in the leaf. There are still open questions about leaf ontogenesis and further research on cellular signaling and cross-talk is necessary to know the molecular mechanisms underpinning leaf shape diversity.

2.4 FORM AND FUNCTION AND THE FUTURE

We have seen how leaf initiation and subsequent growth present key windows within the developmental sequence for variation and increased complexity, and also how leaf shape is closely tied to the vein patterning laid down in the first phase of leaf growth. We will now look into examples of how the leaf form is suited to particular leaf functions with special attention to the venation structures.

There is a clear pattern across plant groups of increased vein length per area (VLA) with higher temperatures and also with aridity (Sack and Scoffoni 2013) suggesting that while there are many solutions to the problem of thriving under high temperatures, one way we see plants evolving strategies to succeed in this environment is through increased vein density.

The leaf is fundamental to the plant's success as it encompasses the majority of light harvesting complexes necessary for photosynthesis and is the interface for gas exchange which establishes the gradient for water movement throughout the entire plant. Leaves with stomata are the main sites of evaporation from the plant however, the leaf form also offers the largest number of sites of resistance to water conductivity in the plant, encompassing up to 30% of resistance though an area of relatively short distance for water transport (compared to stem, root transport length) (Sack and Holbrook 2006). Here we link back to the previously mentioned tradeoff of the high structural investments of xylem tissue vs. maximizing photosynthesizing tissues which themselves rely on the vascular network for irrigation, nutrient and sugar transport. The evolution of xylem tissue was transformative as it offered a more efficient pathways for water movement over the transmembrane route through living cells allowing for larger maximum plant sizes. Another transformative event was the evolution of xylem vessels which allowed vasculature to branch and taper and made way for a myriad of sustainable leaf shapes (Boyce et al. 2009) for maximizing photosynthesis in the lamina.

When considering the constraints to photosynthetic rate, hydraulic conductance is one of the first places to look because the ability of the hydraulic system to sufficiently irrigate photosynthesizing cells and conduct water within the conduits is critical for the stomata to remain open for CO_2 uptake. Brodribb and colleagues show the relationship of highest maximum photosynthetic rates with increasing leaf vein densities, and even more convincingly that maximum photosynthetic rate is tightly linked with a high hydraulic conductance (Brodribb, Feild, and Sack 2010). The relationship between maximum photosynthetic rate and leaf hydraulic conductance is supported several times in the literature (Brodribb, Feild, and Jordan 2007; Nardini and Salleo 2000; Santiago et al. 2004; Sack and Tyree 2005)

While at the same time as needing conduits for consistent irrigation, the mesophyll cells comprising the non-vascular leaf tissue also need conduits for transport of sugars either towards them early in life when they are carbon sinks, or for export at maturity when they act as carbon sources for remote areas of active growth. Similar rules of transport seem to mostly apply to sugar flux in phloem; the larger the distance between active cells and minor veins, the more resistance there is to move sugars thus limiting flux rates. The role of phloem tissue changes throughout the transition of source to sink, as minor veins that were immature during the leaf sink phase, become functionally relevant and their dispersal pattern fundamental in the loading and export of photosynthate during the source phase (Turgeon 2006). This suggests that vein density, and in particular minor vein density, is linked to the rate of photosynthate export which could have 1) impacts on photosynthetic rate in the mesophyll source cells based on feedback mechanisms and 2) have overall impacts on plant size, fecundity, and lifespan due to growth rate and substrate availability.

Previously we discussed the association between leaf shape and leaf temperature, owing to the finding that leaves tend to conform to a standard temperature across latitudes of 21.4°C (Helliker and Richter 2008). A standard leaf temperature across 50° latitude speaks volumes to the ways both physiological and morphological adaptations have allowed plants to thrive in climates as inhospitable as northern boreal forests and also to occupy every niche of the tropical forests. Plant architecture and leaf shape, particularly the rate of gas exchange for evaporative cooling, are well-documented morphological trait adaptations that contribute to this widespread distribution (W. K. Smith 1978; Miller 1972; Gottschlich 1982; as well as the experiments on leaf temperature outlined in the first section).

That said, there are a host of other adaptations that can contribute to a plant's success in a particular environment. Some morphological traits that we readily link to specific environments include for example: the surface area and thickness differences between sun and shade leaves, needle leaves in seasonally cold dry climates, and the small leaves of desert plants(David M Gates, Alderfer, and Taylor 1968; Sprugel 1989; McClendon and McMillen 1982). However, there are exceptions in each of those climates to other leaf morphologies that achieve the same goals of maintaining leaf temperature and avoiding damage by heat or cold (Wright et al. 2017). These exceptions often have other morphological traits we have come to associate with specific climates; hirsuteness and succulence in deserts, pigmentation and chloroplast density in shade or low light environments, thick cuticle in seasonally dry climates and deserts. In addition to morphological traits there are also physiological adaptations that allow plants to inhabit different climates. Some examples include additional carbon fixation pathways such as crassulacean acid metabolism (CAM) for dry environments, modifications in sensitivity to stress hormones such as abscisic acid, and adjustments to developmental programs in order to alter phenology to ‘bypass’ extremes.

Gaining a more complete understanding of how plants inherently acclimate and over time adapt to new environments can be approached by merging the science of two fields: 1) the paleo botanical approach of identifying morphological traits from fossils for climate reconstruction, and 2) the physiological approach for testing mechanistic hypothesis suggested from correlations. This merged approach would not only help to piece together what has happened in the past but also give insight into what we can expect in the future. Ecosystems provide health, wellbeing, structure, and balance that we depend on to an almost unmeasurable degree. Predicting their fate and deterioration in the face of climate change could help diagnose conditions and inform conservation efforts. As well as bridging these academic fields for a direct benefit to preserving natural resources and richness, the function of leaf traits in the environment could also have profound effects on crop breeding and agriculture.

As molecular tools including CRISPR-Cas are refined to be excellent gene editors capable of precisely targeting and swapping favorable genes into crops for higher yield or stress tolerance, the fact remains that the basis for gene editing comes from large scale screenings that cannot provide an understanding of how traits work together, or how physiological mechanisms layer to result in improved performance or stress tolerance. A physiological approach to trait selection for breeding offers advantages including a better annotated genetic map for understanding plant traits and using field-based evidence as markers of success (Reynolds and Langridge 2016). The potential for technology and information available today to advance crop development depends on closing the knowledge gap between physiologists and breeders, and between physiology and ecology. A call to bring advancements in physiology research into agricultural breeding has been made repeatedly (Shunmugam et al. 2018; Jackson et al. 1996) and physiological approaches are likely a beneficial tool to enhance the quickly amassing genomic data.

In many ways it is an exciting time for plant research. The technology available to researchers allows for making rapid advancements at scales ranging from intracellular signaling to global satellite imaging. What is missing is more intersection of these scales at pivotal places to get broader views and wholistic understanding to things as complex as a leaf developing under water stress in a rapidly changing climate. Using data gathered from the past to predict the future is not new. Paleobotanical information and ecologically based meta-analyses are providing highly suggestive theories about how plants adapt to stressful environments. However, assumptions about function of leaf traits for example, have generally not been tested. The utility of understanding the functional contribution of traits, based in mechanistic experimentation, promises to inform future choices in conservation efforts, predict future ecosystem health, and provide functional bases for selective breeding of new crops.

Chapter 3. EFFECTS OF WATER DEFICIT ON LEAF FUNCTIOING IN TOMATO

3.1 ABSTRACT

The correlation between leaf margin-shape and latitude has generated several hypotheses in the paleobotanical literature ranging from herbivory avoidance to increased water use and increased photosynthetic capacity. The leaf is the primary platform for photosynthesis, and so the shape of this structure should have a direct impact on its functioning. Leaf shape is largely determined by venation patterning, hardwired in many species, due to biomechanical limitations determining structure and physiological limitations determining resource movement. A subset of wild and domesticated tomato leaflets with a range of margin shapes was used to test the hypothesis that leaf margin shapes have different physiological functions. Our experiments show that both wild and domesticated plants, toothed edges had higher carbon assimilation rates and when subjected to water deficit this pattern became stronger. The largest decrease in leaflet-edge water potential between well-watered and water-deficit plants occurred in the toothed-edged leaves suggesting margin-shape, and not domestication-type, underlies this pattern.

Keywords: *Lycopersicum*, photosynthesis, leaf shape, water use, drought

3.2 INTRODUCTION

Correlations between leaf shape and climate metrics have caught the attention of scientists for well over a century (Bailey and Sinnott 1916; Givnish 1979; Nicotra et al. 2011; Royer et al. 2012). Some correlations are so strong, in fact, that they are indices for reconstructing deep-time climate records from leaf fossils. Specifically, leaf margin shape is a trait often used as a temperature proxy, though it can also be a useful metric to show resource availability such as precipitation or groundwater supply (Wolfe 1971; Bailey and Sinnott 1915; Sack et al. 2012; Wilf 1997). Thus, leaves with teeth are most abundant in temperate climates, which incidentally are areas with plentiful growing-season precipitation, while entire leaves are known to occupy the consistently warm regions including the wet tropics and also deserts. Because the fossil record reflects the dispersal of plants that have evolved as their climates shifted, understanding the importance of leaf shape to environmental adaptation could yield insights into future species ranges as climate changes, as well as provide valuable leads for agricultural breeding.

The leaf is the primary platform for photosynthesis, and so the shape of this structure should have a direct impact on its functionality. Leaf and leaflet development and the subsequent shapes they could produce are well described in the literature (N. Dengler and Kang 2001; Tsukaya 2005; Chitwood et al. 2013) and are strongly tied to the development of the initiating leaf veins. The leaf lamina fills in as photosynthetically active bridges between and around the major conduits, resulting in the shapes and margin patterns we recognize. In a mature leaf or leaflet, the venation patterning is critical for supporting these photosynthesizing tissues, and thus their arrangement and proportions have different maximum photosynthetic outputs (Sack and Scoffoni 2013; Scoffoni et al. 2011). Leaf shape is therefore intimately tied to venation patterning, as a theoretical limit exists for how much lamina could be supported, both mechanically and physiologically, by vein tissue(Niklas, Cobb, and Spatz 2009). The difference between toothed and entire margins depends on whether the veins branching from the leaflet midrib are free ending at the margin resulting in a tooth, or loop inward and reconnect to existing vasculature, which results in an entire margin (Hickey 1973). These contrasting margin-shapes present the question: What is the functional significance of toothed margins?

Significantly tied to venation patterning is bulk flow of water movement across the leaf. Water is essential for plant growth and functioning in a multitude of ways including the maintenance of turgor pressure for leaf expansion, conduction of the transpiration stream allowing for water and carbon dioxide flux, for solute uptake at the root, and metabolic processes such as photosynthesis. To take in CO_2 from the atmosphere the plant engages in a tradeoff of water molecules lost from the leaf pore (stoma). Just as there are resistances for CO_2 entering and being consumed within the leaf (leaf boundary layer, stomatal opening, concentration gradients of CO_2 in the inner-leaf air space, cell wall and plasma membrane, and the chloroplast membrane), a similar though reverse pathway exists for water exiting the leaf. Water exits the xylem through pores and moves through the leaf tissues either via the cell wall matrix or within the photosynthetic cells on its path to the inner-leaf airspace where it evaporates and exits the leaf through stomata, and enters the atmosphere beyond the boundary layer. A system experiencing prolonged water deficit disrupts water and CO_2 flux within leaves, affecting species' ability to thrive and having disruptive and costly impacts on agriculture.

Of the hypotheses explaining leaf shape mentioned above, only a handful of studies have experimentally tested, and fewer still have articulated an underlying purpose for the presence of leaf teeth. One reason for limited research may be the broad scope of the question; those who recognize a global leaf margin pattern may not be seeking to address the physiological basis for the leaf shape, and vice versa.

Results presented here aim to build an understanding of the physiological contributions of leaf margin shape. This research is addressed through an agriculturally important genus, *Solanum*, as tomato is a model species and water-intensive crop that is commercially grown in arid regions with high economic value (Guan, Biswas, and Wu 2017). They therefore represent a useful tool in understanding how the leaflet margin contributes to overall leaf function, how its behavior responds when exposed to water deficit, and are a potential benefactor of the implications from this study. Using a subset of tomatoes that have toothed and entire leaflets from both wild and domesticated lines with several precisely articulated genetic differences (Eshed and Zamir 1995; Chitwood et al. 2013), the margin-shape function can be illuminated. Just as Royer and Wilf (2006) observed previously in woody species, here we hypothesized that the toothed leaf area would have higher photosynthetic rates per area owing to the vascular arrangement of the toothed region. To assess this, we compared gas exchange rates on discreet areas of each leaflet, and compared the magnitude of these differences among leaf margin types and between domestication backgrounds. We then hypothesized that due to the inherent differences in vein patterns between toothed and entire margins, a water deficit would impact these two leaflet shapes disproportionally based on their ability to conserve water status and maintain functioning. Because toothed margins correspond to temperate areas of ample water availability, we predicted the redundancy of looping veins in entire leaves would better conserve water under deficit. To address this, we tested water potential in edge lamina and middle lamina for each leaflet. Our findings instead suggest that toothed leaves have enhanced productivity in the leaf margin, particularly and unexpectedly, under water deficit in the wild lines tested and a similar pattern was found in the domesticated lines.

3.3 MATERIALS & METHODS

3.3.1 Plant Tissue

Four tomato leaflet shapes were selected for this study based on shape, evolutionary and breeding history, and previous data available on the species. *Solanum habrochaites* and *Solanum pimpinellifolium* are from wild populations native to South America while *Solanum lycopersicum* c.v. m82 and *Solanum lycopersicum x Solanum pennellii* IL 4_3 are domesticated and developed lines used widely for research. Contrasting leaf shape pairs were constructed by selecting one toothed and one entire from among wild and domesticated lines (toothed, entire), see figure 3.1. Seeds of *Solanum lycopersicum x Solanum pennellii* IL 4_3 were obtained from Dan Chitwood (Michigan State University), those and all others are available from the Tomato Genetics Resource Center (University of California, Davis).

				
Genotype	Solanum habrochaites	Solanum pimpinellifolium	Solanum lycopersicum c.v. M82	Solanum lycopersicum c.v. M82 x S. pennellii IL4_3
Domestication-type	WILD	WILD	DOMESTICATED	DOMESTICATED
Margin-type	toothed	entire	toothed	entire
In-text name	Wt	We	Dt	De

Figure 3.1. Leaf shapes selected for this investigation include toothed and entire margins that are from both domesticated and wild backgrounds. Throughout this study shapes will be referred to as their in-text name for ease of comparison.

3.3.2 Growth Conditions

Greenhouse: Plants used for gas exchange measurements were cultivated under greenhouse conditions at the University of Washington Biology Department and the Center for Urban Horticulture greenhouses in Seattle, Washington. During experiments the temperature fluctuated between 16°C (night) to 28°C (day) and light intensity from 0 (night) to 1,200 (midday) µmol m^{-2} s^{-1}. Seeds were sterilized in 50% bleach for two minutes and germinated on a heat mat (Pro Gro, Brookfield, Wisconsin) for up to 10 days until primary cotyledons had expanded. Seedlings were transferred to 6” square pots filled with Sunshine Mix #4 (SunGro Horticulture, Agawam, MA USA) and watered to capacity daily. General Purpose 20-10-20, Miller Iron Chelate D 10% Fe, Cal-Mag 17-5-17 and Magnesium Sulphate Heptahydrate (Plant Marvel Nutriculture, Chicago Heights, IL, USA; Irrigation Mart, Ruston, LA, USA; Canadian Agri Products, Plano, TX, USA) were applied weekly. During experiments the temperature alternated? between 16°C (night) to 28°C (day) and light intensity from 0 (night) to 1,200 (midday) µmol m^{-2} s^{-1}. After 21 days, a uniform subset of plants was selected and randomly assigned to treatment groups of well-watered (WW) or water-deficit (WD) as described in Gilbert et al 2011 and briefly summarized here. Pots with soil were submerged in water and drip-dried for two hours before being weighed to find the maximum soil water content (SWC). Pots were weighed every day after sunset, then watered up to 85% of SWC for well-watered (WW) control and 45% SWC for the water deficit (WD) treatment. Treatments were maintained for three weeks in a random block design with water treatment levels and plant type.

Growth Chamber: Leaflets for leaflet water potential and solute concentration measurements were germinated and grown in a walk-in growth room at 25°C with 16h light/8h dark, with light supplied at 525 µmol m^{-2} s^{-1} via T5 compact fluorescent lamps. Pots were watered daily to capacity with the same fertilizer balance and schedule as described above.

3.3.3 Leaf Gas Exchange Measurements

Measurements took place in locations above between May and November of successive years 2014-2017. Gas exchange was measured with a LI-6400 photosynthesis system (LI-COR, Lincoln, Nebraska) set at saturating conditions of 1000 µmol m^{-2} s^{-1}, 25°C, 425ppm of CO_2, RH 35-70%, and flow rate 350 µmol s^{-1}. Light level was chosen to ensure saturation of photosystems without imposing irradiance stress based on light curves taken pre-experiment (data not shown). Following methods described first by Royer and Wilf (2006), a portion of leaflet including the lamina from the edge to the midrib was enclosed in the IRGA chamber, and allowed to reach steady state before logging. Next, approximately 2 mm of leaf margin was sealed with petroleum jelly (Unilever, London, UK) on the top and bottom of the leaf and the same area was reinserted into the IRGA chamber for an additional measurement (Figure 3.2). The leaf was then removed and scanned for area with ImageJ (NIH, Bethesda, Maryland) which was then used to calculate the area and assimilation rate of the leaf margin.

Figure 3.2. Example of leaf area sections for gas exchange measurements. Heavy lines represent inner perimeter of the 2x3cm chamber. Dotted line represents the grease-sealed portion, referred to as ‘edge’.

3.3.4 Water Potential and Osmolarity

To assess drought treatments in the greenhouse, whole-leaf water potential was measured midday using a Scholander Pressure Chamber (Model 1000, PMS Instruments, Co., Corvallis, OR, USA). Within-leaf water potential was measured using an SC-10A Thermocouple Psychrometer (Meter Environment, Pullman, Washington). To simulate a whole-plant water deficit treatment on individual leaves, leaflets were excised and placed under high light (>500µmol m^{-2} s^{-1}) with a fan for 10 minutes before being cut into strips of either leaf edge or leaf middle for sampling. These leaf pieces were then frozen on dry ice, thawed, and pressed to release sap. 20µl of extracted sap was loaded into a freezing point osmometer (Advanced Instruments Inc, MicroOsmometer model 3300, Norwood, Massachusetts) to measure solute concentration (mOsm/kg).

3.3.5 Anatomical

Stomatal density was measured on the abaxial surface of leaflet margins or middle leaflet areas using a surface imprint produced by a layer of clear nail polish and a light microscope (Nikon, Japan). Images were captured with a Motic camera (Motic Instruments, Richmond, British Columbia, Canada). Density was recorded at 200x and averaged from three fields of view per leaf region (margin or middle) on a minimum of 12 individual leaves per species.

Vein scoring was performed on leaves that were chemically cleared with NaOH solution, stained with Safranin, and scanned at 1,200 pixels per inch according to Scoffoni et al 2011. Total veins branching from the leaflet midrib were counted, and veins were scored as either free ending or looped depending on trajectory. A score was calculated as the number of free-ending veins over the total number of branched veins.

3.3.6 Statistical Analysis

Gas exchange measurements were analyzed using a linear regression model with fixed effect variables as shape, domestication type, water treatment and round which referred to the replication of the experiment. Round was treated as a fixed block effect to account for any variation that may have occurred over months of replication of the experiments and data for each leaf type includes at minimum three separate rounds of experiments (described above, May-November of several years). Contrast analysis was used to define significant differences among variables. Other data represent the means of leaflets measured with standard error of the mean.

3.4 RESULTS

3.4.1 Gas exchange and stomatal density

The four leaf-margin shapes chosen for this study were from wild (W) and domesticated (D) lines, grouped as toothed (Wt, Dt) or entire (We, De) with major vein patterns of either free ending or looping, and perimeter to area ratios of greater than or less than 1.5 as summarized in Table 3.1. Carbon assimilation rates in the edge lamina (margin) was higher than in the middle lamina (Figure 3.3). This edge-to-middle difference in carbon assimilation was significantly greater in wild toothed (Wt) species than in wild entire (We) species (Figure 3.3A, edge: middle Wt 288%, We 129% $p<0.0001$). This pattern was also observed in the domesticated lines (Figure 3A, edge: middle Dt 205%, De 112% $p=0.0553$). Stomatal conductance was always greater in edge lamina than in the middle lamina regardless of margin-type (Figure 3.3B). Stomatal density did not vary significantly between leaflet-edge and leaflet-middle regardless of the margin shape and domestication (Table 3.1).

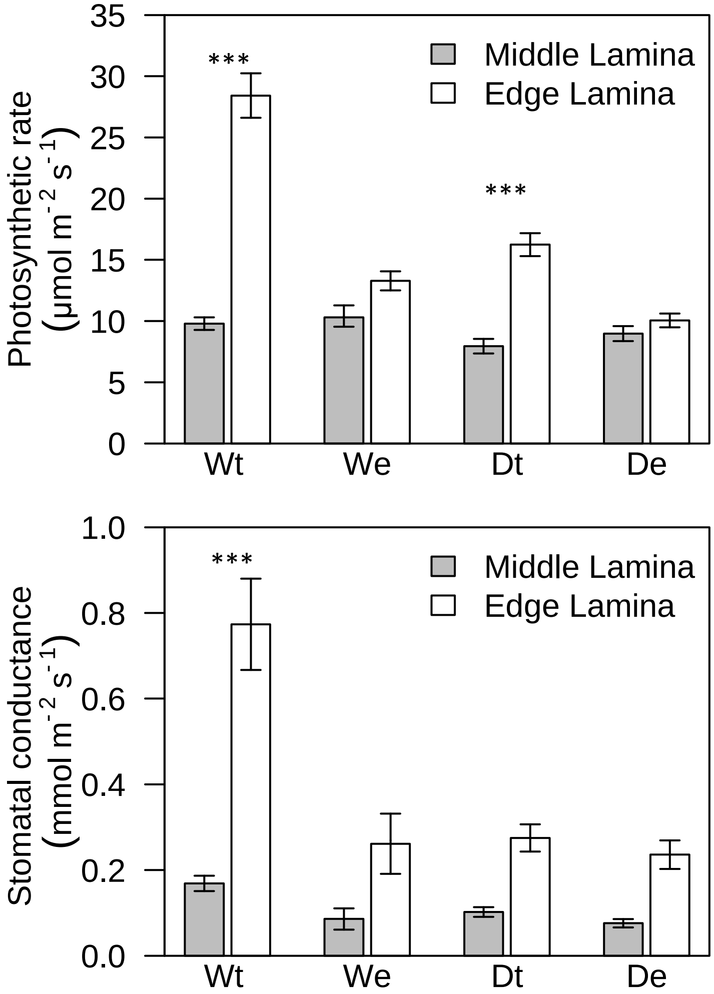


Figure 3.3. A) Photosynthetic rates of well-watered middle lamina (grey bars) and edge lamina (white bars) for each leaflet type. *** denotes $p<0.001$ B) Conductance rates of the same lamina designations as in A (Dt $p=0.0554$).

All well-watered (WW) treatments achieved higher carbon assimilation rates than their corresponding water deficit (WD) treatment (Figure 3.4) the level of water withholding applied was successful in inducing a physiological stress across all species. In the WD treatment there were increased differences in carbon assimilation rates for both domesticated and wild margin-type pairs. For both Wt and Dt the decrease in carbon assimilation rate caused by water deficit was less than in We and De (A 59% and 51% reduction for We and De, 45% and 44% respectively for Wt and Dt. data in Figure 3.4).

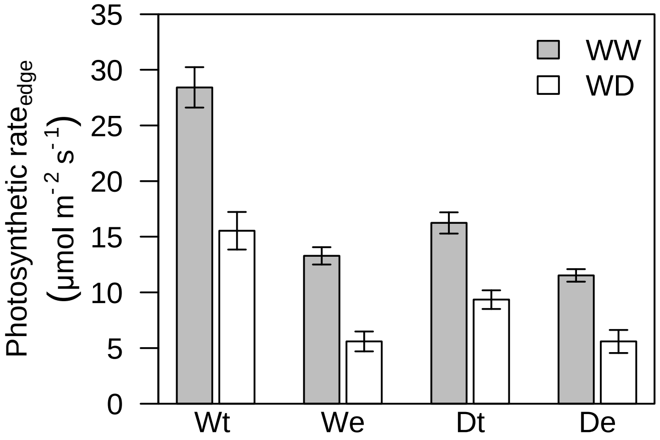


Figure 3.4. Photosynthetic rate of edge lamina in well-watered treatment (WW, dark bars) and water deficit treatment (WD, white bars). *** indicate $p<0.001$ while Dt $p=0.0553$, and all other comparisons $p>0.1$.

3.4.2 Leaf-level water potential and osmolarity

Leaf water potential decreased from the midrib to the edge in every leaf sample measured (Supplemental Figure 3.1). This result was expected, as water flux through the xylem is driven via a pressure gradient (in xylem) and water potential gradient (outside xylem, between photosynthesizing cells and tissues). The magnitude of the gradient from leaf middle to leaf edge was larger according to leaf margin shape and the treatment (Figure 3.5). Water deficient leaves of any margin shape amplified the existing gradient between leaf middle and the leaf edge, though the toothed leaves consistently showed a more pronounced gradient than entire leaves when compared within domestication-type.

For the wild species, solute concentration at the leaf edge as compared to the leaf middle was only significantly different in the water deficit treatment (Table 3.1).

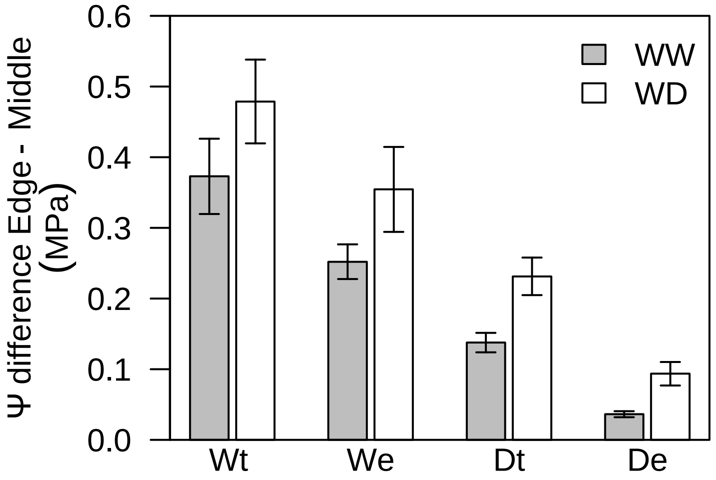


Figure 3.5. Mean differences in water potential between edge lamina and middle lamina for well-watered (WW, dark bars) leaves and desiccated (WD, light bars) leaves. Error bars represent standard error of the mean.

Table 3.1. Characteristics and measurements according to leaf type on vein patterning and margin-type, solute concentration, and stomatal count. Numbers represent the mean with stand error.

	proportion of free		solute concentration mosm/kg				stomatal density mm^2	
plant type	ending veins	Perimeter : Area	WW edge	WW middle	WS edge	WS middle	edge	middle
Dt	0.804	1.85 ± 0.3	--	--	--	--	80 ± 3.6	75 ± 4.5
De	0.292	1.17 ± 0.2	--	--	--	--	68 ± 7.7	70 ± 4.8
Wt	0.867	1.69 ± 0.3	354.4 ± 10.6	348.5 ± 7.9	463.3 ± 9.4	390.0 ± 12.6	71 ± 5.5	85 ± 5.5
We	0.015	1.37 ± 0.1	308.3 ± 12.6	287.3 ± 6.7	381.1 ± 13.1	375.3 ± 16.6	70 ± 6.3	67 ± 6.0

3.5 DISCUSSION

We sought to answer a question raised in the literature on the photosynthetic activity of tooth margins (Royer and Wilf 2006; Peppe et al. 2011; Baker-Brosh and Peet 1997), and whether leaf-margin shape impacted photosynthetic activity differently. We found that carbon uptake rate by edge lamina is disproportionally higher in toothed leaflets than in entire leaflets (Figure 3.3A), regardless of whether the leaflet is wild or domesticated. This pattern holds true when comparing edge lamina to the whole leaf lamina (as opposed to the middle lamina specifically), only to a lesser extent (supplemental Figure 3.2A). However, when considering the stomatal conductance of the same leaf parts there is a consistent pattern across all the sampled leaflets that edge lamina has higher conductance than middle lamina (Figure 3.3B) and also higher than the whole lamina (Supplemental Figure 3.2B). The 'boost' in photosynthesis observed in the leaflet teeth cannot be attributed to increased stomatal conductance alone since increased edge compared to middle conductance was observed in all leaflets, regardless of margin-type. The boost can also not be attributed to stomatal density differences, as the lack of variation in stomatal distribution (Table 3.1) suggests strong developmental control within the entire Solanum genus.

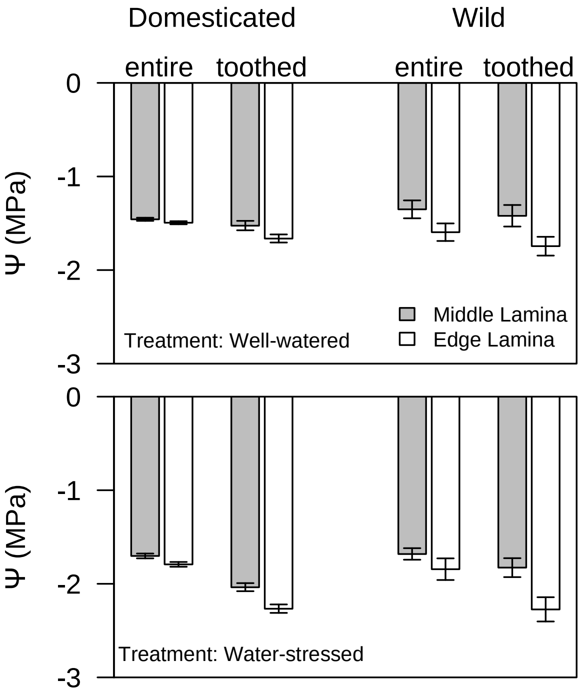
The venation patterns that differ notably between the two leaf-margin types could instead be responsible for the photosynthetic difference. Free ending veins running from midrib to leaf margin are features of toothed leaflets (Table 3.1). This direct piping through the tooth is associated with fewer photosynthetically capable mesophyll cells in this region thereby shifting the ratio of photosynthetically active cells relative to non-photosynthesizing transport cells. This might allow water to be supplied to these cells more directly via reduced dispersal distance, which would ensure continual hydration. Export of photosynthate is possibly enhanced in this region for the same reason, and this would maintain the driving force to power these gradients and prevent feedback inhibition thus resulting in the higher measured rates of CO_2 assimilation. Additionally of importance are the tooth-induced disruptions to the leaf boundary layer, and the evidence for increased transpiration and gas diffusion due to decreased boundary layer resistance (Schuepp 1993; Gottschlich 1982).

While the work presented here on leaf teeth productivity is consistent with previous studies (Peppe et al. 2011; Royer and Wilf 2006), it is important to note that the study organism is an herb and not a perennial woody tree-leaf as the previous work investigated. One reason for the agreement may be that the greatest benefit of leaf teeth was in the early growing stages of the leaf, when the leaf size was comparable to the perennial tomato leaf (Royer et al. 2012). At this stage, the toothed region represents a substantial portion of leaf area and the contribution of increased photosynthetic rate accumulated across this area is considerable. It is critical to point out that these data contradict the observed pattern that toothed leaves of woody species have not been found to thrive in water-limiting habitats from modern day correlations of margin-type and precipitation (Nicotra et al. 2011). In this study on tomato leaves, the toothed leaflets fared better compared to other shapes under water deficit conditions (Figure 3.4, Figure 3.5). This could be due to different water use efficiencies between the plant types (woody, herbaceous), and the different ways hydraulic resistance is controlled outside of the xylem elements, for example the bundle sheath structure or the number of aquaporins (Scoffoni et al. 2017). Due to the shorter stature and transport distance of herbaceous plants, overnight rehydration due to minimal stomatal conductance and positive root pressures could be enough to achieve a water potential close to zero in the leaves thereby rehydrating and repairing drought-induced embolism (Nolf et al. 2016; Tyree and Sperry 1989).

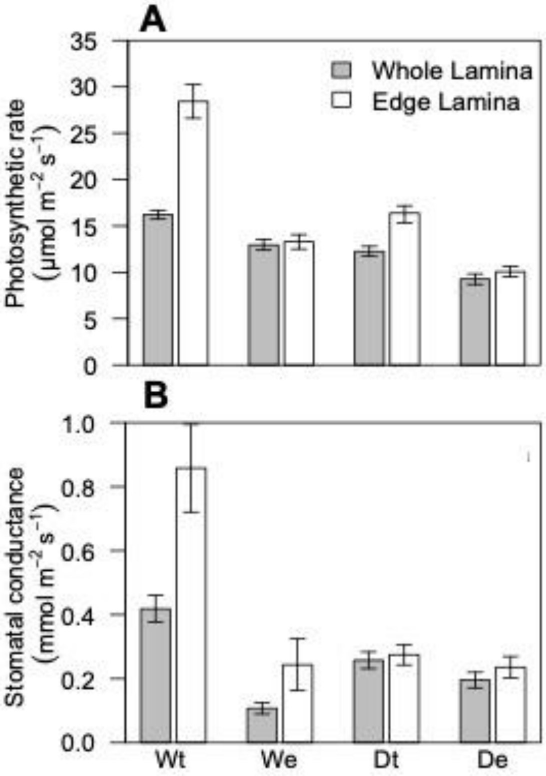
To further explore the question of leaf edge productivity, we next sought to address whether the edge photosynthesis, toothed compared to entire, is differentially impacted during water deficit. Previous work on this question, addressed in woody species, suggests that the 'boost' to photosynthesis provided by leaf teeth comes at a large cost due to water lost from high conductance and increased transpiration. This cost was assumed to be tolerable only in temperate climates early in the season when water is abundant due to heavy precipitation or high groundwater supplied during spring melt (Royer and Wilf 2006; Royer et al. 2009). The implication from those studies is that toothed leaves are poorly adapted to drier conditions, including tropical habitats. In contrast, toothed edges of tomato leaves in this study experienced less of a reduction in photosynthetic rate under water deficit as compared to entire edges (Figure 3.4). This result is inconsistent with previous suggestions that development of leaf teeth is constrained by the need for ample water to support transpiration and hydathode release at the tooth (Royer and Wilf 2006; Royer et al. 2009). A possible explanation for the difference may lie between woody and herbaceous species and the degree of water deficit. The water deficit treatment imposed on tomato plants in this study may have been mild enough to meet the evaporative demand of the lamina, maintaining leaf turgor. However, the treatment was sufficient to reduce stomatal conductance and increase the water potential gradient across the leaf (Figure 3.3B). Alternatively, the toothed tomato leaf is able to maintain high rates of photosynthesis during water deficit because the edge lamina (toothed region) was delivering such a substantial boost in photosynthetic rate that a reduction in rate here did not significantly lower the rate for the entire leaf.

Working in a well-defined subset of tomato species allows for comparisons to be drawn which delineate effects of domestication or leaflet margin shape. By selecting wild species and domesticated subspecies in pairs that represent a toothed and entire leaflet margin-type, patterns between domestication and wild as well as between margin-shapes can be observed. It is important to consider that the domesticated tomatoes Dt and De are close relatives (Li et al. 2018), and the dampening of the shape patterns observed in leaflet-edge photosynthesis and leaflet edge-middle gradient in water potential could be partially explained by this factor. Although wild species achieve higher rates of photosynthesis overall, the effect of the leaflet margin-type outweighs domestication in terms of relative leaflet edge photosynthesis in well-watered and in water deficit conditions. Furthermore, the decrease in water potential along toothed margins does not limit water-limited photosynthetic capacities the way one might expect; low water potential might have been linked to reduced mesophyll turgor, inducing stomatal closure and limiting gas exchange. Instead, what we observed here was that water deficit increased the water potential gradient (edge to middle) within the toothed leaflets more than in the entire leaflets, thus maintaining water flux, stomatal conductance and assimilation.

This indicates that leaf shape plays a significant role in drought response and since this response is most notable in the wild tomato it suggests it may be an inherent trait. Our results highlight the importance of this feature, and its potential value in plant breeding as irrigation continues to be a costly and necessary piece of traditional agriculture. Looking forward, understanding leaf adaptive traits such as leaf and leaflet shape can help explain ecological community composition, and potential vulnerabilities therein, as climate continues to change. Likewise, this approach has implications for global agriculture and food economics as it presents a different selection basis for crop breeding.



Supplemental Figure 3.1. Water potential in sections of lamina from the middle (dark bars) or edge (white bars) in different water treatments. The edge to middle differences are plotted from this data in figure 5.



Supplemental Figure 3.2. A) Photosynthetic rates of well-watered whole leaf lamina (grey bars) and edge lamina (white bars) for each leaflet type. B) Same leaf areas compared for stomatal conductance.

Chapter 4. LEAF HYDRAULICS AND THE COORDINATED RESPONSE TO GAS EXCHANGE IN TOMATO

4.1 ABSTRACT

Leaf vein architecture lays the foundation for the leaf form, and results in a diversity of quantifiably different leaf shapes governed by differences in venation patterning. Leaf veins are important for transporting water upwards through the plant to the sites of evaporation and also to move photosynthetic products from their origins in mesophyll tissue to carbon sinks elsewhere in the plant. Leaf hydraulic conductance summarizes the resistances along this pathway and is closely linked with stomatal conductance. When paired with structural vein traits, it can give insight into plant adaptations to abiotic stresses. Working with a previously established set of toothed and entire tomato leaves comprising wild species and domesticated lines, we used the evaporative flux method to determine the hydraulic vulnerability of the leaves. We paired this with gas exchange data over a range of leaf water potential and also structural vein traits to test whether the species and lines had a coordinated response under water stress and if vein anatomy could explain any species level differences that emerged.

4.2 INTRODUCTION

Plant productivity is largely determined by the rate and balance of physiological processes, often summarized by reporting the photosynthetic rate. Photosynthetic rate is largely determined by rates of stomatal conductance. Further, the intake of atmospheric carbon dioxide by stomata is also largely constrained by the hydraulic transport system that supplies water to the whole leaf including to the sites of evaporation, as this system provides feedback on leaf water status to the stomatal pore thus regulating its aperture (Sack et al. 2013; Scoffoni et al. 2017). Importantly, for CO_2 exchange to occur through stomata, water is lost as vapor into the surrounding atmosphere. Such water must be replaced by an efficient hydraulic transport system or the leaf would desiccate or close its stomata and limit photosynthetic gains. Leaf hydraulic conductance (K_{leaf}) quantifies the transport capacity of this hydraulic system underlying key aspects of leaf function, notably photosynthesis (Sack and Holbrook 2006; Brodribb and Feild 2010; Feild and Wilsony 2012). Parsing the linkages of leaf hydraulics and stomatal and photosynthetic capacity in novel systems such as herbaceous tomatoes will provide insight into the key physiological processes underlying their productivity, thereby providing implications for the extension of hydraulics into agricultural species.

Water flow through plants is characterized by a series of resistances along a pressure gradient, and in leaves flows through pathways both within the vein xylem, and outside through parenchymatous tissues (Sack and Holbrook 2006). Water moves through the xylem driven by the tension from root to leaf at the sites of evaporation, as mediated by the cohesion of water molecules to each other and to the cellulose microfibrils of the xylem cell walls. In times of high evaporative demand, the xylem vessels in the plant are subject to increasingly low pressures, which can cause air pockets to form in the xylem fluid, an event called cavitation. Cavitation impacts water transport through the xylem vessels by breaking the water column and preventing the continuous pull of water through the plant from the roots to the sites of evaporation. The severity of cavitation is based both on the vessel diameters and the number of bundled vessels per vein (cite) as well as the plant's ability to refill air pockets with liquid water (Salleo et al. 2001). Cavitation is most likely to occur in larger conduits that are present in veins with large diameters (leaf major veins, typically those classified as the first, second or third branching order) (Sack and Scoffoni 2013). By contrast, given their larger size, such conduits also have the greatest capacity for flow which scales to influence maximum K_{leaf} (Sack and Scoffoni 2013). Yet, given their importance in influencing K_{leaf} the evolution of greater vein density (vein length per leaf area) of such large vein orders may effectively counteract the impacts of being highly prone to cavitation by providing redundant pathways for maintained flow (Scoffoni et al. 2011). Leaves of eudicotyledonous species have a conserved hierarchical vein architecture that defines the major vein and minor vein arrangement and can be quantitatively characterized through traits including the vein density (vein length per unit leaf area, also VLA), vein diameter, and vein surface area (the vein diameter x vein density x pi). Vein architecture determines the hydraulic pathway and has a significant impact on overall leaf productivity (Sack et al. 2018; Brodribb, Feild, and Jordan 2007). Hydraulic vulnerability is a measure of stress robustness to decreasing leaf water potential, as it represents the resistance of the system to cavitation along the K_x

pathway and/or resistance of the cells outside the xylem (K_{ox}) to dehydration. Indeed, recent studies have highlighted the importance of outside-xylem pathways in determining hydraulic vulnerability under low to moderate dehydration as a mechanism for preventing downstream cavitation within the xylem (Scoffoni et al. outside xylem study). Testing the hydraulic vulnerability can therefore help to describe the system's robustness to water stress, and together with measurements of gas exchange and vein traits can determine the possible limits to the productivity of the leaf system.

Water travels along several routes throughout the leaf either via the xylem (K_x = hydraulic conductance through the xylem), across the bundle sheath and mesophyll cells (K_{ox} = hydraulic conductance across cells outside of the xylem), and finally to the sites of evaporation before diffusing out of the stomatal pore (Caringella, Bongers, and Sack 2015). Stomatal conductance is an important metric of plant productivity, particularly when plants experience water deficit, as the carbon-water tradeoff becomes even more costly.

Leaf hydraulic conductance is a relatively new field of physiological research, and there are limited studies using established methodologies on all plant types, with woody plants being most represented so far (Sack and Tyree 2005). Herbaceous species dominate several ecosystems and as crops cover significant acres of farmland while to date only a handful of studies include data on herbs (Nolf et al. 2016; Sack and Scoffoni 2013). Extending our knowledge on herbs helps to understand not only a different cellular physiology paradigm, but also adds to our understanding of water flux from earth to the atmosphere in these habitats.

Irrigated agriculture is the single largest consumer of global fresh water. Using a previously established subset of wild tomato species (*Solanum pimpinellifolium* and *S. habrochaites*) and inbred lines (*S. lycopersicum* cv M82 and *S. lycopersicum x Solanum pennellii* IL4_3), we tested the following hypotheses: (1) species and inbred lines would differ in K_{leaf}; (2) responses of K_{leaf} to dehydration would be species specific, and associated with species specific responses of g_s and A_{area} to dehydration; (3) if differences in K_{leaf} result from differences in leaf vein traits.

4.3 MATERIALS AND METHODS

4.3.1 Plant material

Four tomato species were selected for this study and grown from seed in a common garden to maximize genetic differences and reduce environmentally-induced plasticity. Seeds of the wild species *Solanum habrochaites (S. habrochaites)* and *Solanum pimpinellifolium (S. pimpinellifolium),* and of the domesticated cultivar *Solanum lycopersicum* c.v. M82 (*S. lycopersicum* c.v. M82) were received from the Tomato Genetics Resource Center (University of California Davis), whereas the seeds of the fourth species and other domesticated cultivar *Solanum lycopersicum* c.v. IL4_3 (*S. lycopersicum* IL4_3*)* were personally received from Dr. Dan Chitwood (Michigan State University). For a given species, three seeds were sown in 2.5 L circular pots (16 cm diameter x 17 cm deep), and following the development of the first set of true leaves, were each transplanted into separate 18.9 L circular pots (17 cm diameter x 30 cm deep) with potting soil (1:1:2:1:1 mixture of washed plaster sand, loam, peat moss, and vermiculite)

Plants were grown at the UCLA Plant Growth Center (minimum, mean and maximum daily values for temperature: 18.8, 22.3, 29.9 $_{o}$C) from April – July 2018. Plants were arranged in twelve randomized blocks spread over two benches, with one individual per species per block (n = 4 individuals per block) and 6 blocks per bench. Plants were irrigated daily with water containing fertilizer (200-250 ppm of 20:20:20 Np:P:K; Scotts Peters Professional water soluble fertilizer; Everris International B.C., Geldermalsen, The Netherlands). After approximately 8 weeks, when plants had > 20 leaves, mature and healthy leaves were chosen for gas-exchange, hydraulic, morphological and anatomical measurements.

4.3.2 Quantification of Kleaf

We measured the leaf hydraulic conductance (K_{leaf}) between July 10 and July 21st 2018 using the steady-state evaporative flux method (EFM, Scoffoni et al. 2011; Sack and Scoffoni 2012). Measurements were made for approximately one primary leaflet (as opposed to the terminal leaflet) per compound leaf, including up to six compound leaves per plant, from six distinct individuals, which resulted in approximately 30 leaves per species. The day preceding measurement of leaf hydraulic conductance (K_{leaf}), plants were transported in pots to the laboratory, watered and enclosed in plastic bags filled with wet paper towels to ensure that plants were fully hydrated. Both hydrated and dehydrated leaves were used to produce vulnerability curves for a range of leaf water potential (Ψ_{leaf}). We dehydrated leaves by allowing plants to naturally dehydrate in pots. Following this dehydration, plants were covered with bags that had been previously exhaled into, and covered with another bag that was filled with wet paper towels for 30 minutes. This facilitates equilibration of the leaf water status by reducing stomatal opening via higher vapor and CO_2 concentration.

The primary leaflet opposite the leaflet used for EFM was used to measure the initial Ψ_{leaf} using a pressure chamber (Plant Moisture Stress model 1000; PMS Instrument). The remaining leaflet was then cut with a razor blade under ultra-pure water at its petiolule, which was then gently wrapped in Parafilm, connected to tubing with a compression fitting that was connected to a water source on a balance (+- 10ug; models XS205 and AB265; Mettler Toledo) that quantified the flow rate into the petiolule every thirty seconds to a computer. Leaflets were held adaxial surface upwards between wood frames strung with fishing line, so that the leaflet was held horizontal and immobile above a large box fan (Lakewood Engineering & Manufacturing Company, Chicago, Illinois, USA). Leaflets received > 1000 umol m^{-2} s^{-1} illumination of white light via light source (model 73828 1000 W, “UV filter”; Sears, Roebuck, Hoffman Estates, Illinois, USA) suspended directly above a pyrex glass container (Corning Incorporated, Corning, New York, USA) filled with water, and the leaf temperature was maintained between 23 and 28 ^{o}C. We allowed leaflets to transpire and stabilize under the EFM for at least 30 minutes, until the flow rate stabilized with a coefficient of variation < 5% for at least five minutes. The 30-minute period was selected to ensure leaves had sufficient time for light acclimation as previous studies have shown that increasing illumination enhances K_{leaf} several-fold for many species (Scoffoni et al. 2008). After stabilization, the average steady-state flow from the previous five minutes (i.e. with < 5% coefficient of variation) was recorded, as was the leaf temperature (Cole-Parmer). We then removed the leaflet from the system, upon which we placed it into a bag that had been previously exhaled into, and then placed into another bag with wet paper towels to equilibrate for 30 minutes. We then measured the final Ψ_{leaf}. We measured the leaf area by tracing the leaflet outline manually onto paper that was scanned and measured using ImageJ (version 1.46r; National Institutes of Health). We calculated K_{leaf} as the flow rate divided by the Ψ_{leaf} driving force (i.e. the water potential of the water delivered to the petiolule [0 MPa] minus measured Ψ_{leaf}), and normalized by leaf area. We also corrected for the dependence of water viscosity on temperature (reference value of 25 $_{o}$C; Weast 1974). Hydraulic vulnerability curves were obtained from plotting K_{leaf} as a function of the most negative Ψ_{leaf} experienced by the leaflet (initial or final).

We determined K_{leaf} in four ways. First, we estimated K_{max}, the maximum leaf hydraulic conductance, as the hydraulic conductance at Ψ_{leaf} of 0.1 MPa based on best fit curves. This is because in our curve selection analysis, the best fit curve for all species was an exponential type curve which can result in unrealistically high values at Ψ_{leaf} 0 MPa (i.e, the intercept). Second, we averaged all K_{leaf} measurements for each species (i.e. 0 – 1.2 MPa Ψ_{leaf}). Third, we averaged K_{leaf} values that occurred for leaves with Ψ_{leaf} of 0 – 0.3 MPa. Fourth, we averaged K_{leaf} values that occurred for leaves with Ψ_{leaf} of 0 – 0.5 MPa. We determined these latter two values given that the species had high vulnerability (i.e. declines in K_{leaf} with relatively little dehydration), and thus would represent more realistically the operating K_{leaf}.

4.3.3 Quantification of diurnal gs and Pmax as a function of Ψleaf

We quantified light-saturated photosynthetic rate (P_{max}) and stomatal conductance (g_s) on 5 individuals per species, selecting one primary leaflet from up to six compound leaves, resulting in approximately 30 leaves per species. Measurements were made from 9:00 to 17:00 in the UCLA plant growth center from July 23 – July 27 2018 using a portable gas-exchange system (LI-6400; LI-COR). Chamber CO_2, temperature, and light intensity (10% blue light) were set to 400 ppm, 25 $_{o}$C, and 1500 umol m_{-2} s_{-1}, respectively. Measurements were taken after allowing equilibration under chamber settings for 10 minutes. P_{max} and g_s values were logged four times at 15 second intervals and then averaged to a final measure. Leaflets were then excised with a razor blade and immediately sealed in a plastic sealable bag (Whirl-Pak; Nasco) that had previously been exhaled into. Bagged leaflets were then placed into another bag filled with wet paper towels. Following 30 minutes of equilibration under these conditions, Ψ_{leaf} was measured using a pressure chamber as previously described.

4.3.4 Quantification of leaf venation traits

We quantified leaf venation traits from one primary leaflet from each of four individuals per species. Leaves were collected in July 2018 and fixed in formalin-acetic acid solution (37% aqueous formaldehyde solution, 50% ethanol, and 13% glacial acetic acid solution). We chemically cleared leaflets with 5% NaOH dissolved in ethanol, stained with safranin, and counterstained with fast-green (Berlyn and Miksche 1976). We mounted leaflets with water between transparency film (CG5000; 3M Visual Systems Division), upon which they were scanned (flatbed scanner; Canon Scan Lide 90; 1200 pixels per inch). From these scans we measured the leaf area, length, width, perimeter, and the numbers and lengths of 1° and 2° veins using ImageJ (version 1.8.0 National Institutes of Health). Tertiary vein lengths were measured for three rectangles per leaf (10-300 mm^2 depending on the leaf size) located in the top, middle and bottom thirds of the leaf. Thus, the vein length per leaf area (VLA) was calculated as the length divided by the leaf area. Further, for the tertiary veins, the VLA was averaged across the three subsampled rectangles. We also measured the vein diameters of 1-3° veins, excluding the bundle sheath, by averaging six measurements (two made in veins in the top, middle and bottom thirds of the leaf).

We quantified leaf minor vein traits using images obtained via digital camera (14.2 Color Mosaic; Diagnostic Instruments) attached to a light microscope (DMRB; Leica Microsystems) at 5x objective. We imaged three rectangles (6 mm^2): one in the top, middle, and bottom thirds of the leaf. The VLA and diameter of minor veins was averaged across the rectangles. Major vein length per area was determined by summing the VLA of 1-3° veins and the minor VLA was determined by summing 4° and higher order veins. Thus, total VLA was determined by summing the major and minor VLA. Further, we estimated the total, major and minor vein surface area per leaf area (VSA) by multiplying VLA by π by vein diameter, and the vein volume per leaf area (VVA) by multiplying VLA by π by the square of half the diameter.

4.3.5 Statistics

Statistical analyses were performed in the R language and environment. Equations for the responses of K_{leaf}, g_s, and P_{max} to Ψ_{leaf} were selected using a maximum likelihood framework (Sack and Holbrook 2006; Sack et al. 2018). The function optimum was used to assess for maximum likelihood and we tested five types of functions to the curves (Sack et al. 2012), where y = K_{leaf}, g_s, or P_{max}, and x is Ψ_{leaf} : linear ($y = ax + b$), logistic ($y = a/(1+(x/x_{initial})^{b})$); two-parameter sigmoidal ($y = a/(1+e^{(-(x-x_{initial})/b)})$); exponential ($y = ae^{-bx}$); and two-parameter exponential ($y = c + ae^{-bx}$).

We tested for significant differences in leaf traits in two ways. First, we implemented one-way analysis of variance (ANOVA) to model the influence of the factor “species” on leaf traits. We then used Tukey’s honest significant difference (HSD) test on such models to assess for significant differences across the four tomato varieties. Yet, given that two of the four are *S. lycopersicum*, whereas the remaining two were distinct wild species, we tested for significant differences between the two wild (*S. pimpinellifolium, S. habrochaites*) species using t-tests. We did not test for significant differences between the two domesticated *S. lycopersium* cultivars, as the mean trait values for most traits for each were largely overlapping in standard error bars, and were clearly not different enough to be considered as distinct species.

4.4 RESULTS

4.4.1 Leaflet hydraulics

Leaflets showed variability in their hydraulic conductance as seen in both the maximum K_{leaf} (K_{max}, Figure 4.3) and in the slope of their vulnerability curves (Figure 4.1). The wild species *S. pim* and *S. hab* showed a steep decline approaching zero conductance with decreasing leaf water potential, while the domesticated lines have a less steep decline and do not approach zero over the same low water potentials sampled. A closer look comparing the extrapolated K_{max} among the pairs (wild, domesticated) revealed that the toothed leaflet of each type had a higher K_{max} than the non-toothed leaflet (Figure 4.3).

4.4.2 Gas exchange

Stomatal conductance of the wild species showed a strong decline with decreasing leaf water potential while the domesticated lines showed little variance in g_s over the same water leaf water potential (Figure 4.1). Light-saturated photosynthetic rate per leaf area reflected the same pattern as g_s. Photosynthetic rate declined steadily and steeply with water potential for the wild species, whereas the domesticated lines declined less steeply (Figure 4.1). Wild species had double the maximum g_s compared to domesticated lines, and higher maximum photosynthetic rates as well (Figure 4.1 curves, values extrapolated from curves Table 4.1).

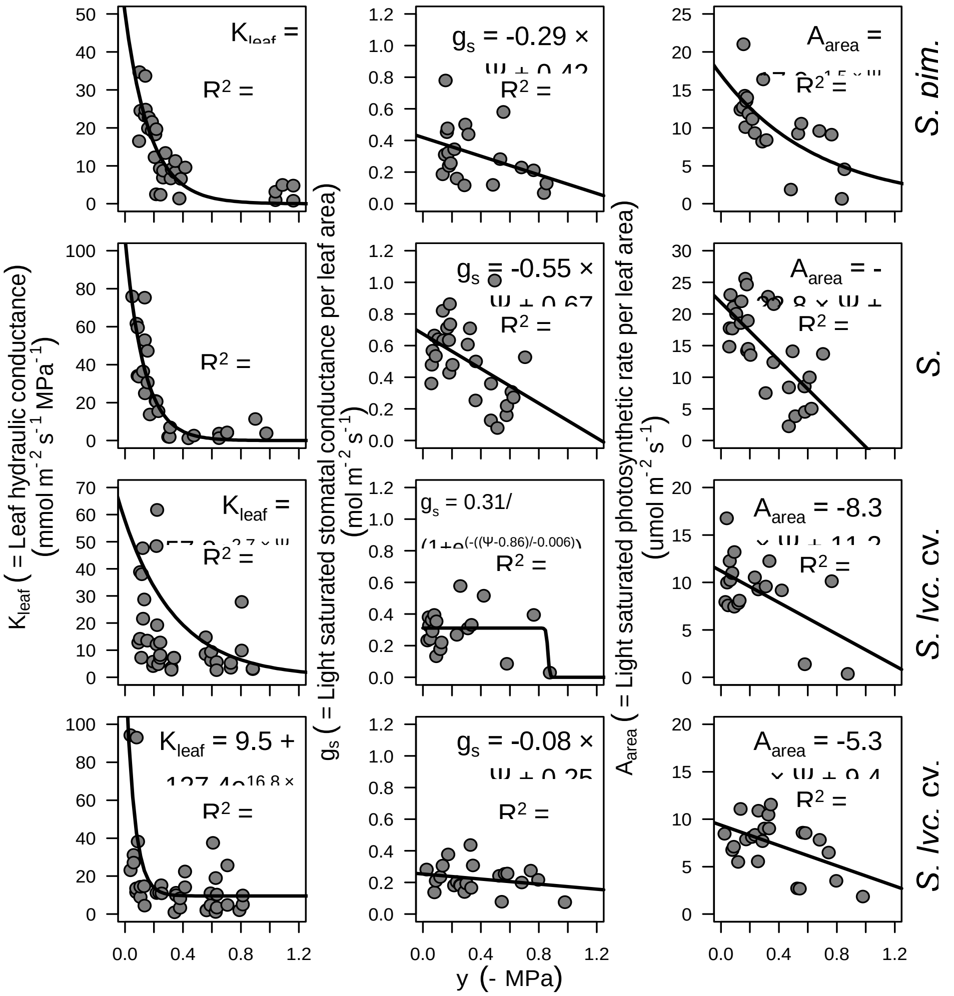


Figure 4.1. Kleaf, gs, and Aarea over a range of water potential for the four tomato leaflets with functions representing a best fit maximum likelihood framework.

4.4.3 Vein traits

Vein length per area (VLA) was overall higher in the wild species than in the domesticated lines across all major vein orders including the minor veins (Figure 4.2). Vein diameter was higher in the domesticated lines than the wild species for the primary and secondary (major) veins, and this pattern was lost with no difference in diameter between species groups in the minor veins (Figure 4.2). After combining into traditional vein groupings, VLA_{major} and VLA_{minor} (as well as VLA_{total}) was higher for the wild species while vein surface area (VSA) revealed different trends among species groups with toothed leaflets having higher VSA_{major} than untoothed leaflets (Figure 4.3). This trend was not reflected in the VSA_{total} due to the VSA_{minor} trend only holding true for the wild toothed species, and not the domesticated toothed line (Figure 4.3).

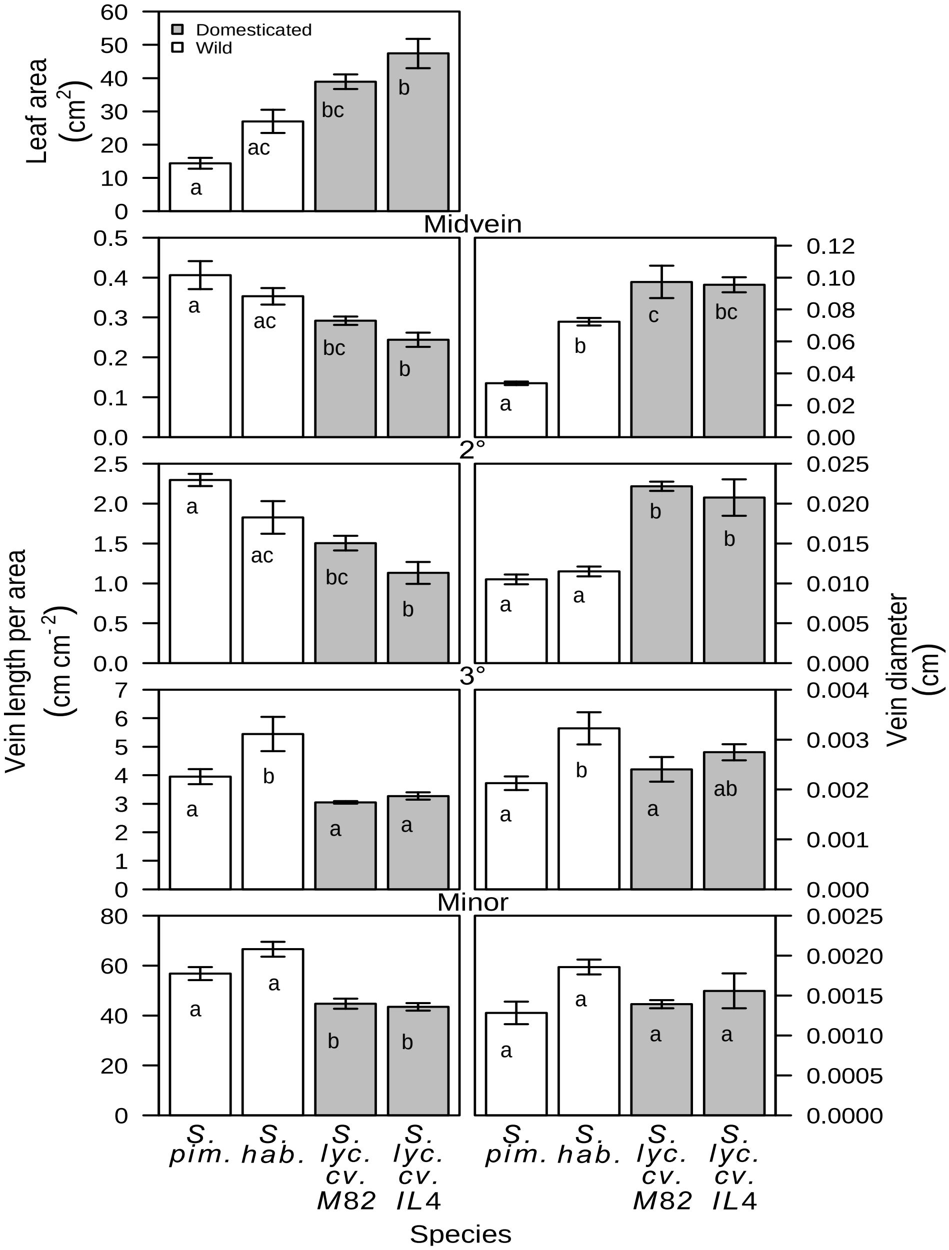


Figure 4.2. Vein length per area (VLA) and vein diameter for the four tomato lines for the orders of major veins: midvein (primary), secondary, and tertiary and also for minor veins. Leaf area also included for comparison. Letters represent significant differences using a one-way ANOVA.

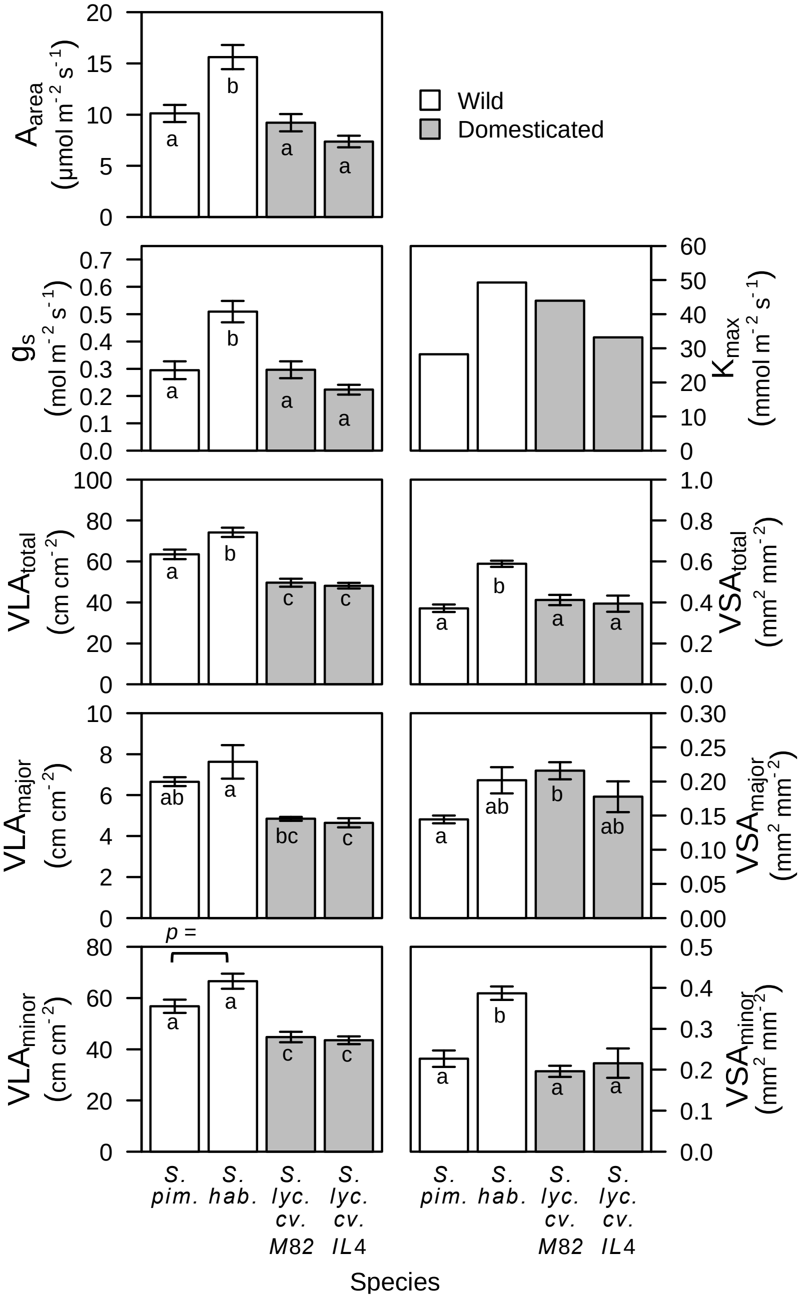


Figure 4.3. VLA as categories ‘total’, ‘major’ or ‘minor’, and VSA (VLA*vein diameter) in the same vein categories. Diurnal means for photosynthesis (Aarea) and stomatal conductance(gs), while the Kmax values represent Kleaf at -0.1MPa (an explanation for this metric can be found in methods: quantification of Kleaf). Letters represent significant differences using a one-way ANOVA.

4.4.4 Global trends

Vein diameter and VLA were previously shown to scale with leaf size across primary vein orders and not with minor veins for a range of leaves occurring around the globe (from Sack et al. 2012). Tomatoes from this study fit well within the global patterns in primary and secondary VLA comparisons ($P<0.05$) and for vein diameter and leaf area tomatoes fit the general trend (Figure 4.4). There was no pattern observed at the level of minor veins.

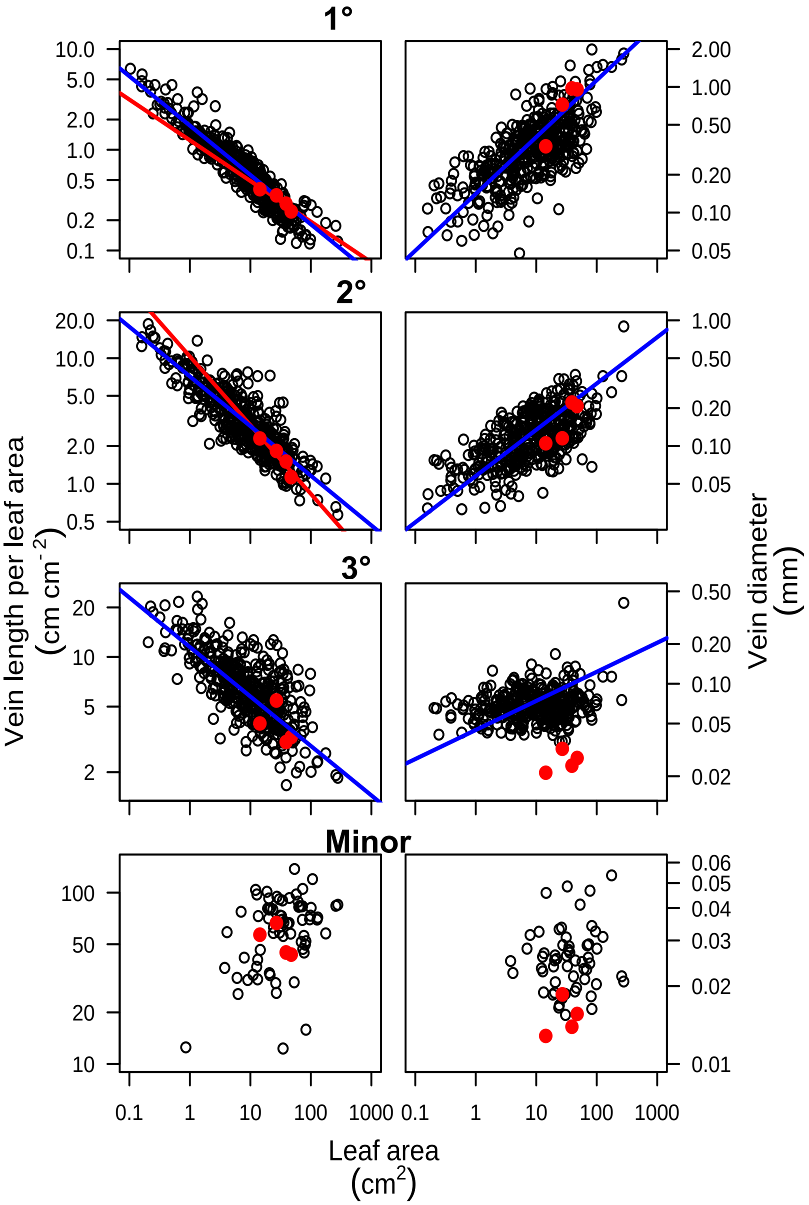


Figure 4.4. The global scaling of VLA and vein diameter from Sack et al 2012 (open circles) with tomatoes from this study (red circles). Red lines indicate a non-significant difference from the global trendline. 1° 2° 3° indicate vein orders.

Table 4.1. Mean leaf traits for four tomato species, including the leaf trait, the unit the trait was quantified, the sample size for each species (i.e., how many leaves were used), the mean value per species ± 1 standard error of the mean, and statistical differences from one way analysis of variance (ANOVA) with Tukey's Honest Significant Difference Post-Hoc Analysis, (first letter followed by the trait value) and from t-tests (second letter) testing for differences between the two wild species (see methods for details). Note, parameter values "@ 0.1 MPa" were extracted from best fit curves of the given trait as a function of Ψ_{leaf} and so significant tests on these traits were not performed. Trait values in bold signify significant differences by t-test between the two wild species.

Leaf Trait Category / Leaf Trait	**Unit**	***n***	**Species**			
			Solanum pimpinellifolium	*Solanum habrochaites*	*Solanum lycopersicum (cv. M82)*	*Solanum lycopersicum (cv. M82 x S. pennellii IL4_3)*
Leaf Morphology						
Leaf area	cm^{2}	4	14.4 ± 1.64; a, a	27.0 ± 3.47; ac, b	38.9 ± 2.22; bc	47.4 ± 4.41; b
Leaf length	cm	4	6.05 ± 0.408; a, a	9.34 ± 0.785; b, b	11.3 ± 0.531; b	11.4 ± 0.592; b
Leaf width	cm	4	3.37 ± 0.181; a, a	4.15 ± 0.651; a, a	6.36 ± 0.306; b	6.72 ± 0.665; b
Vein length per leaf area						
1	cm cm^{-2}	4	0.406 ± 0.0350; a, a	0.353 ± 0.0207; ac, a	0.292 ± 0.0107; bc	0.244 ± 0.0180; b
2	cm cm^{-2}	4	2.29 ± 0.0761; a, a	1.83± 0.205; ac, a	1.50 ± 0.0920; bc	1.13 ± 0.136; b
3	cm cm^{-2}	4	3.95 ± 0.266; a, a	5.45 ± 0.602; ac, a	3.05 ± 0.0444; bc	3.27 ± 0.127; b
Major	cm cm^{-2}	4	6.65 ± 0.217; ab, a	7.63 ± 0.822; a, a	4.84 ± 0.0958; bc	4.65 ± 0.218; c
Minor	cm cm^{-2}	4	**56.8± 2.58; a, a**	**66.6 ± 2.95; a, b (p = 0.047)**	44.8± 2.02; b	43.5 ± 1.50; b
Total	cm cm^{-2}	4	**63.5 ± 2.37; a, a**	**74.2 ± 2.27; b, b**	49.6± 1.99; c	48.2 ± 1.44; c
Vein diameter						
1	cm	4	**0.0338 ± 0.00107; a, a**	**0.0723 ± 0.00235; b, b**	0.0973 ± 0.0101; c	0.0955 ± 0.00469; bc
2	cm	4	0.0105 ± 0.000616; a, a	0.0115 ± 0.000569; a, a	0.0222 ± 0.00183; b	0.0207 ± 0.00229; b
3	cm	4	**0.00213 ± 6.79E-05; a, a**	**0.00323 ± 0.000162; b, b**	0.00240 ± 0.000123; a	0.00275 ± 8.04E-05; ab
Minor	cm	4	**0.00128 ± 0.000140; a, a**	**0.00186 ± 9.32E-05; a, b**	0.00139 ± 5.16; a, a	0.00156 ± 0.000218; a
Vein surface area per leaf area						
1	cm^{2} cm^{-2}	4	**0.0428 ± 0.00271; a, a**	**0.0800 ± 0.00389; b, b**	0.0894 ± 0.0103; b	0.0737 ± 0.00746; b
2	cm^{2} cm^{-2}	4	0.0755 ± 0.00402; a, a	0.0667 ± 0.0102; a, a	0.103 ± 0.00453; a	0.0759 ± 0.0172; a
3	cm^{2} cm^{-2}	4	**0.0262 ± 0.000987; a, a**	**0.0552 ± 0.00665; b, b**	0.0231 ± 0.00144; a	0.0281 ± 0.000287; a
Major	cm^{2} cm^{-2}	4	**0.145 ± 0.00568; a, a**	**0.202 ± 0.0192; ab, b (p = 0.05)**	0.216 ± 0.0126; b	0.178 ± 0.0226; ab
Minor	cm^{2} cm^{-2}	4	**0.227 ± 0.0200; a, a**	**0.387 ± 0.0162; b, b**	0.196 ± 0.0136; a	0.216 ± 0.0359; a
Total	cm^{2} cm^{-2}	4	**0.371 ± 0.0179; a, a**	**0.589 ± 0.0152; b, b**	0.412 ± 0.0250; a	0.394 ± 0.0397; a
Vein volume per leaf area						
1	cm^{3} cm^{-2}	4	**0.000360 ± 1.90E-05; a, a**	**0.00145 ± 8.37E-05; ab, b**	0.00225 ± 5.13E-04; b	0.00178 ± 2.39E-04; b
2	cm^{3} cm^{-2}	4	0.000200 ± 2.18E-05; a, a	0.000195 ± 3.95E-05; a, a	0.000577 ± 0.0000683; b	0.000422 ± 0.000148; a
3	cm^{3} cm^{-2}	4	**0.0000140 ± 2.39E-07; a, a**	**0.0000448 ± 6.76E-06; b, b**	0.0000140 ± 1.49E-06; a, a	0.0000193 ± 3.84E-07; a
Major	cm^{3} cm^{-2}	4	**0.000574 ± 0.0000399; a, a**	**0.00169 ± 0.000115; ab, b**	0.00284 ± 0.000536; b	0.00222 ± 0.00035; b
Minor	cm^{3} cm^{-2}	4	**0.0000748 ± 1.44E-05; a, a**	**0.000180 ± 1.47E-05; b, b**	0.0000687 ± 6.94E-06; a	0.0000901 ± 0.0000249; a
Total	cm^{3} cm^{-2}	4	**0.000648 ± 2.89E-05; a, a**	**0.00187 ± 0.000115; ab, b**	0.00291 ± 0.000543; b	0.00231 ± 0.000340; b
Hydraulic Function						
K_{max} @ 0 MPa	mmol m^{-2} s^{-1} Mpa^{-1}		50.3	106.7	57.8	127.4
K_{max} @ 0.1 MPa	mmol m^{-2} s^{-1} Mpa^{-1}		28.2	49.3	44	33.2
K_{leaf} (0 - 0.3 MPa)	mmol m^{-2} s^{-1} Mpa^{-1}	19, 16, 23, 18	**17.6 ± 2.10; a, a**	**37.8 ± 5.56; a, b**	38.1 ± 10.1; a	41.7 ± 10.3; a
K_{leaf} (0 - 0.5 MPa)	mmol m^{-2} s^{-1} Mpa^{-1}	26, 20, 29, 25	**14.9± 1.79; a; a**	**30.9 ± 5.45; a; b**	30.2± 7.72; a	34.3 ± 8.60; a
K_{leaf} (0 - 1.2 MPa)	mmol m^{-2} s^{-1} Mpa^{-1}	31, 26, 41, 39	**13 ± 1.70; a, a**	**24.9 ± 4.71; a, b**	26.7 ± 6.36; a	22.9 ± 5.25; a
Gas Exchange						
g_s @ 0 MPa	mol m^{-2} s^{-1}		0.42	0.67	0.31	0.25
g_s @ 0.1 MPa	mol m^{-2} s^{-1}		0.39	0.62	0.31	0.25
g_s (0 - 0.3 MPa)	mol m^{-2} s^{-1}	12, 14, 13, 11	**0.346 ± 0.0532; a, a**	**0.611 ± 0.0390; b, b**	0.305 ± 0.0318; ac	0.221 ± 0.0222; c
g_s (0 - 0.5 MPa)	mol m^{-2} s^{-1}	14, 21, 16, 15	**0.336 ± 0.0488; a, a**	**0.577 ± 0.0453; b, b**	0.32 ± 0.0288; ac	0.236 ± 0.0235; c
g_s (0 - 1.2 MPa)	mol m^{-2} s^{-1}	20, 27, 19, 23	**0.310 ± 0.041; a, a**	**0.507 ± 0.045; b, b**	0.296 ± 0.031; a	0.223 ± 0.018; a
P_{max} @ 0 MPa	µmol m^{-2} s^{-1}		17	21.8	11.2	9.4
P_{max} @ 0.1 MPa	µmol m^{-2} s^{-1}		14.6	19.5	10.4	8.84
P_{area} (0 - 0.3 MPa)	µmol m^{-2} s^{-1}	12, 14, 13, 11	**12.9 ± 0.987; a, a**	**19 ± 1.05; b, b**	10.2 ± 0.749; ac	7.94 ± 0.547; c
P_{area} (0 - 0.5 MPa)	µmol m^{-2} s^{-1}	14, 21, 16, 15	**11.8 ± 1.18; a, a**	**16.9 ± 1.31; b, b**	10.2 ± 0.623; ac	8.49 ± 0.505; c
P_{area} (0 - 1.2 MPa)	µmol m^{-2} s^{-1}	20, 27, 19, 23	**10.4 ± 1.04; a, a**	**14.9 ± 1.31; b, b**	9.21 ± 0.85; a	7.37 ± 0.57; a

4.5 DISCUSSION

We extended leaf hydraulic theory to four species of tomatoes. First, we found support for both our first and second hypotheses as species differed in their maximal and mean K_{leaf}, and in their responses of K_{leaf}, g_s, and P_{max}, highlighting the coordination of such traits in constraining physiological processes. Further, variation in vein traits underlie variation in K_{leaf} with more pronounced differences between the two wild species, and convergence within the two domesticated species, thus lending support to our third hypothesis. Lastly, we conclude that tomatoes conform to the pattern of leaf major vein scaling with leaf size, suggesting that the such species also have a conserved leaf developmental program.

4.5.1 Leaf hydraulics

We set out to test whether leaflets with different vein patterns also differed in their hydraulic conductance, and whether there was a coordinated response of gas exchange with hydraulic conductance. The hydraulic vulnerability curves (Figure 4.1) show two major patterns. First, wild species show steep declines approaching zero conductance with decreasing water potential meaning that at low water potential leaf tissue will no longer be able to transport water. Indeed, recent studies have shown that these steep declines signify high vulnerability to water stress, but largely result from loss of outside xylem conductance rather than vein embolism (Scoffoni et al. 2017). Further, regulation of hydraulic conductance by the outside xylem components may serve as a blockade to prevent embolism in the veins. Second, domesticated lines evolved a different mechanism either by a more moderate decline in conductance (*S. lyc* M82) or reaching a steady state of conductance without approaching zero (*S. lyc* IL4_3). At the same low water potentials, -0.8 MPA for example, both domesticated lines show a low level of conductance.

4.5.2 Gas Exchange

K_{leaf}, g_s, and A_{area} showed coordinated responses and differences in these linkages diverged between wild species and domesticated lines (Figure 4.1). With decreasing water potential wild species show a steady decrease in g_s and A_{area}, indicating a close relationship between all three variables. This suggests that the decline in hydraulic conductance results in stomatal closure which reduces gas exchange and limits photosynthesis (Sack and Holbrook 2006). This synchronized decline results in high vulnerability of wild species' productivity to water stress; even small decreases in leaf water potential were enough to trigger these reductions. Indeed, the wild species occupy habitats with less predictable precipitation which would also exacerbate competition for water. Furthermore, we suspect that the wild species have been evolutionarily selected for maximum growth when water is available as such physiology would be advantageous for growth with greater competition under less precipitation (Sinclair, Zwieniecki, and Holbrook 2008). By contrast, the domesticated lines appear to have the ability to maintain hydraulic conductance at low leaf water potential, and also show only moderate declines in g_s and A_{area} as the leaf water potential becomes more negative. The relatively stable g_s could explain the differences between wild and domesticated plants. Perhaps stomatal response dominates K_{leaf}, and failure of domesticated lines to regulate stomata fully allows for low rates of photosynthesis. This suggests that domesticated lines' productivity is less vulnerable to water stress, and would thus reflect their consistent irrigation.

When assessing differences in responses of K_{leaf}, g_s, and A_{area} between wild and domesticated plants, it is important to note that overall the highest g_s and A_{area} are in the wild species, sometimes exceeding double that of the maximum conductance and photosynthetic rates measured in the domesticated lines (Figure 4.1 and Table 4.1). The variability displayed by the wild species may be a result of their sensitivity to decreased leaf water potential being an adaptive, evolutionary response to experiencing water stress. Wild species are competitive with their neighbors for soil resources, and in times of water deficit will compete to use all available water. Though our experiment was carried out in soil-filled pots with no underground competition, it is likely the wild species employed their inherent strategy to use resources competitively and respond quickly to stress signals in order, potentially, to ride out the period of water deficit. Through breeding, domesticated lines often lose the competitive response for soil resources because, agronomically, resources like soil water are supplied. While loss of stomatal sensitivity to water deficit may explain the different stomatal response and maintained K_{leaf}, g_s and A_{area} for these lines (Figure 4.1), it comes with a critical tradeoff of these plants never reaching the exceptionally high rates of g_s and A_{area} that we find in the wild species in conditions that are not resource limiting.

4.5.3 Vein traits

We next looked to see if differences in structural traits help explain the physiological responses we measured above. Differences in K_{max} between the domesticated lines (*S. lyc* M82 and *S. lyc* IL 4_3) was reflected by increased VSA_{major} (Figure 4.3). For the wild species the increased K_{max} of *S. hab* could be attributed to the minor veins in both VSA and VLA which accounts for most of the difference reported in VSA_{total} and VLA_{total} (Figure 4.3). VSA is determined by vein diameter and VLA, so it provides evidence on the resistance to water flux inside the hydraulic pathway. Since a narrow tube offers more resistance, the greater VSA in both the major and minor veins of *S. hab* contributes to the overall greater K_{max} when compared to the other wild species *S. hab* (lower VSA, lower conductance Figure 4.3). Leaflet area varied across all leaflet groups (wild-domesticated, toothed-untoothed) however, VLA was consistently higher across all major vein orders for wild species as compared to domesticated lines (Figure 4.2). Since major veins represent the vein order with the most individual vessels per bundle, this trait likely facilitates the higher maximums for K_{leaf}, g_s, and A_{area} in the wild species at -0.1 MPa (Table 4.1).

Because leaf hydraulic conductance is a measure of conductance along the xylem pathway, measuring xylem traits contributes to our understanding of resistance (the inverse of conductance) to water movement along this part of the pathway. However, there is additional conductance outside of the xylem conduits (K_{ox}) which contributes to the K_{leaf}. Many anatomical factors influence K_x including the thickness of the leaf, the amount of airspace and number of bundle sheath and mesophyll cells from veins to the sites of evaporation (Caringella, Bongers, and Sack 2015). Further, The longer the distance water must traverse from vein to the site of evaporation, the lower the K_{ox} would be (Brodribb, Feild, and Jordan 2007). Importantly, traits such as VLA influence both K_x and K_{ox},. The first would arise from principles of physics: the greater length of vein per area permits greater transport within vein xylem, K_x, whereas the greater length of vein per area also results in greater bundle sheath surface area, and thus VLA indirectly increases K_{ox}. For each vein order the wild species had a higher VLA compared to the domesticated lines (Figure 4.2), though these species also attained higher maximum K_{leaf} as discussed above and in Table 4.1. Herbs that have been measured have notably high K_{leaf} values (Sack and Holbrook 2006) and also high incidence of leaf aquaporins(Sade et al. 2014). All the tomatoes in this study likely had high numbers of aquaporins and were therefore able to regulate K_{ox} at higher water potentials through expression of these channels or by degradation of them at lower leaf water potentials. It stands to reason that wild species exhibit greater control over aquaporin expression than domesticated lines just as many other traits were lost in selection favoring large, sugar-rich fruits.

4.5.4 Global Patterns

Data for tomato leaflets in this study were placed into context of data collected globally for many types of leaves in a wide range of habitats (Sack et al. 2012). We expected the patterns found in the previous study by Sack and colleagues (2012) to hold for tomato leaves, as the inverse relationship between VLA and leaf size is largely a reflection of the economic tradeoff of producing costly vein structures in place of photosynthetically active cells(Boyce et al. 2009; Poorter et al. 2009; Edwards et al. 2014). The positive relationship between vein diameter and leaf size emphasizes the need for vasculature to provide physical structure to display the light-catching lamina. We found that for the largest veins (primary and secondary) the trends in the global data set and tomato leaves are consistent: VLA decreases as leaf size increases, and, vein diameter tends to increase with leaf size for the same vein orders (Figure 4.4). The tomatoes separate from the global trend at the level of tertiary veins. This is likely because leaf in this case is actually a leaflet, whose primary vein more closely matches the secondary veins of non-compound leaves being of comparable size and length. With that in mind, the trends disappearing at the tertiary level is consistent with their being no association in the minor veins of VLA or vein diameter with leaf size(Sack and Frole 2006; McKown, Cochard, and Sack 2010).

4.5.5 Conclusions

Tomato leaflets of wild species show greater hydraulic vulnerability, than domesticated lines, to decreases in leaf water potential. This is consistent with the evolutionary mechanisms in place to use soil resources in a competitive manner. The domesticated lines have lower gas exchange and photosynthetic rates under resource-rich conditions but are able to maintain nominal functioning at low leaf water potential making them less vulnerable to drought. Vein traits can help to tease apart variation in maximum K_{leaf}, g_s and A_{area} by leaflet shape. High flux leading to increases in gas exchange and photosynthesis can be achieved by decreasing internal resistance to water flow, either by increasing the conduit size (*S. hab*, wild species) or by increasing the number of conduits (*S. lyco* cv M82, domesticated line). Lastly, we saw that tomato leaflets generally do scale with global leaf trait patterns which suggests the strength of these patterns from an evolutionary perspective and the importance of vein traits as drivers of physiological limits of the leaf.

Chapter 5. CONCLUSIONS

The purpose of the research carried out for this dissertation was to test hypotheses raised in the literature about the functional significance of leaf shape. Using literature outlined in chapter 1 on leaf fossils, leaf-shape and climate interactions, leaf development, and plant hydraulics, experiments were carried out using a set of tomato leaflets selected for their margin-shape and also their relatedness. The tomatoes in this study were selected for their leaf shape and relatedness and so genotype with toothed margins and another with entire leaf margins were selected from distantly related wild species, and also a toothed and entire leaf shape were selected from introgressed lines of a domesticated cultivar. These experiments revealed several key findings on leaf shape and function relationships detailed in chapters 2 and 3 and summarized here.

We found carbon uptake in toothed edge lamina is disproportionally higher than in entire leaf edges, which agrees with several similar studies on woody species and notably this is the first time this result has been shown in found in herbs. A strong hypothesis for this result in woody plants has to do with the teeth providing a photosynthetic ‘boost’ early in the growing season, since toothed margins are mostly found in a temperate deciduous habitat. This could possibly extend to herbs as well, as many are annuals completing their life cycle in a single growing g season much like deciduous trees, and the quick life cycle may benefit even more from having a resource ‘boost’ that persists not only early in the season but for the whole life of the leaf. The leaf tip is the first part of the leaf organ to reach maturity, and so having many iterations of ‘tips’ (think teeth on a compound leaf) would be a quick way to increase the areas functioning at maturity early on in the leaf’s life.

We found that under water deficit the toothed edges had less of a reduction in photosynthesis when compared to leaves with entire edges even though the water potential in toothed edges experienced steeper declines. Stomatal conductance measurements revealed that toothed edges were similarly impacted by water deficit when compared to entire edges, which lead us to hypothesize that the vein pattern differences between the two margin-shapes could deliver water at different rates, uptake photosynthate at different rates, and could help explain how a steep water potential gradient and high carbon uptake rates are maintained between tooth lamina and middle-leaf lamina.

Leaf hydraulic conductance did not help explain why the toothed margins have higher photosynthetic rates with lower water potential as we predicted, but instead pointed out some interesting patterns in coordinated plant responses of hydraulic conductance and gas exchange when considering domesticated lines versus wild species. In these tomatoes we saw while the maximum K_{leaf} values were higher for the toothed leaflets compared to the entire leaflets, the shape and slope of the vulnerability curves shows the wild species quickly losing conductance at small changes to leaf water potential while the domesticated lines have a slower decline in conductance and do not reach zero over the range of water potentials tested.

We found evidence for a coordinated response in K_{leaf}, g_s, and A_{area} as the fast K_{leaf} declines for wild species was mirrored in g_s and also A_{area} while the domesticated lines g_s and A_{area} declined less steeply, though were never very high to begin with, prompting us to determine wild species more vulnerable to decreases in leaf water potential than domesticated lines. It is difficult to reconcile this with the results gathered from just the leaf edges where toothed shapes showed less vulnerability to water deficit, regardless of whether the leaflet was wild or domesticated. It seems that the functional advantages of having teeth were overridden by the averaged response of the whole leaf measured in the second dataset. This could have been due to unequal water stresses delivered in the two sets of experiments, as the g_s and A_{area} in later experiments where measured over the range of temporary water potentials the plants experienced over the course of a day and the leaf edge experiments were maintained at a water deficit over the course of two weeks. Water stress continues to be a tricky element to simulate for experiments.

The anatomical traits of the venation systems of each leaflet shape did lead us to conclude that toothed leaves have higher VSA in both the major and minor veins, which could be what contributes to their higher K_{leaf} compared to the entire margin of the same background (domesticated, wild). Higher VSA may be costly for the plant in terms of the high investment in non-photosynthetically active tissues, but could see a payoff as seen here in higher hydraulic conductance which has the coordinated response with gas exchange as described above. The wild species had consistently higher VLA with also higher K_{max}, g_s and A_{area} which at first seems counterintuitive given that high VLA includes high bundle sheath extensions in the mesophyll, adding resistance to water flux outside the xylem. A possible explanation for this may lie in aquaporin expression. Wild species may have more control of the expression of these channels, specifically in the tissues causing high resistance outside of the xylem, than the domesticated lines, which could explain high K_{leaf} despite high VLA, and also the higher K_{leaf} values in general.

From this body of research, several new questions came into focus and are presented here as avenues of further research that fall outside the scope of this dissertation. The tomatoes selected for this study were chosen based on consideration of previous research already done in this system, leaf shapes that satisfied the goals of the experiments, and species or varietal relatedness. There are likely other similar sets of leaves outside of *Solanum* that could be tested for their leaf margin functioning in order to support that these results are not specific to this genus and are applicable to a wide array of both ecologically and economically important species. It would likewise be interesting to use other tomato lines and wild species from the tomato genetic resource center, as our leaflets represented shape extremes on the toothed to smooth spectrum, though there exist relatives both quite near (the rest of the *S. lycopersicum* introgression lines) and far (other wild species of tomato that evolved in climates unlike those tested) that could be used to make a more complete dataset of leaf margin functioning. Widening this dataset would help eliminate the question of whether the functional response is a result of genetic underpinnings independent from leaf shape, or truly linked to leaf shape.

Results presented here raise questions about the leaf hydraulics on a very fine scale. The role of minor veins in hydraulic conductance is smaller, perhaps related to some evidence that minor vein functioning changes over the lifetime of leaf beginning with water supply and shifting to sugar export as the leaf matures. The timing of this transition as well as any changes or limitations this poses anatomically could very well affect the results of this study and better inform future research in hydraulics. That minor vein density is independent of leaf size, and that the minor veins are the last to differentiate and mature as the whole leaf matures, makes this an ideal place to research feedback from the environment on leaf traits.

The results here also raise questions about regulation of aquaporins in leaf tissues. Though only referenced here as a possible explanation for increased conductance in an otherwise high-resistance tissue, the idea that aquaporin regulation expression drives a significant portion of K_{ox} and control of this regulation is less prevalent in domesticated species is worth further consideration. Perhaps increased aquaporin expression in the bundle sheath cells could unlock higher K_{leaf}, g_s, A_{area} in the domesticated lines.

Lastly, this research focused largely on flux through the xylem, though considerable attention was focused on the photosynthetic productivity of the leaves. How sugar movement in the phloem may be impacted by temporal increases in carbon uptake and the making of more photosynthate, and also water movement in the xylem, remain questions at large. Short distance transport within sections of the leaf may not be affected by small changes in leaf water status, though the distance between mesophyll cells and the bundle sheath cells as determined by VLA could add resistance to this flow. Once sugar is loaded into the sieve cells for long distance transport through the plant, the pressure-flow model based on an osmotically generated pressure gradient describes a passive mechanism for phloem transport that has been validated in some plants. Because the model requires the associated xylem vessels to have higher or lower water potential (at source and sink, respectively) to drive the pressure gradients for bulk flow through the phloem, if the transpiration stream in the xylem is subject to cavitation due to conduit size and hydraulic conductance is limited or maintained at low leaf water potential the direct effect this has on phloem flow is unknown. This could have major consequences for plant survival, including limiting export of sugars to reproductive sink tissues, and so the direct effects of xylem water movement on phloem movement, and the potential for negative feedback within this system, should be examined further.

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