DISSERTATION | DOCTORAL THESIS

Titel | Title

Beyond Guard Cells: Epidermal and Mesophyll Regulation of Plant Responses to Water Stresses

verfasst von | submitted by

Maryamalsadat Zekri MSc

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

Wien | Vienna, 2026

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 794 685 437
Dissertationsgebiet lt. Studienblatt | Field of study as it appears on the student record sheet:	Biologie
Betreut von | Supervisor:	ao. Univ.-Prof. Mag. Dr. Ingeborg Lang Privatdoz. Dr. Daniel Tholen

Beyond Guard Cells: Epidermal and Mesophyll Regulation of Plant Responses to Water Stresses

Table of Contents

Acknowledegment

First and foremost, I would like to express my sincere and deepest gratitude to my supervisor, Professor Dr. Ingeborg Lang, for her continuous guidance, trust, encouragement, and invaluable support throughout my doctoral research. Her expertise, insightful feedback, and critical perspective have been essential in shaping this work and improving its quality. I am especially grateful for the time she dedicated to discussions, her constructive comments, and her constant willingness to provide guidance whenever it was needed. Her commitment to academic excellence and her thoughtful mentorship has greatly influenced both this research and my development as a researcher.

I would also like to extend my sincere thanks to my co-supervisor, Professor Dr. Daniel Tholen, for his support and valuable contributions during this project. His constructive suggestions, helpful discussions, and thoughtful perspectives have significantly contributed to the progress of this research. I am grateful for his time, expertise, and encouragement, which have helped strengthen this work.

I would also like to express my deepest appreciation to my parents for their unconditional love, encouragement, and lifelong support. They have always supported my ambitions and inspired me to pursue my goals with determination and perseverance. In addition, I would like to thank my family for their continuous encouragement, understanding, and support throughout my studies. Their confidence in me and their constant motivation have meant more than words can express.

My heartfelt gratitude goes to my partner, Mohammad Kianian, whose constant support, patience, and understanding have been invaluable throughout this journey. The process of completing a PhD is often demanding and challenging, and his encouragement, motivation, and belief in me have been a continuous source of strength and reassurance. I am deeply thankful for his presence and for the support he provided during both the difficult and rewarding moments of this journey.

Finally, I would like to acknowledge all those who, directly or indirectly, contributed to the completion of this thesis. The support, encouragement, and inspiration I received from the people around me have made this journey possible and meaningful.

Abstract

Since the foundational work of Gregor Mendel established genetics through the observation of plant phenotypes, research in plant biology has increasingly aimed to link observable traits with their underlying molecular and genetic mechanisms. A deeper understanding of plant anatomical traits is a path for translating molecular findings into practical strategies for improving plant resilience and productivity under adverse environmental conditions. Despite their importance, anatomical traits are often challenging to study due to technical limitations in accurately quantifying structural properties in dynamic and fluctuating environments. Among these traits, stomata represent a critical anatomical structure that regulates the balance between CO_2 uptake for photosynthesis and water loss through transpiration. One particularly interesting yet insufficiently understood phenomenon is the transient opening of stomata following a sudden decline in leaf water potential. This response, known as the wrong-way response (WWR), involves a temporary stomatal opening when the water potential declines and closure would be expected.

The epidermal mechanical advantage hypothesis proposes that this transient opening arises from mechanical interactions between guard cells and surrounding pavement cells. According to this hypothesis, mechanical coupling within the pavement and guard cells plays a key role in determining stomatal dynamics. However, the structural determinants underlying this mechanical coupling remain unresolved. Given that the geometry of interacting components strongly influences the behaviour of mechanical systems, this thesis investigates how the anatomical geometry of pavement cells and stomata contributes to the dynamics of transient stomatal opening. To address this question, the model plant *Arabidopsis thaliana* was used due to its well-characterized genome, availability of genetic resources, and suitability for controlled laboratory experiments. Different Arabidopsis lines were selected based on variation in stomatal size and density. However, differences in additional anatomical features, such as the absence of trichomes in the *tmm1* mutant and limited knowledge regarding mesophyll organization in selected Arabidopsis lines, posed challenges for developing a comprehensive understanding of stomatal behaviour. Therefore, physiological responses of glabrous Arabidopsis line were compared with control plants, alongside detailed analyses of key stomatal traits including size and density. The first study demonstrates trichomes were shown to influence leaf temperature, although their contribution to maintaining photosynthetic performance under drought conditions was limited. The second study introduces a cryo-fixation–based methodological approach to investigate mesophyll anatomy under drought stress. This technique allows visualization of mesophyll organization and cellular architecture while minimizing artifacts caused by dehydration during conventional microscopic preparation. Using this method, structural modifications of both palisade and spongy mesophyll tissues were observed under short- and long-term drought stress. The third study focuses on the transient stomatal opening. The results provide empirical support for the epidermal mechanical advantage hypothesis and demonstrate that pavement cell size significantly influences both the magnitude and duration of this response. These findings

highlight the importance of epidermal geometry in governing stomatal dynamics during rapid changes in plant water status.
Together, the results presented in this thesis demonstrate that plant responses to variable water availability are shaped by anatomical traits across multiple leaf tissues, including the epidermis and mesophyll. These findings underscore the need to move beyond an exclusive focus on guard cells and instead adopt a more integrated perspective that considers how diverse leaf structures collectively regulate plant water relations and stress responses.

Zusammenfassung

Seit die grundlegende Arbeit von Gregor Mendel die Genetik durch die Beobachtung von Pflanzenphänotypen begründete, hat die Forschung in der Pflanzenbiologie zunehmend darauf abgezielt, beobachtbare Merkmale mit ihren zugrunde liegenden molekularen und genetischen Mechanismen zu verknüpfen. Ein tieferes Verständnis pflanzlicher anatomischer Merkmale ist ein Weg, molekulare Erkenntnisse in praktische Strategien zur Verbesserung der Widerstandsfähigkeit und Produktivität von Pflanzen unter widrigen Umweltbedingungen zu übertragen. Trotz ihrer Bedeutung sind anatomische Merkmale häufig schwierig zu untersuchen, da technische Einschränkungen eine genaue Quantifizierung struktureller Eigenschaften in dynamischen und schwankenden Umgebungen erschweren. Unter diesen Merkmalen stellen Stomata eine entscheidende anatomische Struktur dar, die das Gleichgewicht zwischen CO_2-Aufnahme für die Photosynthese und Wasserverlust durch Transpiration reguliert. Ein besonders interessantes, jedoch unzureichend verstandenes Phänomen ist die vorübergehende Öffnung der Stomata nach einem plötzlichen Abfall des Blattwasserpotentials. Diese Reaktion, bekannt als Wrong-Way-Response (WWR), beinhaltet eine vorübergehende Öffnung der Stomata, wenn das Wasserpotential abnimmt und eigentlich ein Schließen erwartet würde.
Die Hypothese des epidermalen mechanischen Vorteils besagt, dass diese vorübergehende Öffnung aus mechanischen Wechselwirkungen zwischen Schließzellen und umgebenden Pavementzellen entsteht. Nach dieser Hypothese spielt die mechanische Kopplung innerhalb der Pavement- und Schließzellen eine Schlüsselrolle bei der Bestimmung der Stomatadynamik. Die strukturellen Determinanten, die dieser mechanischen Kopplung zugrunde liegen, sind jedoch noch ungeklärt. Da die Geometrie interagierender Komponenten das Verhalten mechanischer Systeme stark beeinflusst, untersucht diese Arbeit, wie die anatomische Geometrie von Pavementzellen und Stomata zur Dynamik der vorübergehenden stomatären Öffnung beiträgt. Um diese Frage zu adressieren, wurde die Modellpflanze Arabidopsis thaliana verwendet, da sie ein gut charakterisiertes Genom, verfügbare genetische Ressourcen und eine Eignung für kontrollierte Laborexperimente aufweist. Verschiedene Arabidopsis-Linien wurden auf Grundlage von Variationen in der Größe und Dichte der Stomata ausgewählt. Unterschiede in zusätzlichen anatomischen Merkmalen, wie das Fehlen von Trichomen im *tmm1*-Mutanten sowie begrenztes Wissen über die Mesophyllorganisation in den ausgewählten Arabidopsis-Linien, stellten jedoch Herausforderungen für die Entwicklung eines umfassenden Verständnisses des stomatären

Verhaltens dar. Daher wurden die physiologischen Reaktionen einer glatten Arabidopsis-Linie mit Kontrollpflanzen verglichen, zusammen mit detaillierten Analysen zentraler stomatärer Merkmale einschließlich Größe und Dichte. Die erste Studie zeigt, dass Trichome die Blatttemperatur beeinflussen, obwohl ihr Beitrag zur Aufrechterhaltung der photosynthetischen Leistung unter Trockenstress begrenzt war. Die zweite Studie führt einen auf Kryofixierung basierenden methodischen Ansatz ein, um die Mesophyllanatomie unter Trockenstress zu untersuchen. Diese Technik ermöglicht die Visualisierung der Mesophyllorganisation und der zellulären Architektur, während Artefakte minimiert werden, die durch Dehydration während der konventionellen mikroskopischen Präparation entstehen. Mithilfe dieser Methode wurden strukturelle Modifikationen sowohl des Palisaden- als auch des Schwammparenchyms unter kurz- und langfristigem Trockenstress beobachtet. Die dritte Studie konzentriert sich auf die vorübergehende stomatäre Öffnung. Die Ergebnisse liefern empirische Unterstützung für die Hypothese des epidermalen mechanischen Vorteils und zeigen, dass die Größe der Pavementzellen sowohl das Ausmaß als auch die Dauer dieser Reaktion signifikant beeinflusst. Diese Befunde unterstreichen die Bedeutung der epidermalen Geometrie für die Steuerung der Stomatadynamik während schneller Veränderungen des pflanzlichen Wasserstatus.

Zusammen zeigen die in dieser Arbeit präsentierten Ergebnisse, dass pflanzliche Reaktionen auf variable Wasserverfügbarkeit durch anatomische Merkmale über mehrere Blattgewebe hinweg geprägt sind, einschließlich der Epidermis und des Mesophylls. Diese Befunde unterstreichen die Notwendigkeit, über einen ausschließlichen Fokus auf Schließzellen hinauszugehen und stattdessen eine stärker integrierte Perspektive einzunehmen, die berücksichtigt, wie verschiedene Blattstrukturen gemeinsam die Wasserbeziehungen der Pflanze und ihre Stressreaktionen regulieren.

1. Introduction

There are traces of plant life almost everywhere on our planet, from dense tropical forests to arid deserts or cold steppes. As sessile organisms, plants developed the ability to modify their structure, anatomy, and function in response to both short-term and long-term environmental fluctuations (Taiz et al., 2015). These modifications allow plants to survive in harsh environments and ensure the successful transmission of their genetic material to the next generations. With the intensifying consequences of climate change, specifically water scarcity (Caretta, 2022), it is more important than ever to understand to what extent plants anatomy and its alteration in response to fluctuating environment contribute to physiological responses. Considering that anatomical traits are shaped by evolutionary and developmental factors arising from hydraulic and photosynthetic demands, structural diversity among leaves is expected to reflect fundamental differences in physiological function and stress responsiveness (Brodribb and McAdam, 2011; Zwieniecki and Boyce, 2014). Since the plant anatomy, from cell geometry to whole-plant structure, is the result of underlying molecular processes, understanding anatomical alterations in response to the surrounding environmental provides deeper insight into these molecular mechanisms and strengthens the foundational knowledge for plant engineering. Among the anatomical features that play a crucial role in plant-environment interactions are the features associated with gas exchange and water regulation. In particular, stomata and the surrounding epidermal cells provide a dynamic system, mediating the balance between carbon uptake and water loss. Investigating how the structural properties of the epidermal components influence stomatal behaviour is therefore essential for understanding stomata responses to environmental fluctuations. The main focus of my work is to elucidate how the anatomical properties of epidermal cells contribute to transient stomatal opening, a phenomenon that remains poorly understood in plant physiology.

Transient stomatal opening, commonly called the wrong-way response (WWR), describes the increase in stomatal aperture following a decline in plant water potential, before stomata subsequently close to maintain hydraulic balance (Buckley, 2019; Zait et al., 2024). This behaviour has been observed following both increased evaporative demand and reduced water supply, and is typically followed by a “right-way response” (RWR) characterized by a reduction in stomatal conductance (Buckley et al., 2011; Zait et al., 2024). Although stomatal closure in response to drought and vapor pressure deficit (VPD) has been extensively characterized at molecular, cellular, and whole-plant levels (Mott, 2007; Bauer et al., 2013; Yaaran et al., 2017; Lei et al., 2022), the mechanisms specifically controlling transient stomatal opening remain comparatively poorly resolved. This knowledge gap is notable given that WWR occurs in the time of a decline in leaf water potential and can therefore have important consequences for plant hydraulic safety and carbon–water balance under fluctuating environmental conditions. One of the main and most argued explanations proposed to define WWR is the “epidermal mechanical advantage” hypothesis. This hypothesis, rooted in early work with pressure-probe measurements of guard and epidermal cell turgor, suggests that changes in guard cell volume cannot fully explained by stomatal movements because they

occur in the mechanical context of surrounding epidermal cells, which exert an opposing constraint on guard cell expansion (Franks et al., 1998). In this framework, the epidermis has a mechanical advantage over guard cells, so that changes in epidermal turgor can strongly modulate, or even dominate, the resulting stomatal aperture. When leaf water status is perturbed, differential changes in turgor between guard cells and epidermal pavement cells can therefore drive the unexpected stomatal responses, including transient opening during an initial phase of hydraulic disturbance (Drake et al., 2013; Wang et al., 2018; Nieves-Cordones et al., 2022). Spatially explicit models of stomatal mechanics and hydroactive feedback suggest that, in the absence of active metabolic responses, purely hydraulic and mechanical interactions between guard cells and their epidermal context can generate the WWR (Buckley, 2005; Buckley, 2019; Pichaco et al., 2024). In such hydropassive scenarios, rapid changes in water status may cause a decline in neighbouring epidermal cells turgor, temporarily reducing the constraining force of the epidermis on guard cells and allowing stomata to “pop open” despite an overall decrease in leaf water potential. In a recent review by (Tan et al., 2025), the authors noted that the cells surrounding guard cells are involved in maintaining the length of the stomatal apparatus, however they also highlighted the substantial gap in our understanding of the mechanical role of the epidermis in guard cell dynamics. Subsequent activation of feedback processes, such as osmotic adjustment in guard cells and ABA-dependent signalling, then shifts the system towards a right-way response and sustained closure. These modelling efforts provide a mechanistic rationale for the epidermal mechanical advantage hypothesis and underscore the need to treat the stomatal complex as an integrated biomechanical system rather than a guard cell–autonomous unit (Buckley, 2019).

Recent works support a key role of epidermal anatomy and mechanics in shaping stomatal dynamics (Sapala et al., 2018; Sapala et al., 2019; Liu et al., 2021; Tan et al., 2025; Manandhar et al., 2026) and indicate that the stomatal behaviour depends on the relative size and geometry of guard cells and neighbouring pavement cells (Franks and Farquhar, 2007; Drake et al., 2013; Lawson and Blatt, 2014). In angiosperms that exhibit strong mechanical coupling between guard cells and epidermis, decreased epidermal turgor at high VPD can accelerate stomatal transient opening, consistent with predictions derived from mechanical advantage theory (Pichaco et al., 2024). These findings highlight that stomatal kinetics emerge from the interplay of guard cell properties with pavement cell dimensions, wall stiffness, and packing, rather than from guard cell behaviour alone. However, direct experimental tests of how these anatomical traits translate into transient opening behaviour under hydraulic stress remain unclear, and existing studies provide only fragmentary validation of the epidermal mechanical advantage hypothesis (Buckley et al., 2011; Pichaco et al., 2024; Zait et al., 2024). Additionally, the duration and amplitude of transient stomatal opening have been reported to differ depending on whether WWR is triggered by increased evaporative demand or by reduced water supply (Brodribb and McAdam, 2011; Buckley et al., 2011; Binstock et al., 2023; Pichaco et al., 2024; Zait et al., 2024). Perturbations driven by VPD predominantly affect the demand side of the hydraulic continuum, leading to rapid changes in transpiration and leaf water potential (Buckley et al., 2011; Merilo et al., 2017), whereas soil drying primarily constrains

water supply to the leaf and declining soil water potential reduces root water uptake and hydraulic conductivity along the soil-plant pathway. Mechanistic models and experimental observations suggest that these distinct perturbation modes can produce different patterns of turgor redistribution among mesophyll, epidermal, and guard cells, and thus generate condition-dependent differences in WWR dynamics (Buckley et al., 2011; Koehler et al., 2023). These findings strengthen the view that WWR arises from whole-tissue and whole-plant hydraulic and mechanical interactions, rather than from a simple response of guard cells to leaf water status. Comparative studies across species and genotypes with divergent leaf structure, differing in stomatal size and density, pavement cell geometry, mesophyll porosity, and trichome abundance, therefore, provide a powerful approach to investigate the anatomical and biomechanical determinants of WWR. Integrating quantitative anatomical characterization with measurements of transient stomatal behaviour under precisely defined perturbations of plant water status can help identify which structural features most strongly influence WWR duration, amplitude, and recovery. In this regard, we hypothesize that Arabidopsis lines differing in the geometry of pavement and guard cells will show a distinct pattern of WWR, reflecting the influence of leaf anatomical traits on the duration, amplitude, and recovery of this physiological process.

Although the primary aim of this project was to investigate whether anatomical traits of epidermal cells, including pavement cell shape and size, as well as stomatal characteristics, contribute to the dynamics of the WWR, the emerging evidence for the roles of mesophyll architecture and surface structures (Tomás et al., 2013; Amada et al., 2017; Amada et al., 2020; Baillie and Fleming, 2020; Zhu et al., 2024), underscores the need to move beyond guard cells. In this regard and because the *A. thaliana* lines used in this work were previously selected based on differences in stomatal size and density, the absence of trichomes in one of the lines (*tmm1*) introduced uncertainty regarding how this morphological feature might influence the plant's response to hydraulic stress. In addition, the mesophyll architecture of the selected lines and their potential modification under water-limiting conditions remained largely unresolved. Investigating the mesophyll modification posed a particular challenge because conventional anatomical methods typically involve invasive sample preparation steps that can alter the native structural organization of the leaf tissue. To overcome these limitations, we conducted two separate studies. First, we evaluated the role of trichomes in plant performance under hydraulic stress. Second, we developed a novel imaging approach to visualize mesophyll structure with minimal sample manipulation, enabling a reliable comparison of mesophyll anatomical properties between control and drought-stressed plants. Therefore, this approach provided a comprehensive view of the leaf anatomy in the studied lines, providing a solid foundation for subsequent molecular analyses.

1.1. Studied anatomical features

1.1.1. Stomata size and density

The stomata on the leaf surface serve as the primary structures through which plants interact with their surrounding environment. Stomata are microscopic pores, formed by two

specialized cells called guard cells (Chaffey, 2014; Taiz et al., 2015). In mature guard cells, the cell wall exhibits pronounced spatial heterogeneity, with the pore-facing wall significantly thicker and mechanically stiffer than the opposite side, and with radially oriented cellulose microfibrils around the stomatal aperture (Chaffey, 2014). As turgor pressure increases, the expansion of the thinner, outer walls causes the guard cells to bend apart, resulting in the opening of a stomatal pore in between. A reduction in turgor pressure reverses this deformation and results in stomatal closure. Stomata serve as the primary gateways for gas exchange, regulating the trade-off between CO_2 uptake for photosynthesis and water loss via transpiration (Hetherington and Woodward, 2003; Jones, 2013; Zait et al., 2024). The importance of this function becomes even more pronounced under water stress, when plants must conserve water while still allowing CO_2 uptake for photosynthesis. Therefore, water potential in plants is the key factor, controlling stomata opening/closure, whereas other factors such as light, temperature and CO_2 (in surrounding air and inner leaf) also affect stomata responses. The closure of stomata in response to a decline in water potential, whether in response to dry air or dry soil, has been extensively studied (Hetherington and Woodward, 2003; Drake et al., 2013; Giday et al., 2013; Dow and Bergmann, 2014; Buckley, 2019; Jalakas et al., 2021; Caine et al., 2023; Driesen et al., 2023b; Haworth et al., 2023; Crawford et al., 2025). Under sufficient water availability, the soil has a higher total water potential (it is less negative) than the plant and its cells. As a result, water flows from the soil into the root cells (D'Ario et al., 2021; di Santo et al., 2025; Farolfi et al., 2025). Within the plant, the total water potential in the xylem is higher (less negative) than in surrounding cells, largely because the cells have a more negative osmotic potential; therefore, water moves from the xylem into the cells. In dry soil, soil water potential becomes more negative, and the water is held more tightly to the soil particles. Therefore, the flux of water into the roots is reduced. As a result, the water supply from the soil may no longer be sufficient to keep up with the water loss through transpiration, and plant water potential declines and becomes more negative. The drop in water potential promotes the production of abscisic acid (ABA) (Manzi et al., 2015; McAdam and Brodribb, 2018; Cotelle and Leonhardt, 2019; Negin et al., 2019). ABA in guard cells triggers signalling pathways (including an increase in cytosolic Ca^{2+}), thereby activating anion channels and K^+ efflux channels, leading to a loss of solutes from guard cells. By decreasing the solute concentration in guard cells, water exits from the guard cells, turgor pressure decreases, and stomata close (Merilo et al., 2017; Cotelle and Leonhardt, 2019).

Stomatal closure is also triggered by dry air, a response governed by the VPD. VPD represents the difference between the moisture levels within the leaf (typically assumed to be water-saturated) and the relatively dry ambient air. When the air is dry, this difference widens, creating a steep vapor pressure gradient that increases the evaporative demand on the leaf. If this demand exceeds the supply capacity, it can lead to hydraulic failure: a situation in which the tension in xylem water causes air bubbles to form (embolisms), blocking water transport, resulting in an even more severe water deficit. To mitigate this risk, one of the primary physiological responses to high VPD is stomatal closure, which restricts water vapor exit to

preserve the plant's internal hydraulic integrity. High VPD is sensed in part through its effects on leaf water status and cell turgor, which act as signals for stomatal regulation. Elevated VPD stimulates ABA synthesis in mesophyll cells (McAdam and Brodribb, 2018; Jalakas et al., 2021), the newly synthesized ABA is transported to the guard cells, activates signalling pathways promoting stomatal closure, analogous to those engaged by ABA produced during a soil water deficit.

Key stomatal features, such as size (Drake et al., 2013; Giday et al., 2013) and density (Pathare et al., 2020; Sakoda et al., 2020a; Caine et al., 2023; Haworth et al., 2023) define the capacity of leaves to adjust gas exchange to environmental conditions. Genetically modified plants exhibiting increased stomatal density demonstrated enhanced photosynthetic rates and greater biomass accumulation (Tanaka et al., 2013; Sakoda et al., 2020b). Stomatal density has changed over evolutionary timescales in response to variations in atmospheric CO_2 concentrations (Franks and Beerling, 2009; Haworth et al., 2021). In many plants species, a reduction in stomata density is the mechanism to reduce water lose and enhance water-use efficiency (WUE) under drought stress (Chua and Lau, 2024). However, there are conflicting reports exist regarding changes in stomatal density in plants exposed to drought stress. Under drought stress, rice cultivars exhibiting reduced stomatal density showed decreased net photosynthesis, while drought-resistant cultivars, with high photosynthetic rates under drought, showed an increased stomatal density on the upper leaf side (Adaxial surface) (Lertngim et al., 2023). Drought-induced reductions in stomatal density in *Populus balsamifera* were associated with improved water-use efficiency (Hamanishi et al., 2012). However, basil (*Ocimum basilicum* L.) leaves grown under drought stress developed higher stomata density with higher WUE than well-watered plants although the plants yield was reduced (Driesen et al., 2023a). Moderate drought stress was associated with increased stomatal density, whereas severe drought stress resulted in a reduction in stomatal density, and WUE exhibited a positive relationship with stomatal density, increasing under moderate water deficit but declining under severe drought conditions (Xu and Zhou, 2008). While, stomata density increased in tomato (*Lycopersicon esculentum* Mill.) and sweet pepper (*Capsicum annuum* L) by high humidity (Bakker, 1991), Arabidopsis showed increased stomata density under low humidity (Tulva et al., 2024).

It has been suggested that stomata size is the key factor in the regulation of transpiration (Giday et al., 2013) explaining that smaller stomata have shorter diffusion paths and lower pore volumes, provide them faster opening and closure kinetics in response to fluctuating water status (Rui et al., 2024) which is considered advantageous under rapidly fluctuating environmental conditions (Drake et al., 2013). In the studies mentioned above, Xu and Zhou (2008) reported an inverse relationship between stomatal size and stomatal density. This finding is consistent with the study by Driesen et al. (2023b), where basil leaves grown under drought stress exhibited smaller stomata alongside an increase in stomatal density.

The trade-off between stomatal density and stomatal size represents one of the main evolutionary strategies developed by plants in response to fluctuating environment (Xu and Zhou, 2008; Franks and Beerling, 2009; Lei et al., 2022; Caine et al., 2023; Haworth et al.,

2023; Liu et al., 2023), enabling high gas exchange capacity while maintaining an efficiently fast response to changes in the surrounding environment (Zwieniecki and Boyce, 2014). However, this trade-off becomes more complex when considering that larger guard cells can modify pore dimensions more efficiently through partial opening, thereby exerting stronger control over stomatal aperture and transpiration (Zwieniecki and Boyce, 2014). The complexity is further highlighted by findings in rice, where lines with the smallest stomata at high density had higher net assimilation rate in response to elevated CO_2, whereas under drought conditions, lines combining the smallest stomata with the lowest density were better able to cope with limited water, exhibiting higher water-use efficiency (WUE) and greater biomass per plant than other lines (Pitaloka et al., 2022). These observations, which indicate that adjustments in stomatal size and density are species-specific and influenced by varying environmental stimuli and stress intensities, raise important questions about the mechanisms underlying stomatal development and the functional significance of stomatal traits in environmental adaptation. As discussed above, addressing these questions may require incorporating additional leaf structural traits, such as epidermal and mesophyll properties, and broadening the focus beyond guard cells alone.

1.1.2. Trichomes

Trichomes indirectly affect stomatal responses by adjusting inner leaf temperature (Gasparini et al., 2021). Trichome density has been proposed as an adaptive trait that can increase or decrease in response to climatic conditions, helping plants cope with variation in temperature and precipitation (Zhu et al., 2024). Moreover, trichome layers adjust the leaf energy balance by affecting the heat-diffusion resistance and modifying leaf temperature. While dense trichome coverage reduces leaf temperature in warm environments by increasing reflectance (Chen et al., 2022), trichomes may also serve as an insulating boundary layer under cooler conditions, elevating leaf temperature and subsequently enhancing photosynthetic performance (Amada et al., 2023). Although it has been suggested that trichomes play a role in the leaf energy balance, allowing for enhanced assimilation rates (Gasparini et al., 2021), the available evidence so far suggest that their role is relatively small (Amada et al., 2017) and depends on multiple factors such as elevation, the thickness of the trichomes layer, light intensity, and stomata dynamics (Amada et al., 2023). Despite the small, but positive role of trichomes on photosynthesis and carbon assimilation, it has been shown to have a non-significant (Amada et al., 2017) or negative effect (Amada et al., 2023) on water use efficiency. Trichomes and other surface structures may participate in stomata responses through their effects on boundary layer conductance, evaporative fluxes, and leaf water relations. By increasing boundary layer thickness or altering leaf energy balance, trichomes can modify the microclimate directly above the epidermis, thereby influencing the rate of change in leaf water potential during transitions in ambient humidity or radiation. Furthermore, trichome-bearing and glabrous plants often differ in epidermal and mesophyll development, implying coordinated anatomical adjustments that could in further affect mechanical interactions at the stomatal complex.

1.1.3. Mesophyll

Beyond epidermal structure, evidence increasingly points to a significant contribution of mesophyll architecture and internal anatomy to stomatal responses to water status. The anatomy of the mesophyll plays a key role in determining the efficiency of photosynthesis by affecting the CO_2 diffusion in substomatal cavities (Terashima et al., 2010; Tholen et al., 2012; Théroux-Rancourt et al., 2021a). The structure of palisade and spongy layer (Tomás et al., 2013), the density of the parenchyma cells (Flexas et al., 2012), and organization of intercellular air spaces (Borsuk et al., 2022) define the diffusion of CO_2 through the leaf, and consequently the photosynthetic rate. The variations in mesophyll cell size, packing density, intercellular airspace, and the architecture of palisade and spongy layers can influence the speed and magnitude of leaf water potential changes and thereby affect leaf function in response to water availability (Buckley et al., 2015; Fujii et al., 2023). In addition, mesophyll tissue has been shown to influence stomatal development and patterning (Baillie and Fleming, 2020; Pathare et al., 2020), further highlighting the importance of incorporating mesophyll characteristics into studies of stomatal responses.

Given the central focus of my work on stomatal responses and considering that the selected lines already differ in stomatal density and size, an important question concerned the organization and architecture of the mesophyll in these lines. As noted above, stomatal density has been reported to correlate with mesophyll organization. Therefore, we asked how the anatomical properties of the mesophyll change under water stress in lines with altered stomatal characteristics. Addressing this question may provide a more comprehensive understanding of leaf anatomical variation across these lines and enable a more complete interpretation of the correlation between mesophyll organization and epidermal dynamics.

In this context, a key challenge is to obtain high-resolution image of internal leaf anatomy under hydrated and stressed conditions. Conventional histological techniques and clearing methods often require fixation, sectioning, or chemical treatments that can distort the native geometry of mesophyll tissues (Tholen et al., 2008; Sage and Sage, 2009). These distortions are particularly problematic for mesophyll tissues, where intercellular airspace fraction and cell packing density are critical determinants of hydraulic conductance and stomatal signalling. Therefore, we developed a method for *in situ* imaging of the mesophyll and apply this technique to the selected Arabidopsis lines under both control and water-stress conditions in order to characterize potential anatomical differences.

2. Structure of the Research Studies

This thesis consists of three complementary research studies. When taken together, they demonstrate how leaf anatomical traits affect plant responses to water fluctuations and stomatal dynamics.

In the first publication we aimed to investigate how stomatal traits (size and density) along with absence/presence of trichomes influence photosynthetic efficiency in *A. thaliana* under well-watered and drought-stressed conditions. By comparing the three different Arabidopsis lines with different stomatal traits and the absence/presence of trichomes, this experiment showed that higher stomata density and the presence of trichomes enhanced photosynthetic performance under optimal water availability. However, under drought stress, the control line of Arabidopsis (Col-8) maintained the photosynthetic yield more efficiently than lines with inherently high stomata density or without trichomes. This result highlights that the anatomical modifications induced by drought stress is a more critical determinant of photosynthetic resilience than the plant's inherent anatomy. Furthermore, this study identifies a significant correlation between anatomical plasticity and photosynthetic yield, establishing variations in stomatal density as a primary driver of carbon assimilation efficiency.

My contributions to this paper included the cultivation and maintenance of the experimental plants under controlled conditions, the collection of physiological and anatomical data through gas-exchange measurements and microscopy, and the subsequent data analysis using statistical software and modelling approaches. Additionally, I drafted the full manuscript, coordinated the submission process to the journal, and managed subsequent revisions based on reviewer feedback to facilitate successful publication.

Maryam Alsadat Zekri and Ingeborg Lang. 2024. Lack of trichomes and variation in stomata properties influence the quantum efficiency of photosynthesis in Arabidopsis. Environmental and Experimental Botany. 227: 105948. 10.1016/j.envexpbot.2024.105948

The second publication introduced a novel cryo-fixation method to analyse mesophyll anatomy in drought-stressed Arabidopsis leaves because any contact with water of the dehydrated tissue must be avoided. However, conventional light microscopy usually requires sample preparation steps such as dehydration or chemical fixation, which can introduce artifacts and alter cellular or tissue morphology. This problem becomes even more severe when the plants' hydraulic status is the target of the study. Thus, despite the importance of phenotypic markers, anatomical studies face significant challenges. We used an innovative tool for scanning electron microscopy and cryo-fixation without any water contact of the samples on the five Arabidopsis lines. We applied short- and long-term drought stress conditions to gain an understanding of the mesophyll alterations of the studied lines under the water stress. Interestingly, the results showed that, in short-term drought stress plants increased the leaf thickness, particularly in the palisade layer, while long-term drought stress resulted in the shrinkage of the spongy mesophyll.

My contributions to this paper included supervising our master's student (Carina Leimhofer) during image acquisition, as well as the cultivation and maintenance of the Arabidopsis plants

under controlled conditions. I also conducted the data analysis using statistical software and modelling approaches. Additionally, I drafted the full manuscript, coordinated the submission process to the journal as the corresponding author, and managed subsequent revisions based on reviewer feedback to facilitate successful publication.

Maryam Alsadat Zekri, Carina Leimhofer, Nicole Drexler, and Ingeborg Lang. 2025. A rapid freezing method to determine tissue layer thickness in drought-stressed leaves. Journal of Microscopy. 297: 316-24. DOI: 10.1111/jmi.13272

In the third study, we investigated the dynamics of the **stomatal 'wrong-way response'**, specifically examining how epidermal cell anatomy modulates these transient opening events. Five Arabidopsis cell lines with contrasting stomatal size, stomatal density, and pavement cell geometry were analysed in this comprehensive study. The WWR was induced using a sudden decrease in air humidity and leaf excision. Stomatal conductance was measured at high temporal resolution using infrared gas analysis, and methodological corrections were applied to account for chamber mixing and water desorption effects. Epidermal anatomical traits were characterized using scanning electron microscopy, with quantitative extraction of pavement cell size and shape parameters. The correlation between epidermal traits and transient stomatal opening highlighted the significant role of epidermal cell size in constraining the guard-cell function. These findings strengthen the idea that stomatal responses cannot be fully understood by examining guard cells alone but instead require holistic analysis of the surrounding epidermal tissue.

My contributions to this paper encompassed the cultivation and maintenance of Arabidopsis plants under controlled environmental conditions, as well as data acquisition, which included gas exchange measurements, acquisition of scanning electron microscopy (SEM) images, and detailed anatomical analyses. I also assisted with data analysis using statistical software and modelling approaches to interpret the physiological and structural datasets. Additionally, I drafted the full manuscript and participated in the submission process to the journal, ensuring compliance with editorial guidelines and facilitating peer review.

Maryam Alsadat Zekri, Daniel Tholen, Lukas Koller, Klara Voggeneder, Ingeborg Lang, Guillaume Theroux-Rancourt. 2026. Beyond Guard Cells: Epidermal Anatomy Determines Transient Stomatal Opening Under Water Stress. Physiologia Plantarum (Submission ID c9616141-c9d4-4790-9759-a0a9d91c2a15)

2.1. Lack of trichomes and variation in stomata properties influence the quantum efficiency of photosynthesis in Arabidopsis

M. A. Zekri*[1] and I. Lang[1]
[1] Dept. of Functional and Evolutionary Ecology, Faculty of Life Sciences, University of Vienna, Djerassiplatz 1, Vienna A-1030, Austria
*Corresponding author: Maryam.alsadat.zekri@univie.ac.at

Environmental and Experimental Botany 2024 Vol. 227 Pages 105948
DOI: https://www.sciencedirect.com/science/article/pii/S009884722400306X

Received 20 June 2024
Revised 17 August 2024
Accepted 18 August 2024
Available online 22 August 2024

Citations:

- Kang Wang, Yongkang Wang, Xiaoyan Tang, Na Liao, Danni Yang, Wenjie Wang, Lingyun Yuan, Jinfeng Hou, Jianqiang Wu, Chenggang Wang & Guohu Chen. 2025. Genome wide identification and characterization of EPFL genes and its potential association with male sterility in *Brassica rapa*. Scientific Reports volume 15, Article number: 24033. https://doi.org/10.1038/s41598-025-09555-1
- Xiao, Yao, Zhong-Wei Zhang, Xin-Yue Yang, Lin-Bei Xie, Li-Ping Chen, Yang-Er Chen, Ming Yuan, Guang-Deng Chen, and Shu Yuan. 2025. Effect of Singlet Oxygen on the Stomatal and Cell Wall of Rice Seedling Under Different Stresses. International Journal of Molecular Sciences 26, no. 17: 8382. https://doi.org/10.3390/ijms26178382
- Wang, Yan, Ning Peng, Binbin Liu, Yingbin Yang, Chao Yue, Wenfang Hao, and Junhao He. 2025. Physiological and Flavonoid Metabolic Responses of Black Locust Leaves to Drought Stress in the Loess Plateau of China. Forests 16, no. 4: 695. https://doi.org/10.3390/f16040695

Authors contribution

MAZ conceived the project, acquired the imaging and physiological data, analysed the data, and wrote the first draft of the manuscript with contributions from IL. MAZ and IL addressed the revisions and approved the final version of the manuscript.

Environmental and Experimental Botany 227 (2024) 105948

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Research paper

Lack of trichomes and variation in stomata properties influence the quantum efficiency of photosynthesis in Arabidopsis

Maryam Alsadat Zekri *, Ingeborg Lang

Dept. of Functional and Evolutionary Ecology, Faculty of Life Sciences, University of Vienna, Djerassiplatz 1, Vienna A-1030, Austria

ARTICLE INFO

Keywords:
Arabidopsis
Stomata density
Stomata size
Trichomes
Fv/Fm′
Drought stress

ABSTRACT

This study investigates how the absence of trichomes and variations in stomatal properties affect the quantum efficiency of photosynthesis in *Arabidopsis thaliana* during drought stress. We analyzed three genotypes: Col-8 (with trichomes and lower stomatal density), *epf1epf2* (with higher stomatal density), and *tmm-1* (lacking trichomes and altered stomatal characteristics) to determine the influence of these anatomical traits on photosynthetic performance. Under well-watered conditions, *epf1epf2* and *tmm-1* exhibited higher photosynthetic efficiency (Fv′/Fm′) compared to Col-8. During drought stress, Col-8 maintained stable Fv′/Fm′, while *epf1epf2* and *tmm-1* experienced significant reductions. Our findings indicate that the presence of trichomes and higher stomatal density positively impacts photosynthetic efficiency under optimal watering while the presence of trichomes becomes less crucial under drought stress. Efficient adjustment of stomatal density and size under drought conditions plays a more significant role. These insights emphasize the importance of considering anatomical traits in breeding programs to enhance drought resistance and photosynthetic performance in plants.

1. Introduction

Plants exhibit a remarkable ability to adapt to their environment through modifications in their anatomical features. Various anatomical properties, such as flower structures, leaf characteristics, and root features, contribute to a plant's ability to adapt to fluctuating environmental conditions. Among these features, stomata and trichomes play pivotal roles in mediating plant responses to environmental conditions. Modifications in stomata and trichomes have been suggested as strong adaptive mechanisms to environmental stresses (Amada et al., 2020, Gasparini et al., 2021). Stomata, on one hand, allow CO_2 to enter the leaf mesophyll for photosynthesis and, on the other hand, facilitate water loss from the leaf. These dual functions make stomata crucial for determining a plant's water-use efficiency (WUE), which is the amount of biomass produced per unit of water taken up by the plant. The physical properties of stomata change significantly in response to environmental factors. For instance, reduced stomatal density has been observed under high CO_2 levels and low light intensity (Bertolino et al., 2019, Westbrook and Mcadam., 2021, Xu et al., 2016), and a reduction in stomatal size has been recorded under low water availability (Taylor et al., 2012, Xu and Zhou, 2008). Lower stomatal density has been associated with higher drought resistance by restricting transpiration in *Populus nigra* (Li et al., 2021) and improving water-use efficiency in barley (*Hordeum vulgare*) (Hughes et al., 2017) and rice (*Oryza sativa*) (Caine et al., 2023, Pitaloka et al., 2022a). Conversely, the olive cultivar (*Olea europaea* L.) with higher stomatal density showed improved photosynthesis under water scarcity, correlated with higher CO_2 supply and assimilation (Ennajeh et al., 2010). In optimal environments without stresses such as drought, higher stomatal density and small stomatal size result in higher photosynthetic rates due to increased stomatal conductance (Lertngim et al., 2022). However, under drought stress, the quantum efficiency of photosynthesis decreases less severely in plants with lower stomatal density and smaller stomata (Pitaloka et al., 2022a). Nevertheless, there is a lack of knowledge explaining how higher stomatal density and smaller stomata, advantageous under optimal conditions, respond to drought stress and the extent to which alterations in stomatal density and size contribute to photosynthetic efficiency.

Trichomes, specialized elongated epidermal cells, are another physical property of plant leaves that increase surface roughness and enhance boundary layer thickness, thereby reducing its conductance although the mechanisms by which trichomes improve plant resistance to environmental stresses are not fully understood. It has been suggested that trichomes have no direct effect on total leaf gas exchange (Amada

* Corresponding author.
E-mail address: maryam.alsadat.zekri@univie.ac.at (M.A. Zekri).

https://doi.org/10.1016/j.envexpbot.2024.105948
Received 20 June 2024; Received in revised form 17 August 2024; Accepted 18 August 2024
Available online 22 August 2024

et al., 2017, Pierce et al., 2021) while recent studies have revealed that trichomes indirectly affect gas exchange by influencing light absorbance and leaf temperature (Gasparini et al., 2021). For example, It has been demonstrated that in cold environments, trichomes increased leaf temperature, which eventually improved photosynthesis yield (Amada et al., 2020).

To quantify plant stress, we measured the quantum efficiency of photosynthesis, a commonly used measure of the operating efficiency of photosystem II. This is calculated in light-adapted leaves as Fv′/Fm′ = (Fm′ - F0′) / Fm′. Fm′ represents the maximum chlorophyll fluorescence when all photosynthetic reaction centers are closed after a pulse of saturating light. F0′ is the minimum chlorophyll fluorescence in light-adapted leaves, which is usually smaller than its equivalent, F0 (the minimum chlorophyll fluorescence in dark-adapted leaves), due to non-photosynthetic quenching. Generally, when plants suffer from environmental stresses such as drought, the damage to the photosynthetic reaction centers results in more light energy escaping from the leaf as fluorescence, thereby decreasing Fv′/Fm′.

This study focuses on the model plant *Arabidopsis thaliana* to investigate how variations in stomatal properties and the presence or absence of trichomes influence photosynthetic efficiency, particularly under drought stress. Moreover, non-photochemical quenching (NPQ) and non-regulated non-photochemical quenching (PhiNO), which represent two alternative pathways for the dissipation of excess light energy within the leaf, along with the electrochromic shift (ECS), an indicator of the efficiency of electron transport within the photosynthetic apparatus, have been measured to assess plant stress. By comparing these genotypes under well-watered and drought-stressed conditions, we aim to illustrate the relationships between these anatomical traits and photosynthetic performance. Understanding these relationships is crucial for improving crop resilience to environmental stressors. Insights gained from this study could inform breeding programs aimed at enhancing drought resistance and optimizing photosynthetic efficiency in plants.

2. Material and methods

2.1. Plant material

Seeds of three genotypes of *A. thaliana* _Col-8, *epf1epf2*, and *tmm-1*_ were obtained from the Nottingham Arabidopsis Stock Centre. Based on the NASC database, the genotypes were selected based on distinctive anatomical features. Col-8 was selected as the wild-type with lower stomata density and larger stomata than *epf1epf2* and *tmm-1* in an optimal condition. *tmm-1* (Too Many Mouths) mutant has clusters of stomata with significantly higher stomata count than wild type (Nadeau and Sack, 2002). Similarly, *epf1epf2* shows higher stomata density and smaller stomata (franks et al., 2015) than Col-8. While Col-8 and *epf1epf2* develop leaves with trichomes, *tmm-1* exhibits glabrous leaves without trichomes (Zhang et al., 2021). Ten plants of each genotype were grown in separate pots (13 cm in diameter) containing a mix of 50 % indoor plant soil (Kranzinger GmbH, Austria), 33 % quartz sand, and 17 % Perlite®. The plants were cultivated under stable conditions in a growth room (Conviron MTPS 120, Manitoba, Canada). All pots were fully saturated with water and left for one day before sowing the seeds. After germination, the pots were watered daily for four weeks. The growth room conditions were set to a day/night temperature of 23/18 °C, 60 % relative humidity (RH), and a light intensity of 130–150 μmol m^{-2} s^{-1}. The photoperiod was set to 14 hours of daylight.

2.2. Stress experiment

At the end of the four-week cultivation period, irrigation was ceased for 24 hours to drain excess water. Five pots were then selected as the control group (Group One) and continued to be irrigated daily as before. In the other five pots (Group Two), irrigation was stopped, and these plants were designated as stressed plants by drought. The environmental conditions in the growth room remained unchanged. A soil moisture meter (Aicevoods, model PH3) was used to measure relative soil humidity. Two weeks after the treatment, MultispeQ V2.0 (PHOTOSYNQ INC., USA) was used to measure Fv′/Fm′, the quantum yield of photosynthesis (ΦII), NPQ, PhiNO, ECS, and leaf thickness in 20 randomly selected leaves. The LI-600 compact Porometer with Pulse-Amplitude Modulation (PAM) fluorometers (Germany) was used to measure stomatal conductance and boundary layer conductance in 20 leaves per genotype. To avoid the diurnal and circadian effect on the measurements (Dodd et al., 2007, Xu et al., 2022), we conducted all the measurements in the morning, three hours after the start of daylight.

Additionally, to illustrate the trichomes, microscopy images of the leaf surface were obtained using a Nikon Eclipse Ni microscope from six-week-old leaves in control plants.

2.3. Stomata density and size

To calculate stomatal density and size, five leaves were selected, and nail polish imprints were made of the abaxial (lower epidermis) and adaxial (upper epidermis) surfaces. Three random spots on each imprint were imaged using a Nikon Eclipse Ni microscope, and the number of stomata was counted with ImageJ/Fiji (version 2.14.0.1.54 f). The length of all stomatal apertures in these three random spots was measured using ImageJ/Fiji following the method described by Savvides et al. (2011) (Savvides et al., 2011).

2.4. Statistical analysis

The normality of the data was tested using the Shapiro–Wilk test, which revealed a non-normal distribution for all data sets. Consequently, the nonparametric Kruskal-Wallis test was applied for statistical analysis using the R package (2023.09.0), as it is the nonparametric equivalent of a one-way ANOVA. In this study, the Kruskal-Wallis test was used to evaluate the null hypothesis that drought stress does not significantly change stomatal density, stomatal size, or Fv′/Fm′ after two weeks of drought stress. Additionally, detected differences between group means, identified through the Kruskal-Wallis test, were further analyzed using Tukey's post hoc test to determine significant differences in the R package (2023.09.0). Generalized linear regression was conducted in the R package (version 4.3.2) to illustrate the linear relationship between stomatal anatomical properties and Fv′/Fm′ using the equation Y = a + bX, where 'a' represents the y-intercept, 'b' is the slope, and Y and X are the coordinates.

3. Results

At the end of the treatment, soil humidity in the control pots was 89 %, while in pots where irrigation has been ceased, it reached to 32 %, representing optimal and low soil humidity, respectively (Rivero et al., 2014).

Image acquisition of leaf surfaces showed the present of trichomes in Col-8 (Fig. 1a and d) and *epf1epf2* (Fig. 1b and e) and the lack of trichomes in *tmm-1* (Fig. 1c and f).

Fluorescence measurements showed that Fv′/Fm′ in control *epf1epf2* and *tmm-1* was significantly higher than in control Col-8 ($p<0.001$) (Fig. 2a). Within each genotype, drought stress did not significantly change Fv′/Fm′ in Col-8, whereas in *epf1epf2* and *tmm-1*, drought stress significantly reduced Fv′/Fm′ (Fig. 2a). The quantum yield of photosynthesis in control Col-8 was significantly lower than in control *epf1epf2* and *tmm-1* ($p<0.001$), but after drought stress, a significant increase in quantum yield in Col-8 ($p<0.05$) led to no difference between the stressed genotypes (Fig. 2b). Drought significantly decreased NPQ in Col-8 ($p<0.05$), while it caused an increase in *epf1epf2* ($p<0.1$) (Fig. 2c). While, PhiNO decreased in stressed Col-8, *epf1epf2*, and *tmm-1* ($p<0.05$, $p<0.01$ and $p<0.01$, respectively) (Fig. 2d), ECS decreased only in Col-8 ($p<0.05$) (Fig. 2e).

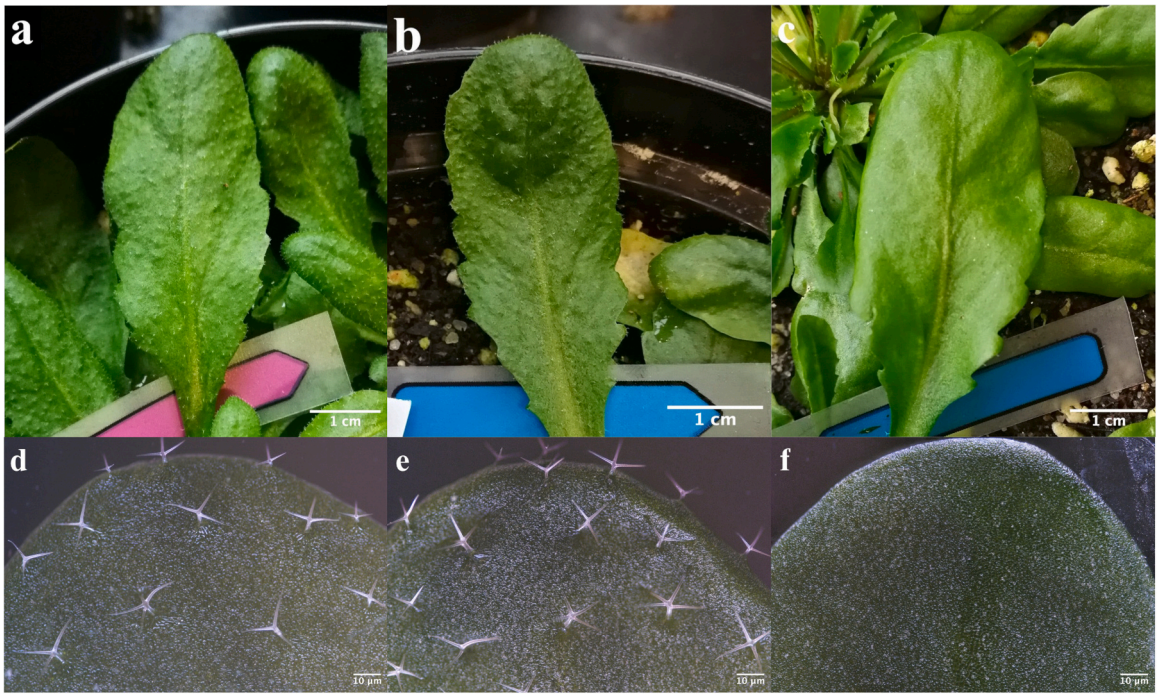

Fig. 1. - The illustration of trichome in Arabidopsis leaves: Col-8 (a and d) and *epf1epf2* (b and e) exhibit trichomes, whereas *tmm-1* (c and f) shows a lack of trichomes. Images a-c display macroscopic leaf images (scale bar: 1 cm), and images d-f present light microscopy images of leaves (scale bar: 10 µm).

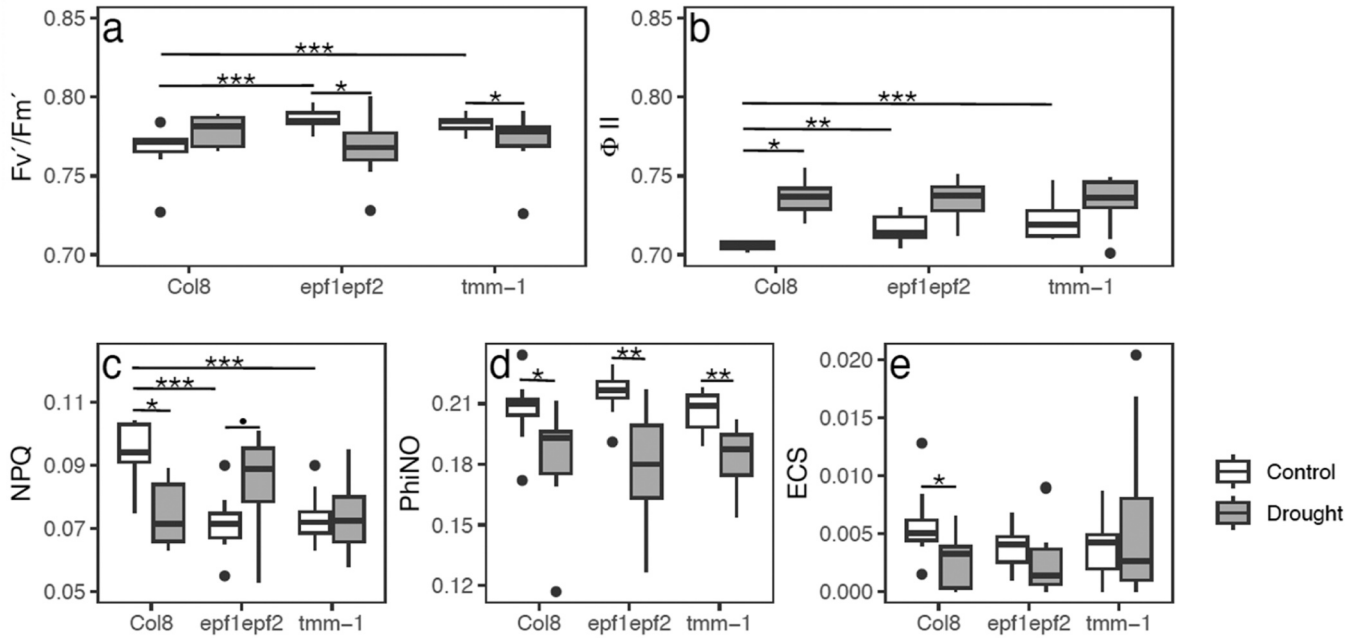

Fig. 2. - a) the efficiency of photosynthesis (Fv/Fm), b) the quantum yield of photosynthesis (**ΦII**), c) non photochemical quenching (**NPQ**), d) non-regulated non-photochemical quenching (**PhiNO**), and e) electrochromic shift (**ECS**) in control and stressed plants of Col-8, *epf1epf2*, and *tmm-1*. ***=pvalue <0.001, **=pvalue <0.01, *=pvalue <0.05, •=pvalue <0.1. Number of measurements: 20 leaves.

Gas exchange measurements with the Prometer indicated that stomatal conductance and boundary layer conductance were not different between control genotypes (Fig. 3a and b).

Well-watered *epf1epf2* and *tmm-1* exhibited higher stomatal density ($p<0.001$) than Col-8. After drought stress, *epf1epf2* showed higher stomatal density than Col-8, while there was no difference between Col-8 and *tmm-1* (Fig. 4a). Within the genotypes, drought stress increased stomatal density in Col-8 ($p<0.01$) but reduced it in *epf1epf2* ($p<0.01$) and *tmm-1* ($p<0.01$) (Fig. 4a). Well-watered *epf1epf2* and *tmm-1* had smaller stomatal size compared to well-watered Col-8 ($p<0.001$). After drought stress, *epf1epf2* showed the smallest stomatal size, smaller than Col-8 and *tmm-1*($p<0.001$), while *tmm-1* showed the largest stomata compared to Col-8 and *epf1epf2* (Fig. 4b). Within the genotypes, drought stress reduced stomatal size in Col-8 and *epf1epf2* ($p<0.001$), while it increased stomatal size in *tmm-1* ($p<0.001$) (Fig. 4b).

Additionally, drought stress significantly increased the inner leaf temperature in *tmm-1* ($p<0.01$) (Fig. 4c). Although the leaf thickness in stressed Col-8 increased significantly compared to control plants

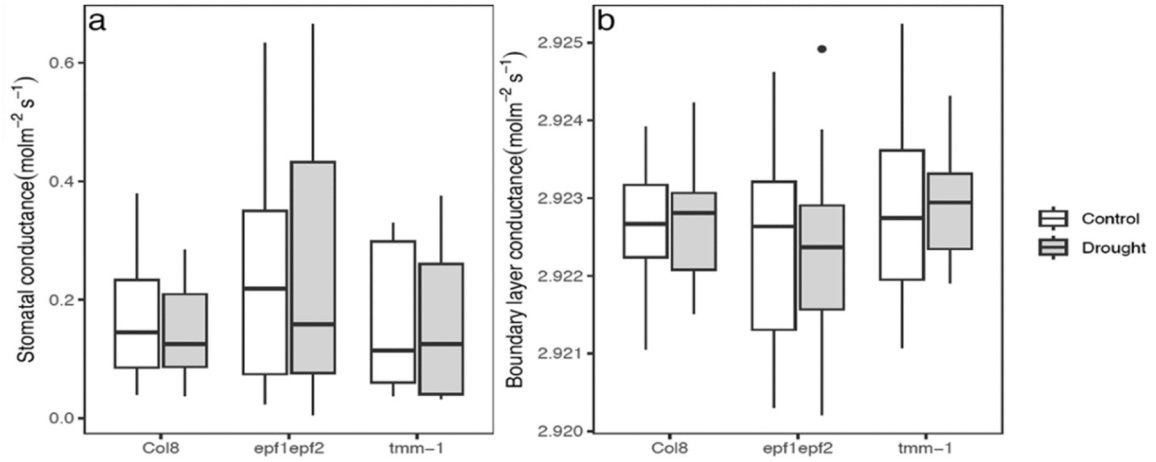

Fig. 3. - gas exchange measurements showed a) stomatal conductance and b) boundary layer conductance in well-watered and stressed Col-8, *epf1epf2*, and *tmm-1*. ***= pvalue<0.001, **= pvalue<0.01, *= pvalue<0.05. Number of measurements: 20 leaves.

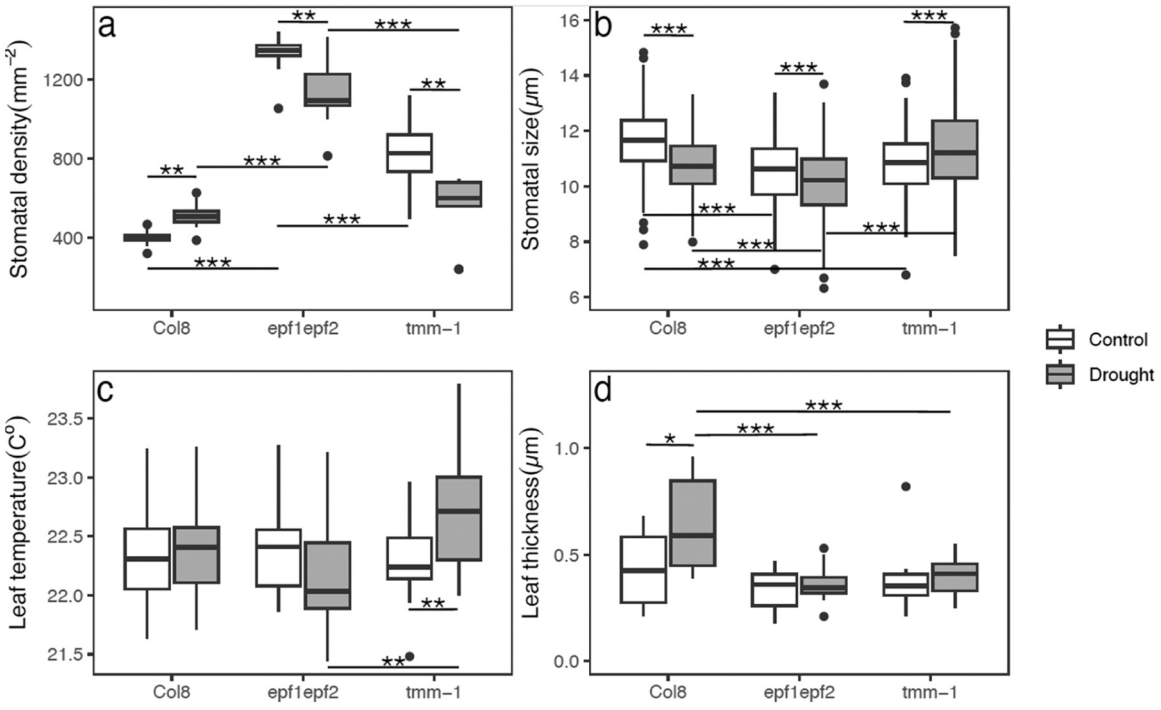

Fig. 4. - a) the total number of stomata was counted to calculate stomata density (mm^{-2}) in stressed and control plants. Number of measurements: 5 leaves. b) size of the stomata was calculated in stressed and control plants. Number of measurements: 5 leaves. ***= pvalue<0.001, **= pvalue<0.01, *= pvalue<0.05.

(p<0.05), it remained unchanged by drought stress in both *epf1epf2* and *tmm-1* (Fig. 4d).

Since Fv′/Fm′ in Col-8 did not change by drought stress, we used generalized linear models (glm) for *epf1epf2* and *tmm-1* to show the correlation between Fv′/Fm′ and stomatal density. The linear regression model showed a significant correlation between decreasing stomatal density and decreasing Fv′/Fm′in *epf1epf2* ($r^2 = 0.65$, $p < 0.001$) (Fig. 5a) and *tmm-1* ($r^2 = 0.84$, $p < 0.001$) (Fig. 5b).

4. Discussion

In this study, we investigated the effects of variations in stomatal properties on photosynthesis in *A. thaliana* under both well-watered and drought stress conditions. Additionally, we examined how the presence of trichomes contributes to the photosynthesis under these conditions. Our results demonstrated the significant contribution of these anatomical traits in photosynthetic performance and stress response of different Arabidopsis genotypes.

The lifespan of Arabidopsis typically ranges from 7 to 8 weeks from seed germination to seed production. However, this duration is reduced to 5–6 weeks under drought stress conditions (Rivero et al., 2014). Considering the 2–3 weeks required for seed germination and plant development, we deemed a 2-week treatment period sufficient to observe anatomical variations in Arabidopsis.

Our study showed that under well-watering regime, the inner leaf temperature of *tmm-1* was not significantly different from Col-8 and *epf1epf2*. However, under drought stress, the inner leaf temperature of glabrous *tmm-1* increased significantly. Meanwhile, we observed the same reduction in Fv′/Fm′ in stressed *epf1epf2*, which possessed trichomes, even though the leaf temperature was significantly lower than

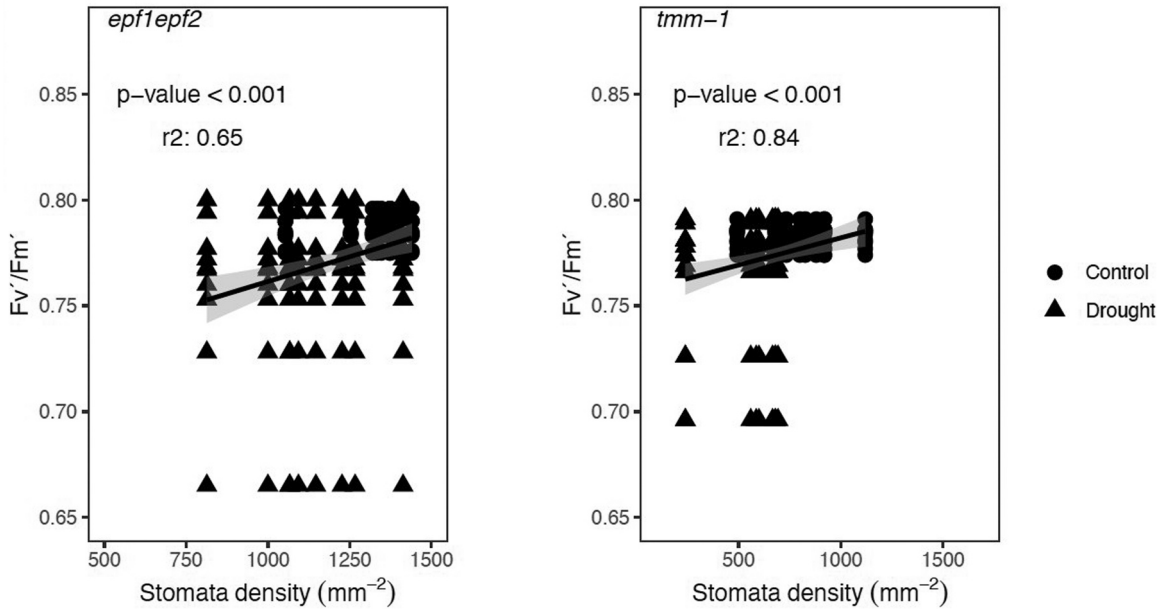

Fig. 5. - Generalized linear model showed the correlation between Fv′/Fm′ and stomata density in *epf1epf2* and *tmm-1*.

in stressed *tmm-1*. Therefore, we concluded that under drought stress, the advantage of having trichomes diminished, as *epf1epf2* did not show a significant different Fv′/Fm′ compared to *tmm-1*. This suggests that although trichomes contribute positively to leaf cooling under water scarcity, their role in drought stress response might be limited. The similarity in boundary layer conductance between control and drought-stressed plants aligns with Benz and Martin (2016), who reported that trichomes had a negligible effect on boundary layer conductance and gas exchange (Benz and Martin, 2006). Given that boundary layer conductance showed no variation among the Arabidopsis plants in this study, we hypothesized that trichomes regulate internal leaf temperature by modulating radiation and the absence of trichomes in the glabrous leaves of *tmm-1* likely caused increased heating due to radiation, as supported by observations in the hairy and glabrous leaves of tomato (*Solanum lycopersicum*) (Gasparini et al., 2021). However, our observations suggest that the heating caused by the absence of trichomes did not lead to the reduction in Fv′/Fm′.

The stomatal responses to drought stress further underscore the complex interplay between anatomical features and physiological performance. Col-8 plants increased stomatal density under drought stress, a response not mirrored by *epf1epf2* and *tmm-1*, which instead showed reduced stomatal density. This increase in stomatal density in Col-8 could be a compensatory mechanism to already developed smaller stomata to maintain CO_2 uptake and mitigate stress impact on photosynthesis. Our findings indicated that under well-watered conditions, Col-8 developed larger stomata in lower density compared to *epf1epf2* and *tmm-1*. However, drought stress caused higher stomatal density, and smaller stomata which are often considered advantageous under drought stress to optimize water-use efficiency (WUE) and photosynthetic yield (Lertngim et al., 2022). While photosynthesis in *epf1epf2* and *tmm-1* was impaired through reduced PhiNO, Col-8 plants, despite showing decreases in ECS, NPQ, and PhiNO, were able to maintain photosynthetic efficiency by increasing stomatal density and leaf thickness. In this context, the increases in stomatal density and leaf thickness may represent structural adaptations that optimize and sustain photosynthetic efficiency. These changes collectively suggest that the plant is enhancing its resilience to drought by improving structural robustness. This observation aligns with Driesen et al. (2023), where basil leaves (*Ocimum basilicum* L.) increased stomatal density and reduced stomatal size as a strategy to cope with water scarcity (Driesen et al., 2023). Therefore, we concluded that Col-8 developed higher stomata density with smaller size in response to drought stress to increase CO_2 assimilation and optimize photosynthesis efficiency. In contrast, the reduced stomatal density in *epf1epf2* and *tmm-1* under drought stress likely contributed to their decreased photosynthetic efficiency, as evidenced by the significant correlation between reduced stomatal density and lower Fv′/Fm′(Fig. 5a and b). In this study, stomatal conductance did not follow the variations in stomatal density. Although higher stomatal density has been correlated with higher stomatal conductance (Caine et al., 2023, Pitaloka et al., 2022b), it has been suggested that stomatal conductance is essentially affected by intercellular air space properties such as CO_2 concentration, temperature, and humidity (Buckley, 2019). Typically, stomatal density is proportional to intercellular air space (Whitewoods, 2021), and hence the intercellular air space proportionally altered by variation in stomata density (Lundgren et al., 2019). Our observations indicated that the photosynthetic efficiency is driven by the variation in stomatal density in conjunction with its proportion to intercellular air spaces, rather than by stomatal density alone. The increased leaf thickness induced by drought stress in Col-8, along with higher stomata density, provides a mechanical advantage to plants by enhancing their ability to retain more CO_2, thereby improving the efficiency of photosynthesis.

The data demonstrated that the presence of trichomes and higher stomata density contribute positively to achieve higher photosynthetic efficiency under optimal watering conditions. However, under drought stress, Col-8 maintained a stable Fv′/Fm′by developing more stomata and thicker leaves. In contrast, both *epf1epf2* and *tmm-1* exhibited a significant decrease in Fv′/Fm′, which regardless to the presence/absence of trichomes, is attributed to their fewer stomata compared to non-stressed plants. This indicates that it is not merely higher or lower stomatal density, but also the direction of variation in stomatal density drives photosynthetic efficiency.

Our results underline the necessity of considering anatomical traits such as trichomes and stomatal properties in breeding programs aimed at enhancing drought resistance and photosynthetic performance in plants. By considering the variation in anatomical characteristics and their role in photosynthesis efficiency before and after environmental stresses, it may be possible to develop crop varieties that can better withstand drought stress while maintaining high photosynthetic efficiency. However, the potential role of trichome density and leaves cuticle has not been address in this study, leaving room for future research to explore how these factors, along with the studied factors in

the present work, may influence the efficiency of photosynthesis and plants stress responses. Since it has been previously suggested that genes involved in trichome and stomata formation may contribute to plant resistance to drought stress (Ruggenthaler et al., 2009, Zhang et al., 2021), this study could be considered as a guideline for future molecular analysis and transcriptomic analysis to reveal the expression of genes involved in stomatal development, trichome formation, and drought response.

Author statement

Please consider our research paper titled "Lack of Trichomes and Variation in Stomatal Properties Influence the Quantum Efficiency of Photosynthesis in Arabidopsis" for publication in Environmental and Experimental Botany.

This manuscript explores the role of two key anatomical features—trichomes and stomata—in plant responses to drought stress. We hypothesized that *Arabidopsis thaliana* genotypes with higher stomatal density and the presence of trichomes exhibit greater photosynthetic efficiency under stress conditions. To test this hypothesis, we measured several indicators of photosynthetic performance—Fv′/Fm′, NPQ, PhiNO, and ECS—along with stomatal number and size before and after drought stress in three Arabidopsis genotypes: Col-8 (with trichomes and low stomatal density), *epf1epf2* (with trichomes and high stomatal density), and *tmm-1* (without trichomes and high stomatal density).

Our findings show that Col-8, which developed higher stomatal density under drought stress, maintained stable Fv′/Fm′, while both *epf1epf2* and *tmm-1* exhibited reduced stomatal density and Fv′/Fm′ under similar conditions. Interestingly, the reduction in Fv′/Fm′ was observed regardless of the presence or absence of trichomes, suggesting that the cooling effect of trichomes under optimal watering conditions does not significantly contribute to drought stress tolerance. Additionally, our results indicate that it is not only the stomatal density but also the variation in stomatal density under drought stress that determines photosynthetic efficiency.

This manuscript has not been published before and is not under consideration elsewhere. We are excited to share these novel findings with Environmental and Experimental Botany.

The co-authors have contributed to the research by consulting on the experiments and assisting in manuscript preparation.

CRediT authorship contribution statement

Maryam Zekri: Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Ingeborg Lang:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maryam Alsadat Zekri reports financial support was provided by Vienna Science and Technology Fund (WWTF). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Microscopy was performed at the Core Facility Cell Imaging and Ultrastructure Research, University of Vienna - member of the Vienna Life-Science Instruments (VLSI). We are grateful to Dr. Danny Tholen at the University of Natural Resources and Life Sciences, Vienna for providing Porometer and Dr. Gert Bachmann at the Department of Functional and Evolutionary Ecology, University of Vienna, for guidance on the instrumentation for photosynthetic measurements. We thank our master's student Maryna Kliemikhina for helping with data collection. We gratefully acknowledge the funding from the Wiener Wissenschaft-, Forschungs- und Technology Fund Vienna Science and Technology Fund (WWTF, LS19–013) and the Vienna Doctoral School of Ecology and Evolution for granting us the Completion Grant (2024).

References

Amada, G., Kosugi, Y., Kitayama, K. & Onoda, Y. 2020. Roles of leaf trichomes in heat transfers and gas-exchange characteristics across environmental gradients.
Amada, G., Onoda, Y., Ichie, T., Kitayama, K., 2017. Influence of leaf trichomes on boundary layer conductance and gas-exchange characteristics in Metrosideros polymorpha (Myrtaceae). Biotropica 49, 482–492.
Benz, B.W., Martin, C.E., 2006. Foliar trichomes, boundary layers, and gas exchange in 12 species of epiphytic Tillandsia (Bromeliaceae). J. Plant Physiol. 163, 648–656.
Bertolino, L.T., Caine, R.S., Gray, J.E., 2019. Impact of stomatal density and morphology on water-use efficiency in a changing world. Front. Plant Sci. 10.
Buckley, T.N., 2019. How do stomata respond to water status? N. Phytol. 224, 21–36.
Caine, R.S., Harrison, E.L., Sloan, J., Flis, P.M., Fischer, S., Khan, M.S., Nguyen, P.T., Nguyen, L.T., Gray, J.E., Croft, H., 2023. The influences of stomatal size and density on rice abiotic stress resilience. N. Phytol. 237, 2180–2195.
Dodd, A.N., Gardner, M.J., Hotta, C.T., Hubbard, K.E., Dalchau, N., Love, J., Assie, J.-M., Robertson, F.C., Jakobsen, M.K., Gonçalves, J., Sanders, D., Webb, A.A.R., 2007. The arabidopsis circadian clock incorporates a cADPR-based feedback loop. Science 318, 1789–1792.
Driesen, E., De Proft, M., Saeys, W., 2023. Drought stress triggers alterations of adaxial and abaxial stomatal development in basil leaves increasing water-use efficiency. Hortic. Res. 10, uhad075.
Ennajeh, M., Vadel, A.M., Cochard, H., Khemira, H., 2010. Comparative impacts of water stress on the leaf anatomy of a drought-resistant and a drought-sensitive olive cultivar. J. Hortic. Sci. Biotechnol. 85, 289–294.
franks, P.J., W. Doheny-Adams, T., Britton-Harper, Z.J., Gray, J.E., 2015. Increasing water-use efficiency directly through genetic manipulation of stomatal density. N. Phytol. 207, 188–195.
Gasparini, K., Da Silva, M.F., Costa, L.C., Martins, S.C.V., Ribeiro, D.M., Peres, L.E.P., Zsögön, A., 2021. The Lanata trichome mutation increases stomatal conductance and reduces leaf temperature in tomato. J. Plant Physiol. 260, 153413.
Hughes, J., Hepworth, C., Dutton, C., Dunn, J.A., Hunt, L., Stephens, J., Waugh, R., Cameron, D.D., Gray, J.E., 2017. Reducing stomatal density in barley improves drought tolerance without impacting on yield. Plant Physiol. 174, 776–787.
Lertngim, N., Ruangsiri, M., Klinsawang, S., Raksatikan, P., Thunnom, B., Siangliw, M., Toojinda, T., Siangliw, J.L., 2022. Photosynthetic plasticity and stomata adjustment in chromosome segment substitution lines of rice cultivar KDML105 under drought stress. Plants (Basel) 12.
Li, H., Yang, Y., Wang, H., Liu, S., Jia, F., Su, Y., Li, S., He, F., Feng, C., Niu, M., Wang, J., Liu, C., Yin, W., Xia, X., 2021. The receptor-like kinase ERECTA confers improved water use efficiency and drought tolerance to poplar via modulating stomatal density. Int. J. Mol. Sci. [Online] 22.
Lundgren, M.R., Mathers, A., Baillie, A.L., Dunn, J., Wilson, M.J., Hunt, L., Pajor, R., Fradera-Soler, M., Rolfe, S., Osborne, C.P., Sturrock, C.J., Gray, J.E., Mooney, S.J., Fleming, A.J., 2019. Mesophyll porosity is modulated by the presence of functional stomata. Nat. Commun. 10, 2825.
Nadeau, J.A., Sack, F.D., 2002. Control of stomatal distribution on the Arabidopsis leaf surface. Science 296, 1697–1700.
Pierce, M.A., Lawren S.A.C.K. & Buckley, T.N. 2021. *The Effect of Trichomes on Leaf Boundary Layer Conductance to Heat Transfer*, University of California, Davis.
Pitaloka, M.K., Caine, R.S., Hepworth, C., Harrison, E.L., Sloan, J., Chutteang, C., Phunthong, C., Nongngok, R., Toojinda, T., Ruengphayak, S., Arikit, S., Gray, J.E., Vanavichit, A., 2022a. Induced genetic variations in stomatal density and size of rice strongly affects water use efficiency and responses to drought stresses. Front Plant Sci. 13, 801706.
Pitaloka, M.K., Caine, R.S., Hepworth, C., Harrison, E.L., Sloan, J., Chutteang, C., Phunthong, C., Nongngok, R., Toojinda, T., Ruengphayak, S., Arikit, S., Gray, J.E., Vanavichit, A., 2022b. Induced genetic variations in stomatal density and size of rice strongly affects water use efficiency and responses to drought stresses. Front. Plant Sci. 13.
Rivero, L., Scholl, R., Holomuzki, N., Crist, D., Grotewold, E., Brkljacic, J., 2014. Handling arabidopsis plants: growth, preservation of seeds, transformation, and genetic crosses. Methods Mol. Biol. (Clifton, N. J.) 1062, 3–25.
Ruggenthaler, P., Fichtenbauer, D., Krasensky, J., Jonak, C., Waigmann, E., 2009. Microtubule-associated protein AtMPB2C plays a role in organization of cortical microtubules, stomata patterning, and tobamovirus infectivity. Plant Physiol. 149, 1354–1365.
Savvides, A., Fanourakis, D., Ieperen, W., 2011. Co-ordination of hydraulic and stomatal conductances across light qualities in cucumber leaves. J. Exp. Bot. 63, 1135–1143.
Taylor, S., Franks, P., Hulme, S., Spriggs, E., Christin, P.A., Edwards, E., Woodward, I., Osborne, C., 2012. Photosynthetic pathway and ecological adaptation explain stomatal trait diversity amongst grasses. N. Phytol. 193, 387–396.
Westbrook, A.S., Mcadam, S.A.M., 2021. Stomatal density and mechanics are critical for high productivity: insights from amphibious ferns. N. Phytol. 229, 877–889.
Whitewoods, C.D., 2021. Riddled with holes: understanding air space formation in plant leaves. PLoS Biol. 19, e3001475.

Xu, Z., Jiang, Y., Jia, B., Zhou, G., 2016. Elevated-CO2 response of stomata and its dependence on environmental factors. Front. Plant Sci. 7.
Xu, X., Yuan, L., Xie, Q., 2022. The circadian clock ticks in plant stress responses. Stress Biol. 2, 15.
Xu, Z., Zhou, G., 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. J. Exp. Bot. 59, 3317–3325.
Zhang, H., Liu, P., Wang, B., Yuan, F., 2021. The roles of trichome development genes in stress resistance. Plant Growth Regul. 95, 137–148.

2.2. A rapid freezing method to determine tissue layer thickness in drought-stressed leaves

M. A. Zekri[*1], C. Leimhofer[1], N. Drexler[2] and I. Lang[1]
[1] Dept. of Functional and Evolutionary Ecology, Faculty of Life Sciences, University of Vienna, Austria, Djerassiplatz 1, A-1030 Vienna, Austria.
[2] Vienna Biocenter Core Facility GmbH, Dr. Bohr-Gasse 3, A-1030 Vienna, Austria
*Corresponding author: Maryam.alsadat.zekri@univie.ac.at

Journal of Microscopy 2025 Vol. 297 Issue 3 Pages 316-324
DOI: https://onlinelibrary.wiley.com/doi/abs/10.1111/jmi.13272

Received: 15 November 2023
Revised: 16 January 2024
Accepted: 22 January 2024
Issue Online: 10 February 2025

Citations:

- Maciel, J. C., C. M. Cabral, J. M. S. Silva, et al. 2025. First Report of a Histological Technique for Observing the Anatomy of the Epidermis of *Melinis minutiflora*. Microscopy Research and Technique88, no. 10: 2771–2782. https://doi.org/10.1002/jemt.70010.
- Findlay, K. 2025. Introduction to special issue '12th International Botanical Microscopy Meeting'. Journal of Microscopy, 297, 255–257. https://doi.org/10.1111/jmi.13388

Authors contribution
MAZ conceived the project. MAZ and CL acquired the imaging data and analysed the data. MAZ wrote the first draft of the manuscript with contributions from IL. MAZ and IL addressed the revisions and approved the final version of the manuscript.

Received: 15 November 2023 | Revised: 16 January 2024 | Accepted: 22 January 2024

DOI: 10.1111/jmi.13272

Journal of Microscopy RMS

THEMED ISSUE ARTICLE

A rapid freezing method to determine tissue layer thickness in drought-stressed leaves

Maryam Alsadat Zekri[1] | **Carina Leimhofer**[1] | **Nicole Drexler**[2] | **Ingeborg Lang**[1]

[1]Faculty of Life Sciences, Department of Functional and Evolutionary Ecology, University of Vienna, Vienna, Austria

[2]Vienna Biocenter Core Facility GmbH, Vienna, Austria

Correspondence
Maryam Alsadat Zekri, Faculty of Life Sciences, Department of Functional and Evolutionary Ecology, University of Vienna, Djerassiplatz 1, A-1030 Vienna, Austria.
Email: maryam.alsadat.zekri@univie.ac.at

Journal of Microscopy_invited paper by Ulla Neumann (RMS Botanical Microscopy meeting 2023)

Funding information
Danny Tholen from the University of Applied Life Sciences Vienna (BOKU); Vienna BioCenter Core Facilities GmbH (VBCF) Electron Microscopy Facility; Austrian Federal Ministry of Education, Science & Research, and the City of Vienna; Vienna Science and Technology (WWTF), Grant/Award Number: LS19_013

Abstract

Plants have been affected by water stress ever since they settled on dry land. In severe and persisting drought, plant leaves are wilting. However, a documentation at the anatomical level of the minute changes that occur before wilting is challenging. On the other hand, understanding the anatomical alteration in plant leaves with respect to water stress provides a stronger basis to study molecular and submolecular processes through which plants enhance drought tolerance. In this work, we applied an affordable method to visualise mesophyll layers of *Arabidopsis thaliana* cell lines without preparation steps that would alter the volume of the cells. We rapidly plunge-froze the leaves in liquid nitrogen, cut them while in the N_2 bath, and immediately imaged the mesophyll cross sections in a scanning electron microscope. We applied a reduction of watering from 60 to 40 to 20 mL per day and investigated two time points, 7 and 12 days, respectively. Interestingly, the overall thickness of leaves increased in water stress conditions. Our results showed that the palisade and spongy layers behaved differently under varying watering regimes. Moreover, the results showed that this method can be used to image leaf sections after drought stress without the risk of artefacts or swelling caused by contact to liquids as during chemical fixation.

KEYWORDS
Arabidopsis thaliana, drought stress, genotypes, mesophyll, plunge freezing, SEM

1 | INTRODUCTION

As sessile organisms, plants are used to changes in the environment and have adopted several mechanisms, ranging from metabolic processes and signal transduction pathways to morphological responses.[1] Both, soil properties (pH, salinity, soil porosity) as well as atmospheric conditions such as exposure to sunlight and CO_2 concentrations greatly influence a plant's productivity.[2] Here, we focused on water deficiency as an abiotic stress factor.

Plants are capable of handling daily fluctuations in water supply and combat drought conditions for some time. There is a threshold when exceeding water or drought stress can damage plant tissue. If the loss of water exceeds its uptake, plants start to wilt. The 'permanent wilting point' is an indicator for the irreversibility of cell damage due to drought stress. In the literature often a

value of −1.5 MPa is found, which means a water loss of 30%.[1] At this stage, the plant's vitality decreases, resulting in higher susceptibility to pathogens, visual morphological changes like reduced growth and yield, and eventually death. Thus, cell turgor and a hydrostatic skeleton is essential for normal plant growth.

The anatomical properties of plants can be modified to facilitate their adaptation to long and/or short-term environmental water fluctuations.[3] Changes in stomata density,[4,5] the thickness of the cuticle,[6,7] rearrangement of parenchyma cells[8,9] or trichome density[10] are some examples of how anatomical properties optimise physiological responses.

Even though plants may change their anatomy to cope with environmental fluctuations, implementation of such changes needs time. Therefore, the numerous observations implying the role of initial reactions and immediate switches in anatomy due to water stress cannot be underestimated. It has been frequently reported that stomata density (SD) and stomata size (SS) have a huge contribution in physiological responses and adaptations of plants.[11–14] Due to higher cell surface to volume ratio, smaller stomata respond faster to humidity changes[11] and stomata density optimises photosynthesis in fluctuating environmental CO_2 concentration and humidity.[15] The general idea implies that intercellular air space is proportional to the stomata density and size. It has been documented that a protein called Stomagen is synthesised in the mesophyll and induces stomata density in the epidermis.[16–18] Therefore, environmental stresses such as drought stress may change the anatomy of the epidermis through affecting the mesophyll dynamics.

The thickness of the leaf mesophyll mainly comprises the layers of palisade and spongy parenchyma. The spongy parenchyma is commonly known as a layer with larger intercellular air spaces, which makes this layer more involved in leaf gas exchange and transportation.[19] The palisade layer is characterised by densely packed and elongated cells with less intercellular space. The palisade cells are more responsible for photosynthesis.[20] The effect of the whole leaf thickness on carbon assimilation has been show before,[21] as well as the role of the volume of intercellular air represented by the thickness of the spongy layer,[22] and the thickness of the palisade layer on photosynthesis.[23] Still, how the anatomy of the mesophyll may alter during drought stress is not yet clear. One possible reason for this ambiguity is that in most studies, the mesophyll thickness was evaluated with light microscopy techniques.

Light microscopy is usually the method of choice because the cells and tissue should be alive to follow the dynamic processes. However, sample preparation and investigation in water is not feasible for drought-stressed tissue due to immediate water uptake by wilted cells. X-ray Microcomputed Tomography (μCT) with high beam intensity, for example, at a synchrotron light source, showed to be very efficient in image acquisition of fresh leaf segments at high resolution within subseconds.[24] However, this technique requires accessibility to the light source facility and is very expensive. To solve this dilemma, we looked for a cryo-immobilisation technique to rapidly freeze drought-stressed leaves and investigate the respective tissue layers in their near-to-native state in a scanning electron microscope, without chemical fixation or staining. That way, we succeeded in the determination of water loss at the tissue level in drought-stressed Arabidopsis leaves. To test our simple preparation method and to investigate morphological changes in leave tissue layers, we froze leaves of four different *A. thaliana* genotypes with varying stomatal traits: clusters of stomata, increased number of stomata, or stomata on both sides of the leave. We aimed to measure the effects of water loss as differences in thickness of (a) the whole leaf, (b) the palisade and (c) the spongy mesophyll. Our main objectives in this work are: (1) to evaluate the accuracy of cryo- immobilisation to investigate the minute changes in size of mesophyll layers after different watering regimes and (2) to investigate the effect of drought intensity (moderate and severe) and duration (short and long-term) on the thickness of the palisade and spongy parenchyma layers. We hypothesised that the different mesophyll layers react differently to water loss. Additionally, we want to test the hypothesis that under moderate stress for a short time, the thickness of the leaf may increase to elevate the photosynthesis rate thereby enabling a plants' resistance to lack of water.[25,26]

2 | MATERIAL AND METHODS

2.1 | Plant material

Four genotypes of *Arabidopsis thaliana* (Col-8 as wild type, *tmm1*, *lcd1-1* and UBP15-overexpression) were ordered from the Nottingham Arabidopsis Stock Centre.[27] Based on the NASC database, the genotypes were selected based on distinctive anatomical features. *tmm1* (*too many mouths* (tmm)) mutant has clusters of stomata[28] with significantly higher stomata count than wild type. In *lcd1-1*, growth is decelerated due to a low cell density (lcd) in the palisade layer.[29] UBP15 is a transgenic line that grows much bigger than the wild type and its leaves are more curled. Epidermis cells and palisade parenchyma cells are increased per area (The Arabidopsis Information Resource (TAIR)). Col-8 was selected as the wild-type or the background line.

Three plants per each genotype were grown in separate pots (diameter = 13 cm) containing 50% indoor plants' soil (Kranzinger GmbH, Austria), 33% quartz sand, and 17%

Perlite® at stable conditions in a growth room (Conviron MTPS 120, Manitoba, Canada) and watered daily with 60 mL water for a cultivation period of 5 weeks. Based on the size of the pots and soil composition, 60 mL was the maximum volume of water that remained in the soil and did not drain from the pots. All the pots were fully saturated with water and left for 1 day before sowing the seeds. The temperature at day was 22.2°C and at night 18°C, relative humidity (RH) was 60% and light intensity 130–150 μmol/m^2 s. The photoperiod of the plant chamber was set to 16 h day length.

2.2 | Stress experiments

After 5 weeks, the plants of the control group were continued to be watered daily with 60 mL water as before (Treatment group 1; control). Treatment group 2 received reduced water of 40 mL daily and treatment group 3 only got 20 mL daily. The relative soil humidity was measured with moisture meter (HH2, Delta-T Devices) after 7 and 12 days. The relative soil humidity after irrigation with 60, 40 and 20 mL, respectively, was ~75%, 50% and 30%, respectively. According to Rivero et al. (2014), the 75%, 50% and 30%, relative soil humidity are correlated to high, mild, and low humidity, respectively.[30] All other parameters in the growth chamber were kept constant. After 7 days of this watering regime, one leaf of each genotype from each treatment group was selected for plunge freezing and consecutive scanning electron microscopy (SEM). This time was considered as short-term stress. After 12 days, which was considered as long-term stress, leaves from all genotypes and all treatment groups were taken again for plunge freezing and imaging. Selection criteria for the leaves were as follows: similar size, stemming from first or second apical nodus, not touching bare soil or the pot (Figure 2).

2.3 | Sample preparation for SEM

High resolution and exact measurements of the hydration status in drought-stressed tissue was essential for this study. We excluded chemical fixation to eliminate the risk of chemically induced morphological changes like swelling or shrinkage.

From each genotype and treatment, two fully developed leaves with no sign of rapture or abnormality were selected and cut from the plant with a razor blade. They were mounted along the leaf distal axis on a custom-built holder for SEM (Figure 1A) and immediately plunged into liquid nitrogen to preserve leave ultrastructure. Subsequently a razor blade was used to create a sharp lateral-section of the leaf while still submerged in liquid nitrogen (Figure 1B). Figure 1C depicted all equipment that are required for sample preparation including holder forceps, long forceps, heater and liquid N flask. Frozen samples were immediately transferred to a Hitachi TM-1000 tabletop scanning EM operated at 15 kV and equipped with a highly sensitive semiconductive BSE detector (https:// www. scribd. com/ document/ 501503982/Manual-TM1000). The low-vacuum condition needed for detectors in the TM-1000 did not require any specimen coating; therefore, no sputter-coating was applied in the present work. Pictures were taken at a magnification of 600× or 800×, respectively. The middle of the leaf, that is, approximately the same distance from the main leaf vain and the leaf edge, was considered to take three images next to each other of each leaf.

2.4 | Tissue traits

We used two leaves per plant and took three images of each leaf. In each leaf, we took three measurements from each layer and also measured the overall thickness. Thus, in each image, the total thickness of the leaf, the palisade layer and the spongy layer were measured in three spots. The border between the palisade layer and spongy layer was drawn by considering all the palisade cells' sizes (the shortest and longest palisade cell) in Fiji (Fiji3, 2022). We calculated the mean of the three measurements in each photo: (1) diameter of whole leaf, (2) thickness of palisade layer and (3) thickness of spongy parenchyma layer (Figure 2). For all parameters, we used the program Fiji (Fiji3, 2022).

3 | RESULTS

One leaf of all four genotypes of *A. thaliana*, namely, *tmm1*, *lcd1-1* and UBP15-overexpression, as well as *Col-8* (wild type) was carefully cut, placed in the SEM-sample holder and immediately plunge-frozen in liquid nitrogen. As an example, Figure 3 shows the leaves of plants of the wild type (Col-8) during the stress experiment watered with 60 mL (Figure 3A), with 40 mL (Figure 3) and 20 mL water, respectively (Figure 3C). The corresponding cross sections of the selected leaves after plunge freezing are imaged directly in SEM without coating (Figure 3D–F).

3.1 | Macroscopic changes

Over the course of the experiment, the plants subjected to drought stress showed in poor development and morphological alterations. In Arabidopsis line *tmm1*, we observed an unspecific, yellowish discoloration at the leaf

FIGURE 1 Sample preparation for EM: (A) custom-designed holder with three plunging spots; (B) the custom-built holder submerged in liquid nitrogen to freeze the mounted leaves; (C) the forceps used to immerse the leaf holder into liquid nitrogen (red arrow), and to hold the razor blade to cut the leaf while submerged in liquid nitrogen (black arrow), the heater to remove ice from the tools (yellow star).

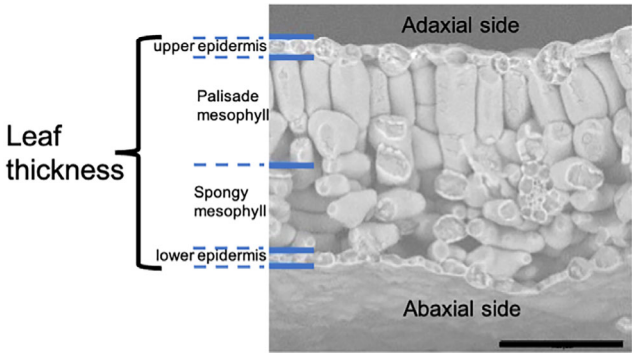

FIGURE 2 SEM micrograph of a cross section of Col-8 genotype of A. thaliana. Individual tissue layers are indicated by blue lines; longest palisade cells define the thickness of palisade layer (e.g. cell in the centre of this image). The thickness of palisade layer, spongy layer and total thickness including the epidermis were measured. Bar = 100 μm.

margins in all treatment groups. Plants of line *lcd1-1* were overall smaller than in the control group and they felt firm and stiff; some leaves showed yellow margins. UBP15 plants were wilting quickly at high water stress (20 mL water).

3.2 | Short-term stress (7 days)

A comparison between the watering regimes of 60 mL (control), and reduced water dosage of 40 and 20 mL, respectively, after 7 days, is given in Figure 4. Already at the start of the experiments in fully watered plants, we noticed a striking difference in leaf thickness between the different genotypes. Col-8 and UBP15 had the thinnest leaves while *tmm1* had the thickest leaves (Figure 4A, left plots). In the lines UBP15 and *tmm1*, this difference resulted mainly from the palisade layer, which is the thinnest and the thickest, respectively (Figure 4B, left plots).

The changes induced by reducing water from 60 to 40 mL in the thickness of the leaf in general, varied between the genotypes with little significance (Figure 4A). However, comparing 60 to 20 mL water dosage, we observed an overall increase in thickness in all lines apart from *lcd1-1* (Figure 4A). In particular, UBP15 and *tmm1* had the thickest leaves when only given 20 mL of water. While in *lcd1-1*, the reduced overall leaf thickness is due to the thinnest palisade layer, the great increase in thickness in line UBP15 resulted from the thick spongy parenchyma layer (Figure 4B and C).

Taking a closer look at each genotype, we see a significant increase in the leaf thickness from 60 to 40 mL and to 20 mL (Figure 4A). In the wild-type genotype (Col8), the increased leaf thickness in moderately watered and severely stressed plants resulted from an increase in spongy parenchyma (Figure 4B) as there is no significant difference in the palisade layer (Figure 4B). The leaf thickness in *lcd1-1* increased slightly from 60 to 40 mL watering but dropped significantly in severely stressed plants (20 mL water; Figure 4A right plots). The decreased leaf thickness of *lcd1-1* rooted from a reduction in thickness of spongy parenchyma in the least watered plants (Figure 4C). Opposite to Col-8 and *lcd1-1*, the overall leaf thickness, palisade and spongy layers of *tmm1* decreased from 60 to 40 mL irrigation but interestingly, increased significantly in the plants that only got 20 mL of water (Figure 4A–C). The leaf thickness of UBP15 increased slightly while watering was reduced so that in severely stressed plants, it is significantly higher than in fully watered plants (Figure 4A–C). In UBP15, both palisade and spongy parenchyma layers increased significantly in severely stressed plants and caused the overall increase in thickness (Figure 4C).

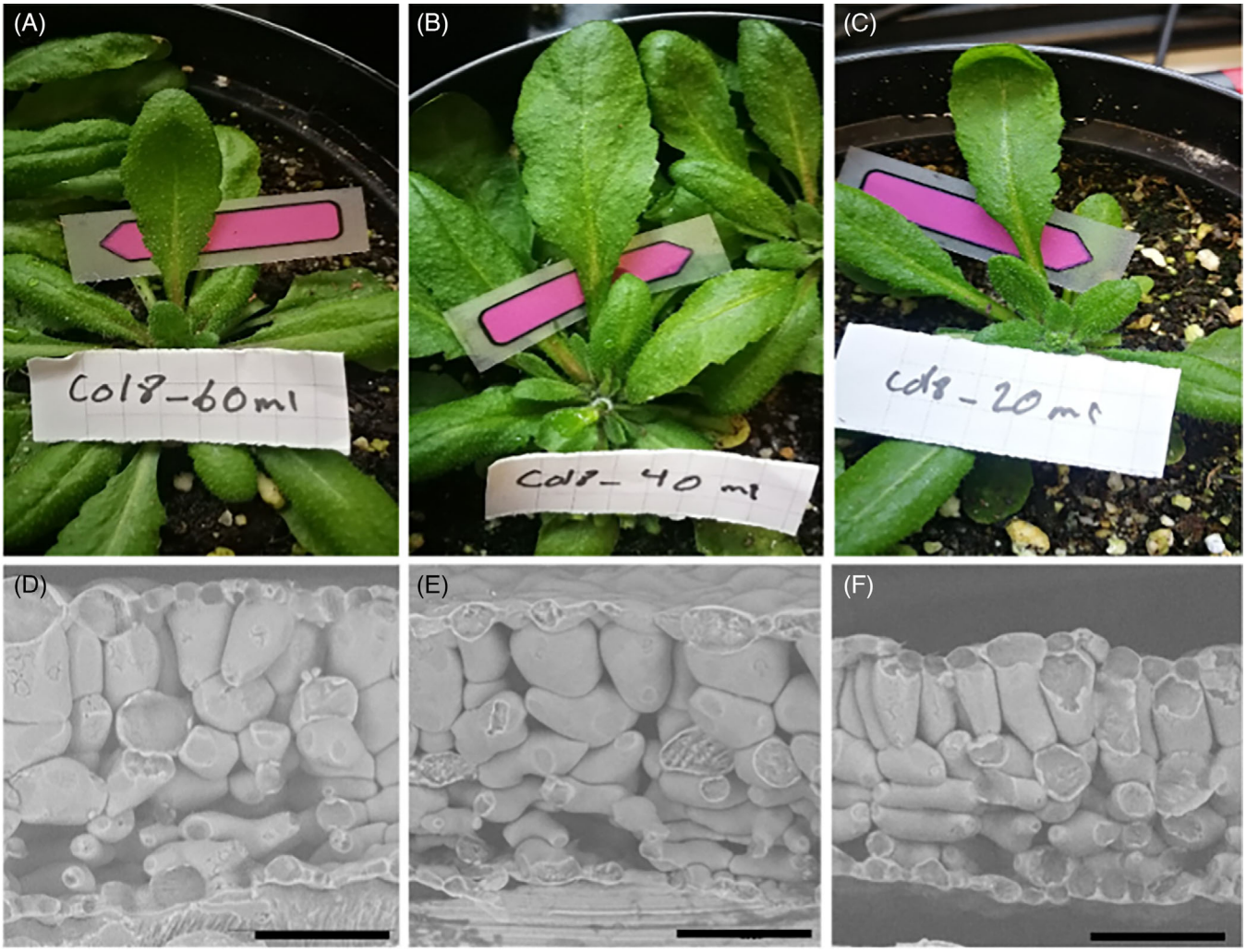

FIGURE 3 Col-8 (wild type) after long-term stress (12 days). A–C shows the Col-8 plants; selected leaves for freezing are indicated. The original watering regime was 60 mL (A), 40 mL (B) and 20 mL (C). D–F: Cross sections of selected leaves A–C. Note the shrinking of the whole leaf and the increasing cell density at the tissue level in the mesophyll. Bar = 100 μm.

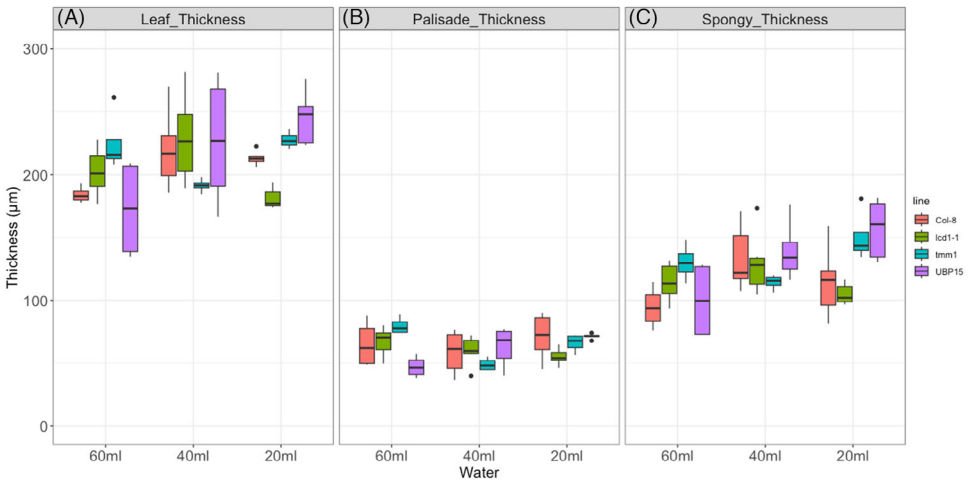

FIGURE 4 Comparison of A.t. genotypes after 7 days of reduced watering ('short-term stress') showing thickness of (A) whole leaf, (B) of palisade parenchyma layer and (C) of spongy parenchyma layer. Water dosage was 60 mL, 40 mL and 20 mL, respectively. $n = 12$ measurements.

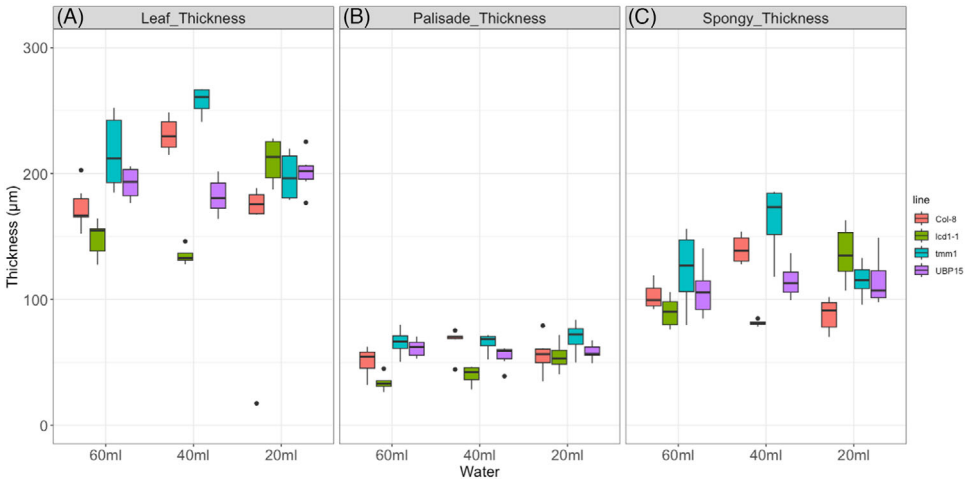

FIGURE 5 Comparison of A.t. genotypes after 12 days of reduced watering ('long-term stress') showing thickness of (A) whole leaf, (B) of palisade parenchyma layer and (C) of spongy parenchyma layer. Water dosage was 60 mL, 40 mL and 20 mL, respectively. $n = 12$ measurements.

3.3 | Long-term stress (12 days)

After 12 days of irrigation with 60 mL water, the overall thickness of the leaves clustered into two groups: *tmm1* and UBP15 had thicker leaves than the genotypes *lcd1-1* and Col8 (Figure 5A, left plots). *tmm1* showed the highest leaf thickness as well as the thickest palisade and spongy parenchyma layers. Line *lcd1-1* had the thinnest leaves overall and also the thinnest palisade layers in all treatments. However, in severely water-stressed leaves (only 20 mL water), the spongy layer of *lcd1-1* line significantly increased in thickness (Figure 5A–C). These two lines also differed most in the thickness of the spongy parenchyma with *tmm1* having the highest and *lcd1-1* the lowest thickness of the spongy parenchyma layer (Figure 5C). Interestingly, under severe water stress, the high overall thickness of the leaf in *lcd1-1* resulted from the spongy layer (Figure 5A and C) while in *tmm1*, the high overall thickness of the leaf resulted from the palisade layer (Figure 5A and B).

Similar to the short-term stress, we see a significant increase in the leaf thickness from 60 to 40 mL and to 20 mL in long-term stress (Figure 5A). The overall leaf thickness of Col-8 increased significantly from 60 to 40 mL watering resulting from both, a significantly thicker spongy and palisade layer (Figure 5A–C). In severely stressed plants that only received 20 mL of water, the thickness of Col8 leaves decreased manly within the spongy parenchyma (Figure 5C), resulting in an overall thickness that is similar to 60 mL watering (Figure 5A, right plots). By contrast, in *lcd1-1*, significant changes only occurred after switching from moderate (40 mL) to severe (20 mL) water stress, resulting in a significant increase in thickness in this line within the spongy parenchyma (Figure 5A and C). Also, in line *tmm1*, the palisade layer did not change by increasing water stress, but the overall leaf thickness increased in 40 mL water dosage as a result of increased thickness of the spongy parenchyma (Figure 5). In *UBP15*, the thickness of parenchyma layers remained almost constant by reducing the water dosage in long-term water stress (Figure 5A–C).

3.4 | Discussion

Drought is a severe challenge for plants, greatly reducing growth and yield. Here we looked at changes at the tissue level of four genotypes of *Arabidopsis thaliana* (*Col-8*, *lcd1*, *tmm1* and UBP15) to evaluate the role of reduced water as well as the duration of this stress. To analyse the minute changes at the tissue level in drying leaves, high- resolution microscopy without re-wetting the samples – as in conventional light microscopy – was essential. In conventional light microscopy at the tissue level, living material is usually prepared on slides in a water film or the samples are fixed and embedded in resin. However, for the present research question, any preparation in water or liquids was impossible as drought-stressed cells could quickly regain full turgor in any liquid or fixative thereby distorting potential changes of cells by water loss and tissue shrinkage. To circumvent this dilemma, all leaves were plunge-frozen and transferred directly to the SEM for imaging without any further treatment or contact to liquids.

Here, the investigated Arabidopsis lines reacted very differently and in particular, the layers of the mesophyll showed distinctive swelling and shrinking patterns. In

general, most microscopic differences observed in the overall leaf thickness originated from the spongy parenchyma. Moderate water stress for a short time increased the leaf thickness in all genotypes except in *tmm1*, which can be related to the lack of trichomes in this genotype. Trichomes play a major role in mitigating the consequences of stress by conserving water in plants,[6] adjusting the leaf temperature[31] and/or protecting against light.[32] An increase in leaf thickness in drought-stressed plants has been observed before.[25,33,34] However, the leaf thickness and specifically palisade thickness of *lcd1-1* decreased significantly under severe stress. Due to the mutation, *lcd1-1* produces less cells per leaf area.[35] Therefore, it is speculated that *lcd1-1* could compensate short-term drought stress but was more susceptible to severe stress. The contrasting genotype to *lcd1-1*, UBP15 with higher cells per leaf area, showed a significantly higher tolerance with almost no changes in leaf thickness over the course of the experiment.

Interestingly, *tmm1* compensated severe drought stress for the short-term treatment very well but was susceptible when the stress persisted. On the other hand, the leaf thickness in this line increased in the medium water dosage and the longer stress period. Opposite to *tmm1*, leaves of *lcd1-1* became thicker under severe stress in long-term while in short-time severe stress, the leaf thickness was less than in fully watered plants. In both *tmm1* and *lcd1-1*, a significant variation in the spongy mesophyll layer was mainly responsible for the observed change in overall leaf thickness. This observation differentiates the thickness of palisade and spongy layers over time. It implies that in short-term drought stress, the palisade layer is more affected by the water stress while in persisting, longer times of stress, the main variation occurs in the spongy layer. Thus, our results showed that the mesophyll plays a major role in drought tolerance of plant leaves, in particular the spongy mesophyll layer in long- and short-term stress. Our data showed that the significant alteration (either increase or decrease) in leaf thickness resulted from changes in both spongy and palisade parenchyma. It is speculated that the elevated thickness of the spongy layer accelerates the rate of the photosynthesis by increasing the mesophyll conductance (g_m).[36] Therefore, developing thicker leaves by increasing the thickness of spongy and palisade layers is considered as an adaptation to drought stress to elevate the water use efficiency in low water availability conditions.[37,38]

Due to their respective and specific leaf anatomy, the chosen genotypes reacted differently, for example, by a delay of this effect. Under severe stress conditions, the genotype UBP15 handled water stress better in short-term stress while *lcd1-1* showed increased mesophyll tissue thickness in long-term stress condition. The *tmm1* genotype could at first increase the water content in this layer when being exposed to drought but shrinkage still occurred during the treatment period. The thick palisade layer in normal, bifacial leaf anatomy, as shown in control plants (Col-8), also supported water loss in the beginning and the middle of the experiment; shrinkage as response to water shortage only occurred at the end of the severely stressed plants (20 mL water dosage).

The chosen genotypes with the respective anatomical differences help to evaluate our results of significant differences in mesophyll properties when plants are exposed to various levels of drought stress. We continue to analyse and relate these results to physiological data. We are aware of the fact that drought stress fuels the phytohormone synthetisation of abscisic acid (ABA), which regulates stomatal closure to avoid water loss.[39] Furthermore, stressed plants produce elevated levels of reactive oxygen species (ROS) in their cells[40] and the carbon metabolism in photosynthesis is also affected by a shortage in water supply.[41] So, further studies regarding ABA, ROS or soluble sugar determination are necessary to link anatomical features and physiological responses triggered by drought conditions.

Here we described a novel technique with a focus on challenging sample preparation to image drought-stressed leaves without manipulating the cell turgor pressure, which is inevitable in most light microscopy techniques. Our approach is an affordable and efficient method to visualise temporal and spatial alteration in mesophyll. We call for more studies applying this technique on other plants' structures such as stems and roots.

ACKNOWLEDGEMENTS

We are grateful to Danny Tholen from the University of Applied Life Sciences Vienna (BOKU) for suggesting this method and providing the specific, vertical SEM-sample holder for plunge freezing of plant leaves. The Vienna BioCenter Core Facilities GmbH (VBCF) Electron Microscopy Facility gratefully acknowledges funding from the Austrian Federal Ministry of Education, Science & Research, and the City of Vienna.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ORCID

Maryam Alsadat Zekri https://orcid.org/0000-0002-3314-468X

REFERENCES

1. Lutz Nover, E. W. W. (2008). *Allgemeine und molekulare Botanik* [Buch], – *[s.l.]*. Thieme.

2. Ng, C. W. W., Zhang, Q., Zhou, C., & Ni, J. (2022). Eco-geotechnics for human sustainability. *Science China Technological Sciences*, *65*, 2809–2845.
3. Haworth, M., Marino, G., Loreto, F., & Centritto, M. (2021). Integrating stomatal physiology and morphology: Evolution of stomatal control and development of future crops. *Oecologia*, *197*, 867–883.
4. Bakker, J. C. (1991). Effects of humidity on stomatal density and its relation to leaf conductance. *Scientia Horticulturae*, *48*, 205–212.
5. Bertolino, L. T., Caine, R. S., & Gray, J. E. (2019). Impact of stomatal density and morphology on water-use efficiency in a changing world. *Frontiers in Plant Science*, *10*, 00225.
6. Chen, M., Zhu, X., Zhang, Y., Du, Z., Chen, X., Kong, X., Sun, W., & Chen, C. (2020). Drought stress modify cuticle of tender tea leaf and mature leaf for transpiration barrier enhancement through common and distinct modes. *Scientific Reports*, *10*, 6696.
7. Liu, L.-L., Deng, Y.-Q., Dong, X.-X., Wang, C.-F., Yuan, F., Han, G.-L., & Wang, B.-S. (2022). ALDH2C4 regulates cuticle thickness and reduces water loss to promote drought tolerance. *Plant Science*, *323*, 111405.
8. Borsuk, A. M., Roddy, A. B., Théroux-Rancourt, G., & Brodersen, C. R. (2022). Structural organization of the spongy mesophyll. *New Phytologist*, *234*, 946–960.
9. Théroux-Rancourt, G., Roddy, A. B., Earles, J. M., Gilbert, M. E., Zwieniecki, M. A., Boyce, C. K., Tholen, D., Mcelrone, A. J., Simonin, K. A., & Brodersen, C. R. (2021). Maximum CO_2 diffusion inside leaves is limited by the scaling of cell size and genome size. *Proceedings of the Royal Society B: Biological Sciences*, *288*, 20203145.
10. Shahzad, M., Khan, Z., Nazeer, W., Arshad, S. F., Ahmad, F., Farid, B., Shahid, M. R., & Riaz, H. (2021). Effect of drought on trichome density and length in cotton (*Gossypium hirsutum*). *Journal of Bioresource Management*, 8(1).
11. Cadart, C., Venkova, L., Recho, P., Lagomarsino, M., & PIEL, M. (2019). The physics of cell-size regulation across timescales. *Nature Physics*, *15*, 993–1004.
12. De Boer, H. J., Eppinga, M. B., Wassen, M. J., & Dekker, S. C. (2012). A critical transition in leaf evolution facilitated the cretaceous angiosperm revolution. *Nature Communications*, *3*, 1221.
13. Franks, P. J., & Beerling, D. J. (2009). Maximum leaf conductance driven by CO_2effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences*, *106*, 10343–10347.
14. Kardiman, R., & Rabild, A. (2017). Relationship between stomatal density, size and speed of opening in sumatran rainforest species. *Tree Physiology*, *38*, 696–705.
15. Westbrook, A. S., & McAdam, S. A. M. (2021). Stomatal density and mechanics are critical for high productivity: insights from amphibious ferns. *New Phytologist*, *229*, 877–889.
16. Jewaria, P. K., Hara, T., Tanaka, H., Kondo, T., Betsuyaku, S., Sawa, S., Sakagami, Y., Aimoto, S., & Kakimoto, T. (2013). Differential effects of the peptides stomagen, EPF1 and EPF2 on activation of MAP Kinase MPK6 and the SPCH protein level. *Plant and Cell Physiology*, *54*, 1253–1262.
17. Kondo, T., Kajita, R., Miyazaki, A., Hokoyama, M., Nakamura-Miura, T., Mizuno, S., Masuda, Y., Irie, K., Tanaka, Y., Takada, S., Kakimoto, T., & Sakagami, Y. (2009). Stomatal density is controlled by a mesophyll-derived signaling molecule. *Plant and Cell Physiology*, *51*, 1–8.
18. Xia, Y., Du, K., Ling, A., Wu, W., Li, J., & Kang, X. (2022). Overexpression of PagSTOMAGEN, a positive regulator of stomatal density, promotes vegetative growth in poplar. *International Journal of Molecular Sciences*, *23*, 10165.
19. Jahan, E., Sharwood, R. E., & Tissue, D. T. (2023). Effects of leaf age during drought and recovery on photosynthesis, mesophyll conductance and leaf anatomy in wheat leaves. *Frontiers in Plant Science*, *14*, 1091418.
20. Gotoh, E., Suetsugu, N., Higa, T., Matsushita, T., Tsukaya, H., & Wada, M. (2018). Palisade cell shape affects the light-induced chloroplast movements and leaf photosynthesis. *Scientific Reports*, *8*, 1472.
21. Herrera, A. (2020). Are thick leaves, large mesophyll cells and small intercellular air spaces requisites for CAM? *Annals of Botany*, *125*, 859–868.
22. Michèle, R. S., & William, K. S. (2002). Mesophyll architecture and cell exposure to intercellular air space in alpine, desert, and forest species. *International Journal of Plant Sciences*, *163*, 937–948.
23. Singhakumara, B., Griscom, H., Ashton, M., & Panditharathna, P. (2008). Changes in leaf structure in relation to crown position and size class for tree species with a Si Lankan tropical rain forest. *Botany*, *86*, 633–640.
24. Théroux-Rancourt, G., Earles, J. M., Gilbert, M. E., Zwieniecki, M. A., Boyce, C. K., Mcelrone, A. J., & Brodersen, C. R. (2017). The bias of a two-dimensional view: Comparing two-dimensional and three-dimensional mesophyll surface area estimates using noninvasive imaging. *New Phytologist*, *215*, 1609–1622.
25. Afzal, A., Duiker, S. W., & Watson, J. E. (2017). Leaf thickness to predict plant water status. *Biosystems Engineering*, *156*, 148–156.
26. Nardini, A. (2022). Hard and tough: the coordination between leaf mechanical resistance and drought tolerance. *Flora*, *288*, 152023.
27. NASC. (1991). The Nottingham Arabidopsis Stock Centre.
28. Nadeau, J. A., & Sack, F. D. (2002). Control of stomatal distribution on the arabidopsis leaf surface. *Science*, *296*, 1697–1700.
29. Barth, C., & Conklin, P. L. (2003). The lower cell density of leaf parenchyma in the *Arabidopsis thaliana* mutant lcd1-1 is associated with increased sensitivity to ozone and virulent *Pseudomonas syringae*. *The Plant Journal*, *35*, 206–218.
30. Rivero, L., Scholl, R., Holomuzki, N., Crist, D., Grotewold, E., & Brkljacic, J. (2014). Handling arabidopsis plants: Growth, preservation of seeds, transformation, and genetic crosses. *Methods in Molecular Biology (Clifton, N.J.)*, *1062*, 3–25.
31. Galdon-Armero, J., Fullana-Pericas, M., Mulet, P. A., Conesa, M. A., Martin, C., & Galmes, J. (2018). The ratio of trichomes to stomata is associated with water use efficiency in *Solanum lycopersicum* (tomato). *The Plant Journal*, *96*, 607–619.
32. Savé, R., Biel, C., & De Herralde, F. (2000). Leaf pubescence, water relations and chlorophyll fluorescence in two subspecies of lotus creticus L. *Biologia Plantarum*, *43*, 239–244.
33. Khan, A., Shen, F., Yang, L., Xing, W., & Clothier, B. (2022). Limited acclimation in leaf morphology and anatomy to experimental drought in temperate forest species. *Biology (Basel)*, *11*, 1186.

34. Sari, J. M., Turnip, M., & Zakiah, Z. (2023). The effect of drought stress on the anatomical characteristics of leaves, stems and roots of *Nepenthes mirabilis* (Lour). *Druce Buletin Kebun Raya*, *26*(1), 1–10.
35. TAIR. (2022). The Arabidopsis Information Resource (TAIR). www.arabidopsis.org
36. Zou, J., Hu, W., Li, Y., Zhu, H., He, J., Wang, Y., Meng, Y., Chen, B., Zhao, W., Wang, S., & Zhou, Z. (2022). Leaf anatomical alterations reduce cotton's mesophyll conductance under dynamic drought stress conditions. *The Plant Journal*, *111*, 391–405.
37. Shuang, L., Abdoul Kader, M., Zhang, Y., Liang, Y., Gao, Y., & Duan, A. (2021). Coordination of leaf hydraulic, anatomical, and economical traits in tomato seedlings acclimation to long-term drought. *BMC Plant Biology*, *21*, 536.
38. Van Rensburg, L., Krüger, H., & Krüger, G. H. J. (1994). Intercellular space variation among air-cured *Nicotiana tabacum* L. genotypes and its relation to their water use efficiency. *South African Journal of Botany*, *60*, 227–230.
39. Lim, C. W., Baek, W., Jung, J., Kim, J. H., & Lee, S. C. (2015). Function of aba in stomatal defense against biotic and drought stresses. *International Journal of Molecular Sciences*, *16*, 15251–15270.
40. Gillespie, K. M., Chae, J. M., & Ainsworth, E. A. (2007). Rapid measurement of total antioxidant capacity in plants. *Nature Protocols*, *2*, 867–870.
41. Ortiz-Marchena, M. I., Ruiz, M., Valverde, F., & Romero, J. (2014). Determination of soluble sugars in arabidopsis thaliana leaves by anion exchange chromatography. *Bio-Protocol*, *4*, e1317.

How to cite this article: Zekri, M. A., Leimhofer, C., Drexler, N., & Lang, I. (2025). A rapid freezing method to determine tissue layer thickness in drought-stressed leaves. *Journal of Microscopy*, *297*, 316–324. https://doi.org/10.1111/jmi.13272

2.3. Beyond Guard Cells: Epidermal Anatomy Determines Transient Stomatal Opening Under Water Stress

Maryam Alsadat Zekri[1], Daniel Tholen[2*], Lukas Koller[2], Klara Voggeneder[2], Ingeborg Lang[1], Guillaume Theroux-Rancourt [2,3]

[1]University of Vienna, Department of Functional and Evolutionary Ecology, Faculty of Life Sciences, Djerassiplatz 1, A-1030, Vienna, Austria

[2]BOKU University, Institute of Botany, Department of Ecosystem Management, Climate and Biodiversity, Gregor-Mendel-Straße 33, A-1180, Vienna, Austria

[3]Biopterre – Bioproducts development center, 1642, rue de la Ferme, Sainte-Anne-de-la-Pocatière (Québec), G0R 1Z0, Canada

*Corresponding author: daniel.tholen@boku.ac.at

Submitted: 10 February 2026

Submission ID: c9616141-c9d4-4790-9759-a0a9d91c2a15

Authors contribution

GTR, DT, IL, and MAZ conceived the project. MAZ acquired gas exchange data, with contributions from KV and GTR. MAZ and LK acquired imaging data. MAZ, DT, and GTR analysed the data. MAZ wrote the first draft of the manuscript, with contributions from GTR, DT and IL. All authors agreed on the final version.

Beyond Guard Cells: Epidermal Anatomy Determines Transient Stomatal Opening Under Water Stress

Maryam Alsadat Zekri[1], Daniel Tholen[2*], Lukas Koller[2], Klara Voggeneder[2], Ingeborg Lang[1], Guillaume Theroux-Rancourt [2,4]

[1] University of Vienna, Department of Functional and Evolutionary Ecology, Faculty of Life Sciences, Djerassiplatz 1, A-1030, Vienna, Austria

[2] BOKU University, Institute of Botany, Department of Ecosystem Management, Climate and Biodiversity, Gregor-Mendel-Straße 33, A-1180, Vienna, Austria

[4] Current address: Biopterre – Bioproducts development center, 1642, rue de la Ferme, Sainte-Anne-de-la-Pocatière (Québec), G0R 1Z0, Canada

***Corresponding author: daniel.tholen@boku.ac.at**

Abstract

The rapid and transient increase of stomatal conductance in response to a sudden water deficit in plants, known as the wrong-way response (WWR), is hypothesized to result from turgor loss of the epidermal cells, which pulls the guard cells apart, leading to the opening of the stomatal pore. Variations in WWR amplitude and duration likely stem from differences in epidermal anatomy and/or cell density, which influence the localized turgor responses driving stomatal movement. This study tested this hypothesis by aiming to induce WWRs through both a sudden increase in the leaf-to-atmosphere vapor-pressure deficit and leaf excision. We investigated the WWR kinetics in five *Arabidopsis thaliana* lines with varying stomatal and pavement cell characteristics. Stomatal conductance was measured at high temporal resolution using infrared gas analysis and epidermal anatomical traits were characterized using scanning electron microscopy. No WWR was detectable following a rapid decrease in air humidity. By contrast, leaf excision consistently triggered a WWR across the majority of leaves and lines examined, enabling robust analysis of response kinetics. While the magnitude of the WWR did not differ among lines, significant variation was observed in the duration of stomatal opening. Lines with smaller pavement cells exhibited a significantly shorter WWR. The rate of stomatal opening following excision was strongly correlated with pavement cell area, path length, and branch length, but not with stomatal size or density. These findings indicate that pavement cell size, rather than cell shape or stomatal dimensions, is a key determinant of the mechanical coupling underlying the WWR.

Keywords: stomata, pavement cells, epidermis, Wrong-Way Response (WWR), *Arabidopsis thaliana*, leaf anatomy

2 Introduction

Stomata are tiny pores on the leaf surface that act as gatekeepers between the atmosphere and the leaf interior, regulating the intake of CO_2 and limiting transpiration. These pores are highly sensitive to environmental conditions that alter the plant's water potential, such as soil drought or declining air humidity. Stomatal closure in response to a water deficit is typically explained by a hydroactive feedback mechanism involving the accumulation of abscisic acid (ABA), which triggers the release of osmotic solutes from the guard cells. This process reduces osmotic pressure in these cells, leading to the closure of the stomata (via the wdm package;Buckley, 2019; Bharath et al., 2021). Nevertheless, stomatal closure is not the immediate response of plants to a decline in water potential. Instead, a transient opening of the stomata immediately after the drop in water potential and before the eventual closure of stomata is typically observed (Darwin, 1898; Ivanov, 1928; Buckley et al., 2011; Cadart et al., 2019). This phenomenon, known as the Wrong-Way Response (WWR), is considered a hydropassive feedback resulting from reduced epidermal turgor pressure, which pulls apart the turgid guard cells, causing the stomata to open (Cowan, 1972; Buckley et al., 2011; Cadart et al., 2019). The role of pavement cells in pulling apart the guard cells following an immediate reduction in water potential has been described as the epidermal mechanical advantage (Buckley, 2019). This concept implies that the transient opening of a stoma in response to the drop in water potential is influenced not only by the physical properties of the stoma itself, but also by those of the surrounding pavement cells. While it is well-documented that physical properties of stomata, such as size and density, play a significant role in their movement (Franks and Beerling, 2009; Buckley et al., 2011; Carlson et al., 2015; Bertolino et al., 2019; Haworth et al., 2021), there remains a gap in our understanding of how physical characteristics of stomata and pavement cells affect the dynamics of transient opening. With our study we aim to address this gap by providing empirical data to shed light on the hypothesis of the epidermal mechanical advantage.

The role of pavement cell geometry in stomatal development (Dow and Bergmann, 2014) and responses to environmental stresses (Clauw et al., 2015) suggests that epidermal anatomy is a significant factor that modulates responses of plants to variations in water potential. In addition, smaller cells with a higher surface-area-to-volume ratio respond faster to osmotic changes (Drake et al., 2013; Giday et al., 2013; Kardiman and Rabild, 2017; Nunes et al., 2020) and it has been suggested that -mechanical pressures impact smaller structures less significantly than larger ones (Bassel et al., 2014). Additionally, stomatal density has been shown to play a role in gas exchange and stomatal conductance (Kardiman and Rabild, 2017; Harrison et al., 2020), aiding in the regulation of water potential.

In this study, we investigated the kinetics of the WWR in five Arabidopsis lines with contrasting epidermal anatomical features. The selected Arabidopsis lines varied in stomatal length, stomatal density, and pavement cell characteristics such as area, circularity, and number of lobes. These traits were analyzed to elucidate their role in the hydropassive response. We hypothesized that Arabidopsis lines with smaller pavement cells and stomata would be more resilient to mechanical forces resulting from water potential perturbations. Additionally, we

examined whether pavement cells with significantly different interdigitation (e.g. a higher lobe count and higher cell convexity) exhibit greater resistance to mechanical pressures like turgor. The observed line-specific differences in stomatal and pavement cell characteristics highlight their crucial role in leaf responses to water stress, suggesting that optimizing these traits is key to enhancing plant water use efficiency and stress tolerance.

2 Material and Methods

2.1 Plant material and growth conditions

Seeds of five *A. thaliana* lines were provided by NASC (NASC, 1991; lines are described in Table 1). 14 plants per line were grown in individual pots (diameter = 13 cm) containing 50% potting soil (Kranzinger GmbH, Austria), 33% quartz sand, and 17% perlite. Plants were grown for five weeks in a growth room (Walk-in FytoScope, Conviron MTPS 120, Manitoba, Canada) with a 16 h photoperiod, 130-150 µmol m^{-2} s^{-1} photosynthetic photon flux density, 22/18°C Day/night temperature, and 60-65% humidity (RH). Only well-grown and fully expanded leaves were used for further analysis (described below).

Table 1. *A. thaliana* lines used in this study and their respective phenotypic traits.

line	NASC catalog number	Notes
Col-8	N60000	Wild type
epf1epf2	N67139	Increased stomatal cell density compared with the wild type
lcd1-1	N6359	Low cell density in the leaf palisade parenchyma, resulting in decreased chlorophyll content per leaf area
SALK069	N569127	Bigger leaves and rosette than wild type
UBP	N70771	Larger and rounder rosette leaves than wild type in early stages, severely downward curled in later stages; sometimes show a knot in the leaf tip region in later rosette leaves, such as the ninth rosette leaf; increased epidermal and palisade cell number across the leaf blade

2.2 Stomatal density and pore length

To extract features of the epidermal cells, detailed images of the epidermis were collected by Scanning Electron Microscopy (SEM). Talbot and White (2013) compared several different tissue preparation methods for SEM and found that fixation by methanol and dehydration in ethanol typically results in the best preservation of tissue size and morphology (Talbot and White, 2013). We therefore adapted this method for our purposes. 10 mature leaves per line were fixed in 100% methanol for 10 minutes, followed by two dehydration steps in 100% ethanol for 30 minutes. Critical point drying (Leica, CPD300) was performed based on the manufacturer's protocol (CPD300, 2012). Three leaves were randomly selected from each line to collect anatomical features. Two pieces were excised from each leaf, one representing the abaxial surface and the other the adaxial surface and mounted on stubs using double-sided adhesive carbon tape. The leaf pieces were cut from the well-expanded portion of the leaf, avoiding the edges, tip, areas near the main vein, and petiole. Since the imaging was

performed under high vacuum, the samples were coated with gold (Small Multi-target Metal Ion Sputtering Coater Machine, Model Number, TMAX_BY_JS11). Three images at different locations on the abaxial and adaxial surface were acquired by SEM (JEOL IT300) (Fig. 1). On each image, three individual squares (5000 x 5000 µm) were selected to measure stomatal density. Stomatal length was defined as the length of the guard cells (Savvides et al., 2011) and was measured on 100 stomatal apertures per leaf (300 stomata apertures per line) using ImageJ (Schindelin et al., 2012).

2.3 Extraction of epidermal cell features

Based on the recommendations of Möller et al. (2017) and Sapala et al. (2019), six different epidermal characteristics were analysed. Lobe count, convexity, and circularity were chosen to characterize the shape of pavement cells, while three additional features, i.e. cell area, branch length, and longest path length were selected to represent cell size (Möller et al., 2017; Sapala et al., 2019). Lobe count indicates the total number of lobes along the cell contour, convexity measures the degree of indentations, and circularity assesses how closely the cell shape approximates a circle. Cell area denotes the total projected area of a cell, branch length refers to the length of the lobes, and longest path length represents the longest straight line that can be drawn through the cell (Sapala et al., 2019).

To extract features of pavement cells, SEM images were analysed using the PaCeQuant plugin of Fiji/ImageJ (Möller et al., 2017). Since this plugin requires high-contrast images, cell boundaries were first manually highlighted in GIMP-2.10 (The GIMP Development Team, 2019) and added to the image as a second layer, which was used in PaCeQuant (Fig. S1). Figure 1 shows representative SEM images of the abaxial and adaxial epidermis of the five Arabidopsis lines.

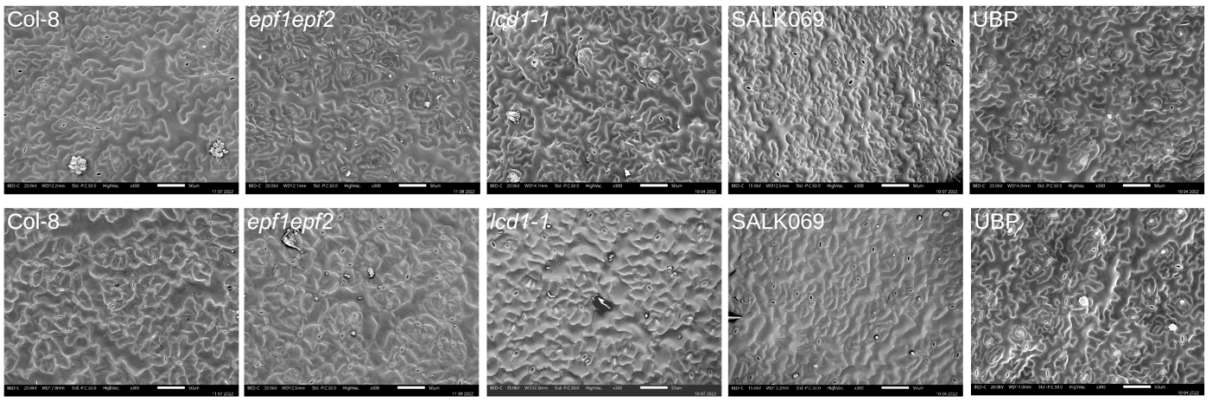

Figure 1. SEM images of abaxial (upper row) and adaxial (lower row) surfaces of five Arabidopsis lines. Scale bar: 50µm.

2.4 Gas exchange measurements and WWR experiments

The plants were watered daily for a cultivation period of six weeks. After the growth period, stomatal conductance to water vapor (g_{sw}) was measured using a portable infrared gas analyzer system (LI-6800, LI-COR Biosciences, Lincoln, NE, USA). The leaf temperature inside the chamber was maintained at 25°C along with a light level of 400 µmol m^{-2} s^{-1} and a

reference CO_2 concentration of 400 µmol mol^{-1}. Air flow rates through the sample chamber were kept constant at 400 µmol s^{-1} during leaf excision but increased to 500 µmol s^{-1} during humidity changes to facilitate faster mixing of air inside the chamber. The humidity of the air entering the leaf chamber was set to a relative humidity of 60%, corresponding to a leaf-to-atmosphere vapor-pressure deficit (VPD) of 1.2 kPa. Before initiating the WWR experiments, leaves were acclimated to these conditions for 10–15 minutes to reach a steady state. We considered gas exchange to be in a steady state when fluctuations in g_{sw} did not exceed 0.1 mol m^{-2} s^{-1} for 5 minutes. Subsequently, the humidity was reduced to ~19% RH, corresponding to a VPD of 2.5 kPa. To study epidermal anatomy, 10 leaves per line were labeled and put aside for subsequent microscopic analysis (see below). Seven leaves were used for the second WWR experiment, in which their petiole was cut just below the lamina to separate the leaf from the plant using a razor blade (called "excision" hereafter) (Fig. 2)

2.5 Correction for lags resulting from chamber mixing and water desorption

After changing the humidity in the leaf chamber, the water differential in the chamber becomes unstable. Zait et al. (2024) characterized the response of artificial plastic leaves and concluded that water concentrations were unstable for 3 minutes post-switch due to the gas turnover time of the chamber (Zait et al., 2024). However, the effect of water desorption from chamber walls can persist even longer and Buckley et al. (2011) fitted a two-phase exponential model to the empty chamber response to correct for both issues. We followed a similar approach by using a 200 µm Polytetrafluoroethylene foil (PTFE; LI-COR Biosciences, Lincoln, NE, USA) representing a leaf as control measurement. We subsequently fitted a smoothed biphasic power law to the relation between time and the difference between sample and reference humidity. Sample water concentrations were corrected using this model and stomatal conductance was recalculated using the gasanalyzer package (Tholen, 2024). Large outliers persisted immediately after changing the VPD, even with the correction. Because the WWR peak is expected to occur several minutes later (Buckley et al. 2011), we discarded data from the first 36 seconds to avoid these initial gas transients.

2.6 The kinetics of the WWR

The kinetics of the WWR were analyzed using g_{sw} values extracted at specific time points (Fig. 2), following Buckley et al. (2011). Time 1 (T_1) represents the moment where g_{sw} reached a steady state before switching to a higher VPD (g_1), time 2 (T_2) indicates the time when g_{sw} reached its maximum during the WWR (g_2), and time 3 (T_3) is when g_{sw} returned to the g_1 value (g_3). Time 4 (T_4) indicates the time when g_{sw} stopped changing (g_4) and at which the petiole was cut from the leaf, time 5 (T_5) indicates the moment where g_{sw} peaked (g_5), and time 6 (T_6) is when g_{sw} dropped back to the level of g_4. The kinetics of the WWR were described by the size (S), length (L), and rate ($V = S/L$) based on Buckley et al. (2011). The size of the WWR represents how much stomatal conductance increased ($g_2 - g_1$ or $g_5 - g_4$) after an increase in VPD or leaf excision, and the WWR length represents how long it took to reach the maximum stomatal conductance after an increase in VPD or leaf excision ($T_2 - T_1$ or $T_5 - T_4$; Fig 2).

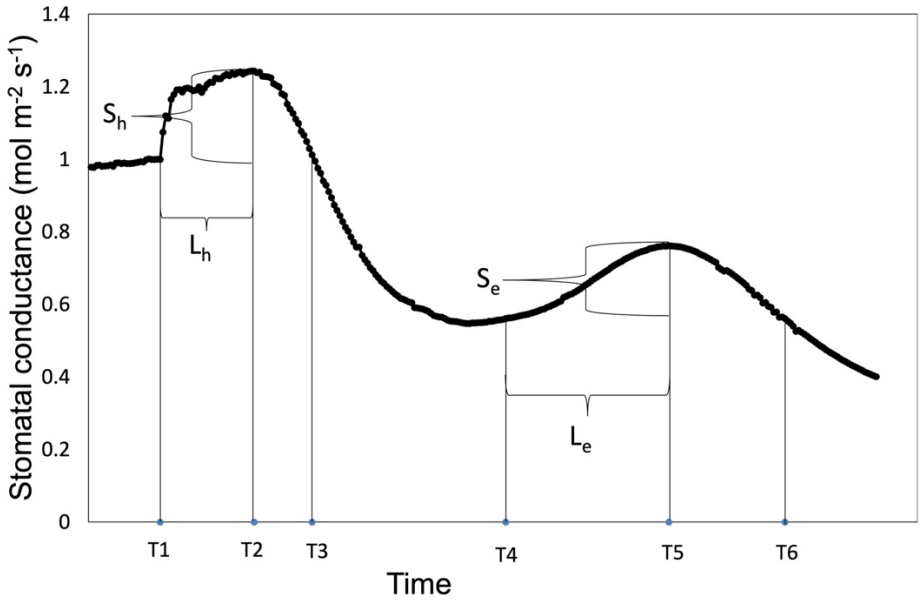

Figure 2. Schematic of the expected stomatal conductance in response to a reduction in humidity and leaf excision at time points T_1 to T_4, respectively. T_1 indicates the time of humidity reduction. At T_2, g_{sw} reaches its highest value. At T_3, g_{sw} drops back to the value of g_{sw} at T_1. At T_4, the leaf was excised. The highest g_{sw} occurred at T_5 after cutting the leaf. At T_6, g_{sw} dropped back to g_{sw} at T_4. Sh: WWR size caused by reducing the humidity, Lh: WWR length caused by reducing the humidity, Se: WWR size caused by excision, and Le: WWR length caused by excision.

2.7 Statistical analysis

Differences between groups were tested using a one-way anova or linear mixed-effect model using R version 4.3.2 (R Core Team, 2024) and the lme4 package (Bates, 2015). Pairwise comparisons between means were done using the emmeans package (Lenth, 2024) using the Tukey method for p-value adjustment. The normality of the data was confirmed using a Shapiro–Wilk test. Correlation significance between anatomical traits and WWR parameters was determined using a weighted Kendall's test (via the wdm package; (Nagler, 2025)) rather than Pearson's correlation because it better captures possible non-linear, monotonic relationships while remaining robust to outliers and heteroscedasticity. Weights were assigned based on the combined relative error of the traits, defined by the product of their respective sample sizes divided by their variances, to mitigate the influence of high-variance outliers on the correlation coefficient.

3 Results

3.1 Stomatal density and size

The stomatal density of *epf1epf2* was typically doubled relative to that of the other lines ($p < 0.001$; Fig. 3). There were no significant differences in total stomatal density among Col-8, *lcd1-1*, SALK069, and UBP15. Average length of the guard cells was generally consistent across lines, except that in UBP15, pores were 5-7% ($p<0.05$) larger compared to *epf1epf2* and *lcd1-1* (Fig. 4).

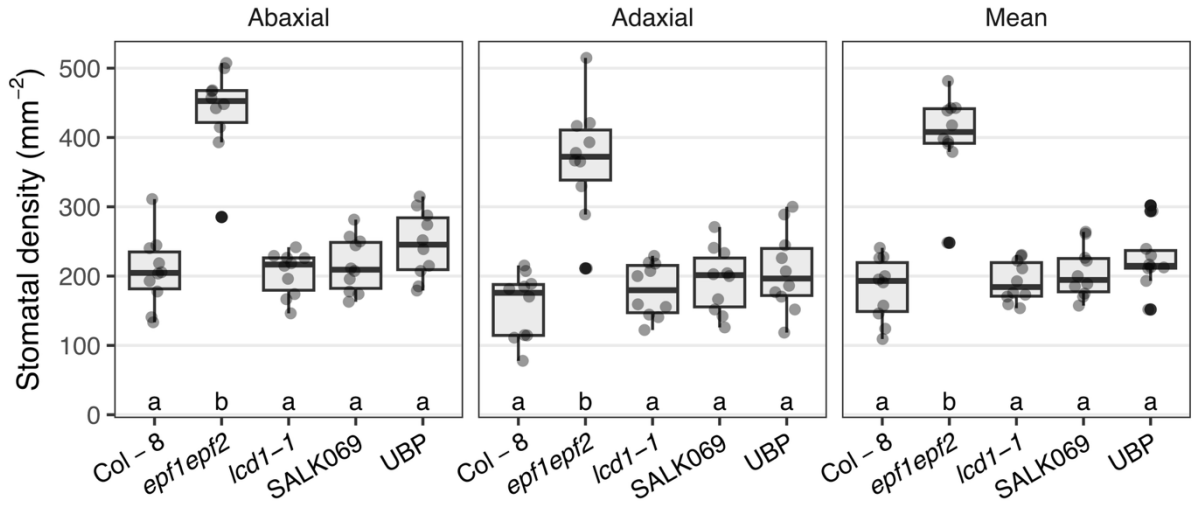

Figure 3. Stomatal density of 5 different Arabidopsis lines on the abaxial and adaxial surface, and in total. Different letters indicate a significant difference according to Tukey's honest significant difference test (n = 10).

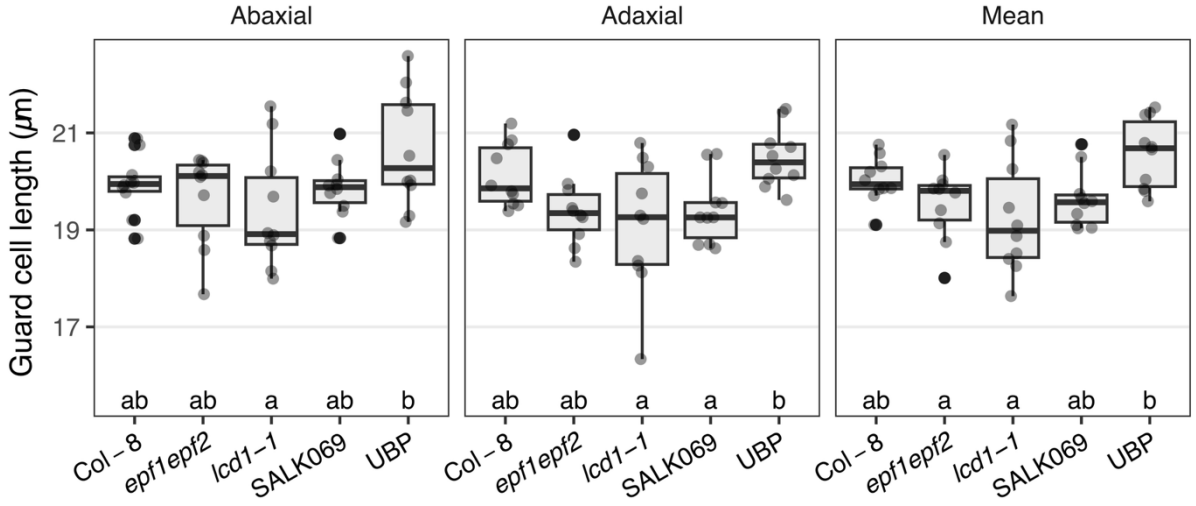

Figure 4. Size of abaxial and adaxial guard cells, and mean size of guard cells from both surfaces, among the five different Arabidopsis lines studied. Different letters indicate a significant difference according to Tukey's honest significant difference test (n = 10)

3.2 Features of pavement cells

Projected areas of the pavement cells on the abaxial side were largest for Col-8 and UBP15, although the difference only achieved significance in comparison with epf*1epf2* (67-68%, $p < 0.01$; Fig. 5). *epf1epf2* cells on this leaf surface also showed higher circularity than Col-8 (32%, $p<0.001$), *lcd1-1* (29%, $p<0.001$), SALK069 (25%, $p<0.01$), and UBP15 (23% higher, $p<0.01$), and shorter branch lengths compared to UBP15 (27%, $p<0.01$) and marginally so when compared to Col-8 (21%, $p = 0.065$). The longest path through a cell was also shorter in *epf1epf2* than in Col-8 (49% shorter, $p<0.001$), SALK069 (40% shorter, $p<0.001$), UBP15 (47% shorter, $p<0.001$), and *lcd1-1* (39% shorter, $p<0.001$). *epf1epf2* had the highest cell convexity, being 7-9% ($p<0.001$) higher than all other lines, and had 44-52% fewer lobes compared to the other lines.

Results for the adaxial leaf side were very similar, although the differences were typically somewhat smaller (Fig. S2).

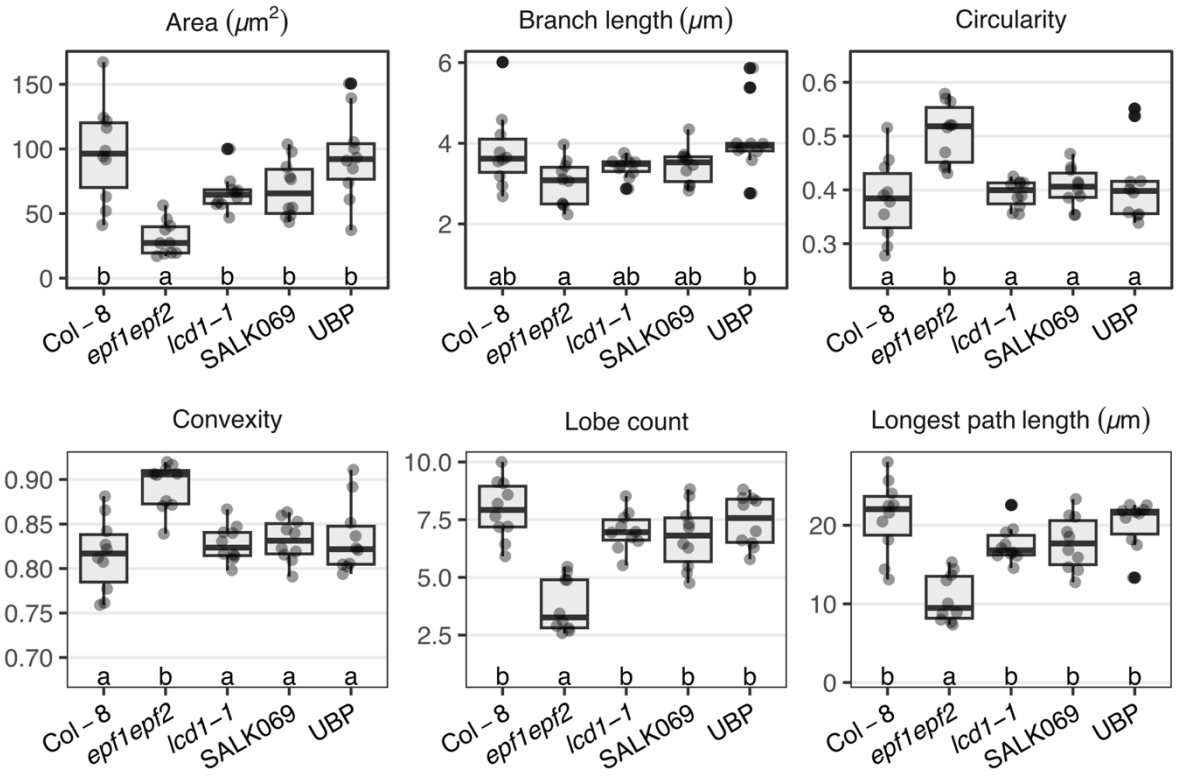

Figure 5. Anatomical characteristics of epidermal cells on the abaxial leaf surface in the five lines of A. thaliana. Different letters represent a statistically significant difference between the lines for each trait analyzed (n = 10).

3.3 WWR parameters

Gas-exchange data showed clear changes in water mole fractions, transpiration rates and stomatal conductance after a change in VPD and after leaf excision. However, after accounting for chamber mixing and water desorption, the increase in g_{sw} in response to a VPD change was negligible in all five lines (Fig. S3). Following leaf excision, a clear WWR was typically observed (Fig. S4). The features defining the WWR were quantified as described in Figure 2. The size of the WWR was comparable across all 5 lines, but differences in response length were observed, with *epf1epf2* having a significantly shorter WWR compared to the Col-8 control plants (Fig. 6). Interestingly, several replicates of SALK069 and *lcd1-1* did not show a WWR, increasing the variance and reducing the size and length of the averaged response (Fig S4).

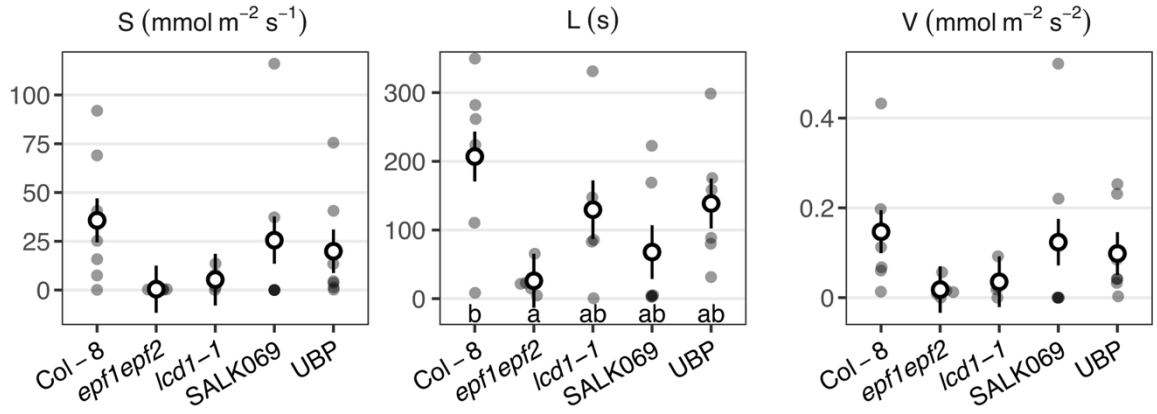

Figure 6. WWR parameters in response to leaf excision (n= 5-7). A) Size of the WWR, relative to steady-state stomatal conductance before excision. B) Length of the WWR. C) Rate of the WWR (*V*), computed as *S*/*L*. Different letters represent a statistically significant difference between the lines for each trait analyzed.

3.4 Relationships between WWR parameters and leaf anatomical properties

The length of the WWR following excision (*L*) was significantly correlated on the abaxial surface to pavement cell area (p=0.038) and the longest path length (p=0.022), but not with branch length (p=0.18), stomatal density (p=0.13), or guard cell length (p=0.96; Fig. 7). The rate of WWR following excision (*V*) presented stronger correlations with abaxial epidermal features. *V* showed significant relationships on the abaxial surface with pavement cells area (p=0.015), longest path length (p=0.019), and branch length (p=0.037; Fig. 7). As for L, there were no correlations with stomatal density (p=0.28) or guard cell length (p=0.89).

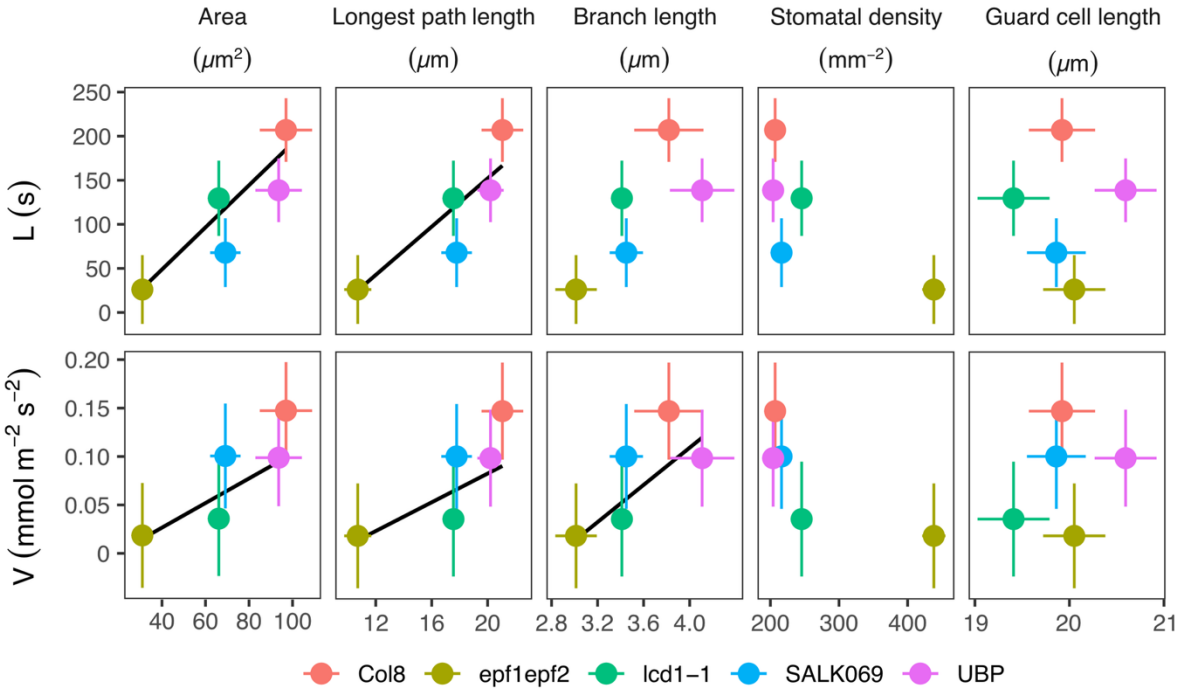

Figure 7. Relationships between the length (L, top row) and the rate (V, bottom row) of stomatal transient opening following leaf excision and anatomical properties on the abaxial

surface for five studied *A. thaliana* lines. Points show mean values for each Arabidopsis line, with horizontal and vertical bars representing standard deviation for each variable. Statistically significant correlations are represented with a black line. No significant correlation was observed with stomatal features.

4 Discussion

In this study, we analyzed sudden stomatal opening in response to a drop in water potential triggered either by a switch from low to high VPD or by leaf excision, commonly referred to as the wrong-way response (WWR). The most widely accepted hypothesis explaining the WWR suggests a passive and ABA-independent pathway following a decrease in water potential: a decline in turgor pressure of the pavement cells pulls the guard cells apart, resulting in transient stomatal opening (Buckley, 2019).

Buckley et al. (2011) observed a WWR after increasing VPD that peaked between 7 and 11 minutes after changing humidity in 20 species with heterobaric or homobaric leaves and herbaceous or woody life forms (Buckley et al., 2011). For different Arabidopsis lines, Zait et al. (2024) reported durations between 8 and 17 min (Zait et al., 2024). At our relatively high flow speeds (500 µmol s^{-1}), the effect of gas-mixing and water desorption from the chamber walls can persist for 10 min after a change in chamber humidity. After appropriate correction for such effects, we found no evidence for an increase in g_{sw} following a change in humidity. This highlights that failing to account for the effects of mixing and water desorption can easily lead to misinterpretation of the size and length of the WWR. We confirmed our results in separate experiments with the same Arabidopsis lines grown in different growth-chambers (with a photoperiod of 10 h and a light intensity of 300 µmol m^{-2} s^{-1}) and measured with different gas-exchange equipment (GFS-3000, Heinz Walz GmbH, Effeltrich, Germany). Since it takes time for the humidity concentration in the gas-exchange chamber to stabilize, a response faster than 36 seconds could have occurred. However, it is very unlikely that the hydroactive response would trigger after such a short timeframe and suppress a WWR (Buckley et al., 2011; Vialet-Chabrand et al., 2017). Transient responses to humidity are not consistently observed in all individuals and species (Buckley et al. 2011). This aligns with our own results and indicates that, under our growth and measurement conditions, the epidermal cells of Arabidopsis may simply not dehydrate rapidly enough following a humidity drop to trigger a WWR. It may be that the time required for the humidity to stabilize at the lower level (less than 40 s) was not abrupt enough, or the high VPD (2.5 kPa) was not substantial enough to trigger substantial epidermal dehydration.

When leaves were excised, some notable differences in the responses of the different lines were observed. The line with the smallest pavement cells, *epf1epf2*, exhibited the shortest WWR (Fig. 6). L, as well as V, were correlated to the size of the pavement cells on the abaxial surface (through cell area and the longest path length within cells; Fig. 7). Therefore, the observed relation between decreasing size of the pavement cells on the abaxial surface and shorter L or smaller V suggests that it is the size, rather than the shape, of the pavement cells that drives the rate of WWR. Thus, smaller cells may exert less backpressure on the guard cells. In agreement with this view, Bassel et al. (2014) demonstrated that when large

embryonic cells of *Arabidopsis* sp. are subjected to pressure, they expand more than smaller cells (Bassel et al., 2014). Similarly, results by Sapala et al. (2018) supported the view that plants confront mechanical stresses through higher cell interdigitation or smaller cell size (Sapala et al., 2018; Bidhendi et al., 2019). This implies that small cells are more resilient to volume changes under mechanical stresses and supports the idea of higher capability of small structures to adjust their anatomy to confront mechanical stresses (Drake et al., 2013; Kardiman and Rabild, 2017; Théroux-Rancourt et al., 2021; Caine et al., 2023).

The observed differences in stomatal responses following the humidity or excision treatments may be related to the nature of the induced water deficit. The decrease in leaf water potential following low humidity is likely more gradual and primarily driven by external environmental factors. The water loss of epidermal cells is influenced by mechanical barriers such as the cuticle (Riederer and Schreiber, 2001), trichomes (Zhu et al., 2024), and, more generally, by the boundary layer conductance (Schuepp, 1993; Jones, 1999). Leaf excision, on the other hand, disrupts the water supply from the soil, causing a severe and abrupt decline in leaf water potential. Although epidermal properties may still affect the rate of dehydration, the latter may also be affected by other tissues in the hydraulic pathway, such as the bundle sheaths cells, which are known to control water flow from the xylem into the mesophyll cells (Sade et al., 2015; Hua et al., 2021). In addition, embolism formation in the xylem may also influence the dynamics of the WWR (Salleo et al., 2000; Buckley et al., 2011). Therefore, the absence of a WWR following a change in humidity may be related to the capacity of the leaf to maintain the hydration status of the epidermis through continued water supply from the mesophyll or xylem. Excision of the petiole would not allow such a mechanism and therefore induce a WWR.

The findings of this study enhance our understanding of stomatal responses and the mechanisms by which plants react to changes in water potential. It also gives further insights into plant adaptability and resilience and supports previous results for other plant species. Shorter WWR durations and slower stomatal opening rates were associated with smaller pavement cells, but these features were independent of stomatal traits. This highlights the distinct roles of pavement cell geometry and stomatal anatomy in epidermal organization and demonstrates how cell packing specifically shapes leaf physiological responses. Understanding these relationships is essential for breeding programs aimed at improving plant resilience to environmental stressors. However, as previously discussed, stomatal responses are influenced by more than just stomata and pavement cells and follow-up studies on the anatomical properties of the mesophyll and vascular tissue in plants with varying WWR kinetics are suggested to generate a more holistic view of the dynamics of stomatal transient opening. In addition, future research should explore the genetic mechanisms underlying driving these morphological traits and evaluate their direct impact on plant fitness in various environments.

Authors contribution

GTR**,** DT, IL, and MAZ conceived the project. MAZ acquired gas exchange data, with contributions from KV and GTR. MAZ and LK acquired imaging data. MAZ, DT, and GTR analyzed the data. MAZ wrote the first draft of the manuscript, with contributions from GTR, DT and IL. All authors agreed on the final version.

AI generative statement

No AI tool was used to generate scientific content, analysis, interpretations, or results. All text was revised and edited by the authors, who take full responsibility for the accuracy and integrity of the content. The authors used the generative AI tool ChatGPT (OpenAI) only for grammar and language improvement. The authors confirm that there is no AI authorship or contribution to the scientific conclusions of the paper.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

Acknowledgement

Microscopy (SEM Image acquisition) was performed at the Core Facility Cell Imaging and Ultrastructure Research, University of Vienna - member of the Vienna Life-Science Instruments (VLSI) and we are grateful to Daniela Gruber for guiding in SEM image acquisition. We gratefully acknowledge the funding from the Vienna Science and Technology Fund (WWTF) [10.47379/LS19013].

References

Bassel, G. W., P. Stamm, G. Mosca, P. Barbier de Reuille, D. J. Gibbs, R. Winter, A. Janka, M. J. Holdsworth, and R. S. Smith. 2014. Mechanical constraints imposed by 3D cellular geometry and arrangement modulate growth patterns in the Arabidopsis embryo. *Proceedings of the National Academy of Sciences* 111 (23):8685-8690. doi: doi:10.1073/pnas.1404616111.

Bates, D., Mächler, M., Bolker, B., and Walker, S. 2015. Fitting linear mixed-effects models using lme4', Journal of Statistical Software. *Journal of Statistical Software, 67(1), pp. 1–48*. doi: doi:10.18637/jss.v067.i01.

Bertolino, L. T., R. S. Caine, and J. E. Gray. 2019. Impact of Stomatal Density and Morphology on Water-Use Efficiency in a Changing World. *Frontiers in Plant Science* 10. doi: 10.3389/fpls.2019.00225.

Bharath, P., S. Gahir, and A. S. Raghavendra. 2021. Abscisic Acid-Induced Stomatal Closure: An Important Component of Plant Defense Against Abiotic and Biotic Stress. *Frontiers in Plant Science* 12. doi: 10.3389/fpls.2021.615114.

Bidhendi, A. J., B. Altartouri, F. P. Gosselin, and A. Geitmann. 2019. Mechanical Stress Initiates and Sustains the Morphogenesis of Wavy Leaf Epidermal Cells. *Cell Reports* 28 (5):1237-1250.e6. doi: https://doi.org/10.1016/j.celrep.2019.07.006.

Buckley, T. N. 2019. How do stomata respond to water status? *New Phytologist* 224 (1):21-36. doi: https://doi.org/10.1111/nph.15899.

Buckley, T. N., L. Sack, and M. E. Gilbert. 2011. The Role of Bundle Sheath Extensions and Life Form in Stomatal Responses to Leaf Water Status *Plant Physiology* 156 (2):962-973. doi: 10.1104/pp.111.175638.

Cadart, C., L. Venkova, P. Recho, M. Lagomarsino, and M. Piel. 2019. The physics of cell-size regulation across timescales. *Nature Physics* 15. doi: 10.1038/s41567-019-0629-y.

Caine, R. S., E. L. Harrison, J. Sloan, P. M. Flis, S. Fischer, M. S. Khan, P. T. Nguyen, L. T. Nguyen, J. E. Gray, and H. Croft. 2023. The influences of stomatal size and density on rice abiotic stress resilience. *New Phytologist* 237 (6):2180-2195. doi:

https://doi.org/10.1111/nph.18704.
Carlson, J. E., C. A. Adams, and K. E. Holsinger. 2015. Intraspecific variation in stomatal traits, leaf traits and physiology reflects adaptation along aridity gradients in a South African shrub. *Annals of Botany* 117 (1):195-207. doi: 10.1093/aob/mcv146.
Clauw, P., F. Coppens, K. De Beuf, S. Dhondt, T. Van Daele, K. Maleux, V. Storme, L. Clement, N. Gonzalez, and D. Inzé. 2015. Leaf Responses to Mild Drought Stress in Natural Variants of Arabidopsis *Plant Physiology* 167 (3):800-816. doi: 10.1104/pp.114.254284.
Cowan, I. R. 1972. Oscillations in stomatal conductance and plant functioning associated with stomatal conductance: Observations and a model. *Planta* 106 (3):185-219. doi: 10.1007/BF00388098.
Darwin, F. 1898. Observations on stomata. *Proceedings of the Royal Society of London* 63 (389-400):413-417. doi: doi:10.1098/rspl.1898.0053.
Dow, G. J., and D. C. Bergmann. 2014. Patterning and processes: how stomatal development defines physiological potential. *Current Opinion in Plant Biology* 21:67-74. doi: https://doi.org/10.1016/j.pbi.2014.06.007.
Drake, P. L., R. H. Froend, and P. J. Franks. 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *J Exp Bot* 64 (2):495-505. doi: 10.1093/jxb/ers347.
Franks, P. J., and D. J. Beerling. 2009. Maximum leaf conductance driven by CO₂ effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences* 106 (25):10343-10347. doi: doi:10.1073/pnas.0904209106.
Giday, H., K. H. Kjaer, D. Fanourakis, and C.-O. Ottosen. 2013. Smaller stomata require less severe leaf drying to close: A case study in Rosa hydrida. *Journal of Plant Physiology* 170 (15):1309-1316. doi: https://doi.org/10.1016/j.jplph.2013.04.007.
Harrison, E. L., L. Arce Cubas, J. E. Gray, and C. Hepworth. 2020. The influence of stomatal morphology and distribution on photosynthetic gas exchange. *Plant J* 101 (4):768-779. doi: 10.1111/tpj.14560.
Haworth, M., G. Marino, F. Loreto, and M. Centritto. 2021. Integrating stomatal physiology and morphology: evolution of stomatal control and development of future crops. *Oecologia* 197 (4):867-883. doi: 10.1007/s00442-021-04857-3.
Hua, L., S. R. Stevenson, I. Reyna-Llorens, H. Xiong, S. Kopriva, and J. M. Hibberd. 2021. The bundle sheath of rice is conditioned to play an active role in water transport as well as sulfur assimilation and jasmonic acid synthesis. *The Plant Journal* 107 (1):268-286. doi: https://doi.org/10.1111/tpj.15292.
Ivanov, L. A. 1928. Zur Methodik der Transpirationsbestimmung am Standort. . *Berichte der Deutschen Botanischen Gesellschaft* 46: 306-310.
Jones, H. G. 1999. Physicochemical and Environmental Plant Physiology, 2nd edn. *Journal of Applied Ecology* 36 (6):1076-1077. doi: https://doi.org/10.1046/j.1365-2664.1999.00459-5.x.
Kardiman, R., and A. Rabild. 2017. Relationship between stomatal density, size and speed of opening in Sumatran rainforest species. *Tree Physiology* 38 (5):696-705. doi: 10.1093/treephys/tpx149.
Möller, B., Y. Poeschl, R. Plötner, and K. Bürstenbinder. 2017. PaCeQuant: A Tool for High-Throughput Quantification of Pavement Cell Shape Characteristics *Plant Physiology* 175 (3):998-1017. doi: 10.1104/pp.17.00961.
Nagler. 2025. wdm: Weighted Dependence Measures
. doi: doi:10.32614.

NASC. 1991. "The Nottingham Arabidopsis Stock Centre. Nottingham Arabidopsis Stock Centre, University of Nottingham, UK. ." Available at: https://arabidopsis.info/.

Nunes, T. D. G., D. Zhang, and M. T. Raissig. 2020. Form, development and function of grass stomata. *Plant J* 101 (4):780-799. doi: 10.1111/tpj.14552.

Riederer, M., and L. Schreiber. 2001. Protecting against water loss: analysis of the barrier properties of plant cuticles. *Journal of Experimental Botany* 52 (363):2023-2032. doi: 10.1093/jexbot/52.363.2023.

Sade, N., A. Shatil-Cohen, and M. Moshelion. 2015. Bundle-sheath aquaporins play a role in controlling Arabidopsis leaf hydraulic conductivity. *Plant signaling & behavior* 10:e1017177. doi: 10.1080/15592324.2015.1017177.

Salleo, S., A. Nardini, F. Pitt, and M. A. L. Gullo. 2000. Xylem cavitation and hydraulic control of stomatal conductance in Laurel (Laurus nobilis L.). *Plant, Cell & Environment* 23 (1):71-79. doi: https://doi.org/10.1046/j.1365-3040.2000.00516.x.

Sapala, A., A. Runions, A. L. Routier-Kierzkowska, M. Das Gupta, L. Hong, H. Hofhuis, S. Verger, G. Mosca, C. B. Li, A. Hay, O. Hamant, A. H. Roeder, M. Tsiantis, P. Prusinkiewicz, and R. S. Smith. 2018. Why plants make puzzle cells, and how their shape emerges. *Elife* 7. doi: 10.7554/eLife.32794.

Sapala, A., A. Runions, and R. S. Smith. 2019. Mechanics, geometry and genetics of epidermal cell shape regulation: different pieces of the same puzzle. *Current Opinion in Plant Biology* 47:1-8. doi: https://doi.org/10.1016/j.pbi.2018.07.017.

Savvides, A., D. Fanourakis, and W. Ieperen. 2011. Co-ordination of hydraulic and stomatal conductances across light qualities in cucumber leaves. *Journal of experimental botany* 63:1135-43. doi: 10.1093/jxb/err348.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona. 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods* 9 (7):676-682. doi: 10.1038/nmeth.2019.

Schuepp, P. H. 1993. Tansley Review No. 59 Leaf boundary layers. *New Phytologist* 125 (3):477-507. doi: https://doi.org/10.1111/j.1469-8137.1993.tb03898.x.

Talbot, M. J., and R. G. White. 2013. Methanol fixation of plant tissue for Scanning Electron Microscopy improves preservation of tissue morphology and dimensions. *Plant Methods* 9 (1):36. doi: 10.1186/1746-4811-9-36.

Théroux-Rancourt, G., A. B. Roddy, J. M. Earles, M. E. Gilbert, M. A. Zwieniecki, C. K. Boyce, D. Tholen, A. J. McElrone, K. A. Simonin, and C. R. Brodersen. 2021. Maximum CO₂ diffusion inside leaves is limited by the scaling of cell size and genome size. *Proceedings of the Royal Society B: Biological Sciences* 288 (1945):20203145. doi: doi:10.1098/rspb.2020.3145.

Tholen, D. 2024. GasanalyzeR: advancing reproducible research using a new R package for photosynthesis data workflows. *AoB PLANTS* 16 (4). doi: 10.1093/aobpla/plae035.

Vialet-Chabrand, S. R. M., J. S. A. Matthews, L. McAusland, M. R. Blatt, H. Griffiths, and T. Lawson. 2017. Temporal Dynamics of Stomatal Behavior: Modeling and Implications for Photosynthesis and Water Use *Plant Physiology* 174 (2):603-613. doi: 10.1104/pp.17.00125.

Zait, Y., A. Joseph, and S. M. Assmann. 2024. Stomatal responses to VPD utilize guard cell intracellular signaling components. *Frontiers in Plant Science* Volume 15 - 2024. doi: 10.3389/fpls.2024.1351612.

Zhu, Y., J. Zheng, H. Kang, N. Hui, S. Yin, Z. Chen, B. Du, and C. Liu. 2024. Spatial variations in leaf trichomes and their coordination with stomata in Quercus variabilis across Eastern

Asia. *Journal of Plant Ecology* 17 (3):rtae023. doi: 10.1093/jpe/rtae023.

3. Discussion

The objective of this doctoral research was to show how plant anatomical features at multiple levels, ranging from epidermal structures such as stomata and trichomes to internal cellular and subcellular structures of tissues and leaves, contribute to a physiological performance under contrasting water availability. Based on the three studies presented in this thesis, the consistent outcome is: **anatomical plasticity** plays a central role in determining plants responses to variations in water availability, yet the functional significance of individual traits is strongly context dependent. This work combines whole-leaf physiological measurements with detailed anatomical analyses, providing more insights to fill the gap between classical physiological studies and modern cell-biological approaches. The results collectively demonstrate that neither stomatal traits, trichomes, nor internal cellular organization alone can explain water responses. Instead, plant response to water stress arises from coordinated adjustments of multiple structures within leaves.

3.1. Stomatal traits as dynamic regulators rather than fixed determinants

The first key contribution of this thesis is that stomatal density and size should be interpreted as dynamic traits meaning that stomatal density and size are plastic depending on environmental conditions, and their modification has the key role in plants responses to a water deficit. We demonstrated that *Arabidopsis* sp., lines with inherently high stomatal density (e.g. *epf1epf2* and *tmm-1*) exhibited higher photosynthetic yield (F_v'/F_m') under well-watered conditions, which aligns with previous studies that higher stomatal abundance enhances CO_2 diffusion and light-use efficiency when water is not limiting (Bertolino et al., 2019; Sakoda et al., 2020a). However, as stomatal density declined, the photosynthetic yield also decreased. Notably, lines with higher initial stomatal densities exhibited a pronounced decline in photosynthetic yield when stomatal numbers decreased below their initial levels, even though stomatal density remained higher than in control plant (Col-8). This observation is consistent with studies showing that altered stomatal density or reduced stomatal conductance can influence primary photosynthetic reactions (Schlüter et al., 2003; Singh and Raja Reddy, 2011). In contrast, no significant change in Fv'/Fm' was observed in stressed wild-type (Col-8) plant. The increase in stomatal density and a reduction in stomatal size, along with stable F_v'/F_m' in stressed wild-type plants (Col-8) supports prior studies demonstrating that a high density of smaller stomata is an adaptive advantage, enabling plants to optimize CO_2 uptake and photosynthetic gas exchange under fluctuating environmental conditions while minimizing water loss through transpiration, thereby preventing drought-induced damage and preserving overall photosynthetic performance (Franks and Beerling, 2009; Pitaloka et al., 2022; Caine et al., 2023; Haworth et al., 2023; Liu et al., 2023). These findings support the view that the adjustment of stomatal density and size enhances stomatal responsiveness and water-use efficiency under stress, which is consistent with recent work in plant model species and crops (Haworth et al., 2021; Lei et al., 2022; Caine et al., 2023; Liu et al., 2023).

3.2. Evaluating the functional role of trichomes under drought stress

The first publication in this thesis examines how trichomes influence plant responses to drought stress (Zekri and Lang, 2024). Trichomes are often assumed to improve drought tolerance by increasing boundary layer resistance and reducing leaf temperature(Amada et al., 2017; Amada et al., 2020). Although this thesis confirms this view by showing higher leaf temperature in trichome-free Arabidopsis line *tmm-1* under drought, the physiological consequences of this elevated temperature were not as expected. Although the leaf temperature was elevated, *tmm-1* did not exhibit a stronger decline in F_v'/F_m' when compared to the trichome-bearing line *epf1epf2*. This observation suggests that trichomes *alone* do not protect the photosynthetic apparatus under water limitation. No significant difference in boundary layer conductance among lines diminished the importance of trichomes under drought stress, which supports the view that internal leaf features and stomatal adjustments may be more effective than the presence of trichomes when water availability is the main constraint (Benz and Martin, 2006; Bickford, 2016; Amada et al., 2017; Buckley, 2019). In the absence of trichomes, the *tmm-1* leaves likely absorbed more radiation, which contributed to increased leaf heating without necessarily impairing photosystem yield. As mentioned before, the study highlights that drought responses were more strongly associated with stomatal adjustments than with trichome presence. For instance, the wild-type Col-8 maintained photosynthetic yield under drought by increasing stomatal density, which enhances CO_2 diffusion into the inner leaf. In contrast, *epf1epf2* and *tmm-1* exhibited reduced stomatal density under drought, which coincided with decreased photosynthetic performance. These findings suggest that internal leaf anatomical traits and stomatal regulation play a more decisive role in maintaining photosynthetic function during water limitation than features such as trichomes. This finding challenges models of leaf energy balance (Amada et al., 2017; Bertolino et al., 2019; Amada et al., 2023; Tan et al., 2025) that rely on single factors and calls for a more holistic interpretation of trichome function that also includes the interactions with internal anatomy and physiological regulation.

3.3. Mesophyll architecture as hidden determinants of stress tolerance

The anatomical and ultrastructural analyses presented in the microscopy-based study (Zekri et al., 2025) provides a deeper understanding of the internal structures of studied Arabidopsis lines. Alterations in mesophyll organization and compartmentalization under water stress indicate structural adjustments in plants in response to environmental stress (Momayyezi et al., 2025; Sun et al., 2025). While an increase in leaf thickness and stomatal density (along with a decrease in stomatal length) in drought-resistant species has been reported (Khan et al., 2022), others observed a decrease in leaf thickness and photosynthesis (Wang et al., 2018). The changes in leaf thickness observed in drought-stressed plants (Khan et al., 2022; Sun et al., 2025), suggest an active modification of leaf internal architecture in response to dehydration. We showed that the observed changes in mesophyll structure, particularly in the spongy parenchyma, were largely responsible for the alterations in leaf thickness under drought. Such modifications may influence mesophyll conductance and internal CO_2 diffusion,

suggesting that anatomical adjustments within the leaf can contribute to maintaining photosynthetic performance during water stress. We therefore propose that increasing the thickness of mesophyll layers may represent a structural adaptation that improves water-use efficiency and photosynthetic capacity under limited water availability. Such modifications could alter chloroplast positioning to increase electron transport efficiency and decrease irreversible photodamage (Kitashova et al., 2021), as illustrated in this study by the pronounced changes in the spongy parenchyma and palisade layer in response to different water regimes (Zekri et al., 2025). In agreement with this finding, light microscopy analysis of palisade and spongy cells revealed a significant re-allocation of chloroplasts after drought stress (Kato et al., 2025). Nevertheless, Kato et al. (2025) reported more pronounced alterations in the palisade layer under drought conditions and therefore suggested that this tissue is more responsive to the drought regime. This conflict may be explained by differences in sample preparation, as their method involved leaf dehydration before imaging. Such dehydration steps can cause structural artifacts, potentially leading to misinterpretation of tissue responses (Nagy-Déri et al., 2011). We established a novel technique to depict mesophyll modifications with minimal manipulation during sample preparation, enabling rapid and low-cost analysis of mesophyll structure. This technique, while requiring skilful handling of leaves and rapid, precise cuts, allows immediate imaging of the mesophyll at the scanning electron microscopy level, thereby providing an accurate representation of its state at the time of sampling.

3.4. Epidermal mechanical advantage and hydropassive behaviour

The final part of this thesis sheds light on the kinetics of the wrong-way response and demonstrates that it is regulated by more than just guard cells, with surrounding pavement cells playing a key role in defining the WWR. Our observations indicate that plants with larger pavement cells (e.g. Arabidopsis lines Col-8 and UBP15) exhibit a more significant WWR, while plants with smaller abaxial pavement cells, particularly *epf1epf2*, show a reduced WWR. This observation suggests that a smaller and more compact epidermal cell network results in greater mechanical stability and can buffer hydropassive stomatal opening. Our work confirms the idea that the epidermal geometry, rather than stomatal traits *per se*, addresses the hydropassive transient opening and is consistent with the concept that pavement cells have a mechanical constraint on guard cells, so that their rapid loss of turgor following a sudden drop in leaf water potential temporarily relieves backpressure and allows stomata to open (Franks and Farquhar, 2007). Collectively, our data provide empirical support for the epidermal mechanical advantage hypothesis (Buckley, 2019) and highlight epidermal cell size and packing as promising anatomical targets for modulating transient stomatal behaviour under water stress.

3.5. Limitations of Gas-Exchange Measurements in Resolving WWR induced by high VPD

In our study of the WWR following a reduction in air humidity, we did not detect a significant stomatal transient opening after correcting for gas-mixing effects, although a significant

response was detected after leaf excision. The absence of a WWR following humidity reduction can be due to the long air-mixing time in the measurement chamber, which was sufficiently long for stomata to adjust, overcome the back pressure from surrounding pavement cells, and initiate closure. In our measurements, gas-mixing effects lasted for almost 10 minutes. Zait et al. (2024) applied a comparable experimental protocol and reported a WWR duration of approximately 9 minutes in wild-type Arabidopsis (Zait et al., 2024). While we do not rule out the possibility of absent WWR under high VPD, we suggest that the prolonged chamber equilibration time may have prevented the transient stomatal opening that typically follows a decline in air humidity. Large variations in chamber gas-mixing times reported by several studies (Buckley et al., 2011; McAdam and Brodribb, 2015; Binstock et al., 2023; Zait et al., 2024) raise concerns about the reliability of standard gas-exchange protocols for quantifying WWR under changing humidity conditions. We argue that the current gas-exchange approach should be applied with more caution, as it may not be able to reliably induce rapid transient stomatal responses. Methodological refinements, including improved chamber design, higher-frequency data acquisition, and explicit modelling of mixing and response kinetics, are likely required to accurately resolve these dynamics.

3.6. Abaxial-Adaxial decoupling and whole-leaf hydraulics

We have also observed that WWR parameters show a stronger link with anatomical traits on the abaxial surface, indicating the functional decoupling between leaf sides and suggesting that spongy mesophyll architecture and abaxial hydraulic connectivity more strongly influence hydropassive responses. Moreover, the rapid freezing technique, which allowed us to capture the *in situ* state of the mesophyll under short- and long-term drought stress (Zekri et al., 2025), showed that the two mesophyll compartments, palisade and spongy parenchyma, respond differently and should be treated as two separate systems in analyses of drought stress responses. This aligns with structural-functional frameworks highlighting the spongy mesophyll as a key driver of internal CO_2 diffusion and hydraulic capacitance (Earles et al., 2018; Théroux-Rancourt et al., 2021b). In this regard, Driesen et al (2023) demonstrated greater sensitivity of adaxial stomata to environmental stimuli, as evidenced by more closed stomata in the adaxial surface compared to the abaxial surface. Pathare et al (2020) showed that adaxial stomata play a more important role in mesophyll conductance. These findings highlight that models or experiments which treat the leaf as a single, homogeneous structure may overlook important functional differences, leading to misinterpretation.

4. Implications and future directions

In this doctoral project, I consider the work as foundational research that establishes the basis for subsequent studies and future investigations. The transient opening of stomata remains a relatively understudied phenomenon, and it was therefore essential to develop a robust foundation and conceptual understanding. Establishing this groundwork enables the next research steps to be defined with greater clarity and rigor. From an applied perspective, this work highlights that:

1. There are risks of targeting individual anatomical traits only, such as increased stomatal density or enhanced trichome coverage. Instead, breeding for drought tolerance should prioritize and **consider the cumulative effect of leaf traits**, anatomical plasticity, and coordinated structural responses in studies of internal leaf organization with respect to environmental stimuli. Evidence from this thesis shows that neither stomatal traits, trichomes, nor internal cellular organization alone can fully explain plant responses to water limitation. Rather, drought tolerance and hydraulic responses emerge from coordinated adjustments across multiple tissues, including the epidermis and mesophyll, which together regulate gas exchange, water relations, and photosynthetic performance. For example, changes in stomatal density did not consistently translate into corresponding changes in stomatal conductance, highlighting that physiological performance depends on interactions between stomatal traits and other anatomical features of the leaf. The results of this work demonstrate that internal leaf structures, such as mesophyll architecture and pavement cell geometry, can significantly influence stomatal behaviour and plant responses to water stress. Consequently, a holistic perspective that integrates epidermal, mesophyll, and whole-leaf structural traits is required to better understand and improve plant drought resilience.
2. Future research should extend this approach to **explore the genetic controls underlying coordinated anatomical adjustments**. The overexpression of the *epf1* gene in rice (Mohammed et al., 2019) and barley (Hughes et al., 2017) resulted in decreased stomatal density and higher water use efficiency and drought resistance. The overexpression of *ZmSDD1* in maize plants reduced stomata density, which has been further correlated with higher drought resistance, WUE, and photosynthetic rate (Liu et al., 2015). The overexpression of STOMAGEN in Arabidopsis resulted in higher stomata density and CO_2 assimilation rate (Tanaka et al., 2013). More studies have examined the genetic control of stomatal density and size; however, the concomitant modifications associated with these genetic manipulations are often overlooked, for example, how such changes are reflected in mesophyll architecture or in the size and shape of epidermal cells. Therefore, and in line with highlight 1, we call for investigations into the genetic regulation of individual anatomical traits alongside assessments of how modifications in that trait may induce changes in other anatomical properties, before evaluating the effects of modifications in that trait on physiological responses.

3. Future studies should extend this work to include **a wider range of species with contrasting epidermal cell anatomy, mesophyll architectures, and leaf hydraulic properties**. Such comparative analyses would provide a robust test of the mechanical advantage of the epidermis over the WWR in stomatal dynamics. Through this, researchers can better understand how physical and hydraulic traits interact to influence transient stomatal behaviour. Extending this approach across angiosperm lineages would illuminate the evolutionary pressures shaping stomatal regulation and clarify how different plant groups respond to rapid environmental fluctuations in water availability. These insights could ultimately improve predictions of plant performance under drought and other water-limited conditions.
4. Beyond the anatomical factors, **additional regulators of cytoskeletal dynamics and cell wall biosynthesis** should be considered into the investigations as they contribute to epidermal patterning and geometry. For instance, proteins that modulate actin–microtubule crosstalk, as well as cellulose synthase complexes and their associated regulators, can influence anisotropic cell expansion and thereby alter final cell geometry. Microtubule-associated proteins define the cell shape and the spatial patterning of cell wall deposition by affecting the organization of the cortical microtubule cytoskeleton (Ruggenthaler et al., 2009). ROP proteins, a family of plant Rho GTPases, contribute to cell shape by defining where lobes and indentations of pavement cells form (Fu et al., 2002; Feiguelman et al., 2017). In addition, KIP-related protein 4 (KRP4) controls epidermal cell size by holding cells in the G1 phase before entry into S phase; larger cells contain lower KRP4 concentrations, whereas smaller cells have higher concentrations (D'Ario et al., 2021). I did not quantify these proteins and therefore suggest measuring their abundance or activity in future work. for example, targeted proteomics techniques and/or fluorescence-based protein localization, could reveal whether these regulators act uniformly across the epidermis or show cell-specific patterns. These approaches provide more information to link epidermal anatomy to underlying genetic regulation.
5. In this study, Arabidopsis lines with contrasting epidermal anatomical characteristics were used to evaluate the mechanical advantage of the epidermis. However, these lines differ significantly in mesophyll architecture, stomatal density and size, trichome abundance, and the anatomical modification under water stress. To minimize the influence of these confounding variables, I recommend applying targeted genetic approaches to modify pavement cell anatomy within a single control background. Based on protein quantification analyses (highlight 4 above), I propose genetic modification and expression of proteins that play a major role in determining pavement cell anatomy in a single Arabidopsis control line. This allows a more direct assessment of how variations in pavement cell shape and size influence epidermal mechanics and, consequently, hydropassive stomatal behaviour. Furthermore, it would enable clearer attribution of observed physiological responses to specific cellular traits, reducing ambiguity arising from whole-leaf structural heterogeneity.

Nevertheless, I still recommend building on the findings of this thesis and applied techniques by incorporating mesophyll architecture, boundary layer conductance, and stomatal density and size into WWR analyses to achieve a more comprehensive characterization of leaf anatomy.

6. Although gas exchange measurements under controlled environmental conditions are a widely accepted and reliable method for analyzing WWR, technical limitations regarding the measurement devices and the need for corrections of data can introduce significant uncertainty into the results. Instrument-specific issues such as chamber leakage, leaf and sensors misalignment or displacement, and the time required for air stabilization can affect measurement accuracy if not carefully controlled. In addition, variability in correction protocols may further contribute to inconsistencies, especially when analyzing stomatal responses to changes in VPD. Therefore, I recommend applying **advanced microscopy techniques to directly visualize WWR**. In this context, our attempts to design a custom-built chamber that simultaneously controls environmental conditions and captures live stomatal responses were unsuccessful. However, novel approaches such as custom-built plugins specifically designed for stomatal response imaging (https://www.miatecs.com/phenom) can be modified through collaboration with the manufacturer to allow visualization of stomatal movements under lowering air humidity. Additionally, there has been a substantial progress in live stomatal imaging by integrating gas exchange measurements with confocal microscopy, a system that could be further adapted to allow precise environmental control during WWR visualization (Crawford et al., 2025). Moreover, monitoring real-time ABA dynamics in guard cells using FRET-based ABA biosensors (e.g., ABAleon) could enable precise and non-invasive ABA quantification of the timing of ABA accumulation and help determine the onset of ABA (Waadt et al., 2014). Through live imaging of transient stomatal opening and tracking the onset of ABA signaling as the initiation point of the hydroactive response, it becomes possible to obtain a precise measurement of WWR duration. Therefore, we can generate robust evidence on the duration of the WWR and address the conflicting reports in the literature regarding this parameter.

References

Amada, G., K. Kobayashi, A. Izuno, M. Mukai, R. Ostertag, K. Kitayama, and Y. Onoda. 2020. Leaf trichomes in Metrosideros polymorpha can contribute to avoiding extra water stress by impeding gall formation. *Ann Bot* 125 (3):533-542 doi: 10.1093/aob/mcz196.

Amada, G., Y. Onoda, T. Ichie, and K. Kitayama. 2017. Influence of leaf trichomes on boundary layer conductance and gas-exchange characteristics in Metrosideros polymorpha (Myrtaceae). *Biotropica* 49 (4):482-492 doi: 10.1111/btp.12433.

Amada, G., K. Yoshiko, K. Kitayama, and Y. Onoda. 2023. Roles of lower-side leaf trichomes in diffusion resistance and gas-exchange characteristics across environmental gradients in Metrosideros polymorpha. *Tree Physiology* 43 (8):1400-1415 doi: 10.1093/treephys/tpad053.

Baillie, A. L., and A. J. Fleming. 2020. The developmental relationship between stomata and mesophyll airspace. *New Phytologist* 225 (3):1120-1126 doi: 10.1111/nph.16341.

Bakker, J. C. 1991. Effects of humidity on stomatal density and its relation to leaf conductance. *Scientia Horticulturae* 48 (3):205-212 doi: 10.1016/0304-4238(91)90128-L.

Bauer, H., P. Ache, S. Lautner, J. Fromm, W. Hartung, Khaled A. S. Al-Rasheid, S. Sonnewald, U. Sonnewald, S. Kneitz, N. Lachmann, Ralf R. Mendel, F. Bittner, Alistair M. Hetherington, and R. Hedrich. 2013. The Stomatal Response to Reduced Relative Humidity Requires Guard Cell-Autonomous ABA Synthesis. *Current Biology* 23 (1):53-57 doi: https://doi.org/10.1016/j.cub.2012.11.022.

Benz, B. W., and C. E. Martin. 2006. Foliar trichomes, boundary layers, and gas exchange in 12 species of epiphytic Tillandsia (Bromeliaceae). *J Plant Physiol* 163 (6):648-56 doi: 10.1016/j.jplph.2005.05.008.

Bertolino, L. T., R. S. Caine, and J. E. Gray. 2019. Impact of Stomatal Density and Morphology on Water-Use Efficiency in a Changing World. *Frontiers in Plant Science* Volume 10 - 2019 doi: 10.3389/fpls.2019.00225.

Bickford, C. P. 2016. Ecophysiology of leaf trichomes. *Funct Plant Biol* 43 (9):807-814 doi: 10.1071/fp16095.

Binstock, B. R., A. Manandhar, and S. A. M. McAdam. 2023. Characterizing the breakpoint of stomatal response to vapor pressure deficit in an angiosperm. *Plant Physiology* 194 (2):732-740 doi: 10.1093/plphys/kiad560.

Borsuk, A. M., A. B. Roddy, G. Théroux-Rancourt, and C. R. Brodersen. 2022. Structural organization of the spongy mesophyll. *New Phytologist* 234 (3):946-960 doi: 10.1111/nph.17971.

Brodribb, T. J., and S. A. McAdam. 2011. Passive origins of stomatal control in vascular plants. *Science* 331 (6017):582-5 doi: 10.1126/science.

Buckley, T. N. 2005. The control of stomata by water balance. *New Phytol* 168 (2):275-92 doi: 10.1111/j.1469-8137.2005.01543.x.

Buckley, T. N. 2019. How do stomata respond to water status? *New Phytologist* 224 (1):21-36 doi: 10.1111/nph.15899.

Buckley, T. N., G. P. John, C. Scoffoni, and L. Sack. 2015. How Does Leaf Anatomy Influence Water Transport outside the Xylem? *Plant Physiol* 168 (4):1616-35 doi: 10.1104/pp.15.00731.

Buckley, T. N., L. Sack, and M. E. Gilbert. 2011. The Role of Bundle Sheath Extensions and Life Form in Stomatal Responses to Leaf Water Status *Plant Physiology* 156 (2):962-973 doi: 10.1104/pp.111.175638.

Caine, R. S., E. L. Harrison, J. Sloan, P. M. Flis, S. Fischer, M. S. Khan, P. T. Nguyen, L. T. Nguyen, J. E. Gray, and H. Croft. 2023. The influences of stomatal size and density on rice abiotic stress resilience. *New Phytologist* 237 (6):2180-2195 doi: 10.1111/nph.18704.

Caretta, M. A., A. Mukherji, M. Arfanuzzaman, R.A. Betts, A. Gelfan, Y. Hirabayashi, T.K. Lissner, J. Liu, E. Lopez Gunn, R. Morgan, S. Mwanga, and S. Supratid. 2022. Water. In: Climate Change 2022: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. *Cambridge University Press, Cambridge, UK and New York, , NY, USA*:pp. 551–712 doi: 10.1017/9781009325844.006.

Chaffey, N. 2014. Raven biology of plants, 8th edn. *Annals of Botany* 113 (7):vii-vii doi: 10.1093/aob/mcu090.

Chen, J.-J., Y. Sun, K. Kopp, L. Oki, S. B. Jones, and L. Hipps. 2022. Effects of Water Availability on Leaf Trichome Density and Plant Growth and Development of Shepherdia ×utahensis. *Frontiers in Plant Science* Volume 13 - 2022 doi: 10.3389/fpls.2022.855858.

Chua, L. C., and O. S. Lau. 2024. Stomatal development in the changing climate. *Development* 151 (20) doi: 10.1242/dev.202681.

Cotelle, V., and N. Leonhardt. 2019. "Chapter Four - ABA signaling in guard cells1." In *Advances in Botanical Research*, edited by Mitsunori Seo and Annie Marion-Poll, 115-170. Academic Press.

Crawford, J. D., D. Mayfield-Jones, G. A. Fried, N. Hernandez, and A. D. B. Leakey. 2025. Stomata in-sight: Integrating live confocal microscopy with leaf gas exchange and environmental control. *Plant Physiology* 199 (4) doi: 10.1093/plphys/kiaf600.

D'Ario, M., R. Tavares, K. Schiessl, B. Desvoyes, C. Gutierrez, M. Howard, and R. Sablowski. 2021. Cell size controlled in plants using DNA content as an internal scale. *Science* 372 (6547):1176-1181 doi: 10.1126/science.abb4348.

di Santo, H., T. Knipfer, and F. H. Barrios-Masias. 2025. Using the water potential curve to identify the transition from water-consumptive to water-conservative behaviour in Cucumis melo. *Functional Plant Biology* 52 (9) doi: 10.1071/FP25048.

Dow, G. J., and D. C. Bergmann. 2014. Patterning and processes: how stomatal development defines physiological potential. *Current Opinion in Plant Biology* 21:67-74 doi: 10.1016/j.pbi.2014.06.007.

Drake, P. L., R. H. Froend, and P. J. Franks. 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *J Exp Bot* 64 (2):495-505 doi: 10.1093/jxb/ers347.

Driesen, E., M. De Proft, and W. Saeys. 2023a. Drought stress triggers alterations of adaxial and abaxial stomatal development in basil leaves increasing water-use efficiency. *Hortic Res* 10 (6):uhad075 doi: 10.1093/hr/uhad075.

Driesen, E., M. De Proft, and W. Saeys. 2023b. Drought stress triggers alterations of adaxial and abaxial stomatal development in basil leaves increasing water-use efficiency. *Horticulture Research* 10 (6) doi: 10.1093/hr/uhad075.

Earles, J. M., G. Théroux-Rancourt, A. B. Roddy, M. E. Gilbert, A. J. McElrone, and C. R. Brodersen. 2018. Beyond Porosity: 3D Leaf Intercellular Airspace Traits That Impact Mesophyll Conductance. *Plant Physiology* 178 (1):148-162 doi: 10.1104/pp.18.00550.

Farolfi, E., A. Kulhánková, F. De Berardinis, S. L. Tarnay, G. A. Gambetta, U. Hochberg, A. Forneck, and J. C. Herrera. 2025. Leaf seasonal osmotic adjustment is not driven by temperature or water deficit. *Plant J* 124 (3):e70569 doi: 10.1111/tpj.70569.

Feiguelman, G., Y. Fu, and S. Yalovsky. 2017. ROP GTPases Structure-Function and Signaling Pathways. *Plant Physiology* 176 (1):57-79 doi: 10.1104/pp.17.01415.

Flexas, J., M. M. Barbour, O. Brendel, H. M. Cabrera, M. Carriquí, A. Díaz-Espejo, C. Douthe, E. Dreyer, J. P. Ferrio, J. Gago, A. Gallé, J. Galmés, N. Kodama, H. Medrano, Ü. Niinemets, J. J. Peguero-Pina, A. Pou, M. Ribas-Carbó, M. Tomás, T. Tosens, and C. R. Warren. 2012. Mesophyll diffusion conductance to CO2: An unappreciated central player in photosynthesis. *Plant Science* 193-194:70-84 doi: 10.1016/j.plantsci.2012.05.009.

Franks, P. J., and D. J. Beerling. 2009. Maximum leaf conductance driven by CO2 effects on stomatal size and density over geologic time. *Proc Natl Acad Sci U S A* 106 (25):10343-7 doi: 10.1073/pnas.0904209106.

Franks, P. J., Cowan, 2 I. R., and G. D. Farquhar. 1998. A study of stomatal mechanics using the cell pressure probe. *Plant, Cell & Environment* 21 (1):94-100 doi: 10.1046/j.1365-3040.1998.00248.x.

Franks, P. J., and G. D. Farquhar. 2007. The mechanical diversity of stomata and its significance in gas-exchange control. *Plant Physiol* 143 (1):78-87 doi: 10.1104/pp.106.089367.

Fu, Y., H. Li, and Z. Yang. 2002. The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during Arabidopsis organogenesis. *Plant Cell* 14 (4):777-94 doi: 10.1105/tpc.001537.

Fujii, S., K. Nishida, T. K. Akitsu, A. Kume, and Y. T. Hanba. 2023. Variation in leaf mesophyll anatomy of fern species imposes significant effects on leaf gas exchange, light capture, and leaf hydraulic conductance. *Photosynthetica* 61 (2):225-235 doi: 10.32615/ps.2023.017.

Gasparini, K., M. F. da Silva, L. C. Costa, S. C. V. Martins, D. M. Ribeiro, L. E. P. Peres, and A. Zsögön. 2021. The Lanata trichome mutation increases stomatal conductance and reduces leaf temperature in tomato. *Journal of Plant Physiology* 260:153413 doi: 10.1016/j.jplph.2021.153413.

Giday, H., K. H. Kjaer, D. Fanourakis, and C.-O. Ottosen. 2013. Smaller stomata require less severe leaf drying to close: A case study in Rosa hydrida. *Journal of Plant Physiology* 170 (15):1309-1316 doi: 10.1016/j.jplph.2013.04.007.

Hamanishi, E. T., B. R. Thomas, and M. M. Campbell. 2012. Drought induces alterations in the stomatal development program in Populus. *Journal of Experimental Botany* 63 (13):4959-4971 doi: 10.1093/jxb/ers177.

Haworth, M., G. Marino, F. Loreto, and M. Centritto. 2021. Integrating stomatal physiology and morphology: evolution of stomatal control and development of future crops. *Oecologia* 197 (4):867-883 doi: 10.1007/s00442-021-04857-3.

Haworth, M., G. Marino, A. Materassi, A. Raschi, C. P. Scutt, and M. Centritto. 2023. The functional significance of the stomatal size to density relationship: Interaction with atmospheric [CO2] and role in plant physiological behaviour. *Science of The Total Environment* 863:160908 doi: 10.1016/j.scitotenv.2022.160908.

Hetherington, A. M., and F. I. Woodward. 2003. The role of stomata in sensing and driving environmental change. *Nature* 424 (6951):901-908 doi: 10.1038/nature01843.

Hughes, J., C. Hepworth, C. Dutton, J. A. Dunn, L. Hunt, J. Stephens, R. Waugh, D. D. Cameron, and J. E. Gray. 2017. Reducing Stomatal Density in Barley Improves Drought Tolerance without Impacting on Yield *Plant Physiology* 174 (2):776-787 doi: 10.1104/pp.16.01844.

Jalakas, P., Y. Takahashi, R. Waadt, J. I. Schroeder, and E. Merilo. 2021. Molecular mechanisms of stomatal closure in response to rising vapour pressure deficit. *New Phytologist* 232 (2):468-475 doi: 10.1111/nph.17592.

Jones, H. G. 2013. Plants and Microclimate: A Quantitative Approach to Environmental Plant Physiology. doi: 10.1017/CBO9780511845727.

Kato, Y., T. Oi, Y. Sato, and M. Taniguchi. 2025. Bundle sheath cell-dependent chloroplast movement in mesophyll cells of C(4) plants analyzed using live leaf-section imaging. *Sci Rep* 15 (1):3447 doi: 10.1038/s41598-025-86153-1.

Khan, A., F. Shen, L. Yang, W. Xing, and B. Clothier. 2022. Limited Acclimation in Leaf Morphology and Anatomy to Experimental Drought in Temperate Forest Species. *Biology (Basel)* 11 (8) doi: 10.3390/biology11081186.

Kitashova, A., K. Schneider, L. Fürtauer, L. Schröder, T. Scheibenbogen, S. Fürtauer, and T. Nägele. 2021. Impaired chloroplast positioning affects photosynthetic capacity and regulation of the central carbohydrate metabolism during cold acclimation. *Photosynth Res* 147 (1):49-60 doi: 10.1007/s11120-020-00795-y.

Koehler, T., F. J. P. Wankmüller, W. Sadok, and A. Carminati. 2023. Transpiration response to soil drying versus increasing vapor pressure deficit in crops: physical and physiological mechanisms and key plant traits. *Journal of Experimental Botany* 74 (16):4789-4807 doi: 10.1093/jxb/erad221.

Lawson, T., and M. R. Blatt. 2014. Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiol* 164 (4):1556-70 doi: 10.1104/pp.114.237107.

Lei, Z., Y. He, X. Li, Z. He, Y. Zhang, W. Zhang, F. Liu, and Y. Zhang. 2022. Domestication has reduced leaf water use efficiency associated with the anatomy of abaxial stomata in cotton. *Journal of Experimental Botany* 74 (3):878-888 doi: 10.1093/jxb/erac447.

Lertngim, N., M. Ruangsiri, S. Klinsawang, P. Raksatikan, B. Thunnom, M. Siangliw, T. Toojinda, and J. L. Siangliw. 2023. Photosynthetic Plasticity and Stomata Adjustment in Chromosome Segment Substitution Lines of Rice Cultivar KDML105 under Drought Stress. *Plants* 12 (1):94 doi: 10.3390/plants12010094.

Liu, C., L. Sack, Y. Li, J. Zhang, K. Yu, Q. Zhang, N. He, and G. Yu. 2023. Relationships of stomatal morphology to the environment across plant communities. *Nature Communications* 14 (1):6629 doi: 10.1038/s41467-023-42136-2.

Liu, S., F. Jobert, Z. Rahneshan, S. M. Doyle, and S. Robert. 2021. Solving the Puzzle of Shape Regulation in Plant Epidermal Pavement Cells. *Annual Review of Plant Biology* 72 (1):525-550 doi: 10.1146/annurev-arplant-080720-081920.

Liu, Y., L. Qin, L. Han, Y. Xiang, and D. Zhao. 2015. Overexpression of maize SDD1 (ZmSDD1) improves drought resistance in Zea mays L. by reducing stomatal density. *Plant Cell, Tissue and Organ Culture (PCTOC)* 122 (1):147-159 doi: 10.1007/s11240-015-0757-8.

Manandhar, A., F. E. Rockwell, N. Watson, A. Graham, and N. M. Holbrook. 2026. Visualizing physical interactions between guard and epidermal cells during light-induced stomatal opening in 2 angiosperms. *Plant Physiology* 200 (1) doi: 10.1093/plphys/kiaf644.

Manzi, M., J. Lado, M. J. Rodrigo, L. Zacarías, V. Arbona, and A. Gómez-Cadenas. 2015. Root ABA Accumulation in Long-Term Water-Stressed Plants is Sustained by Hormone Transport from Aerial Organs. *Plant and Cell Physiology* 56 (12):2457-2466 doi: 10.1093/pcp/pcv161.

McAdam, S. A., and T. J. Brodribb. 2015. The evolution of mechanisms driving the stomatal response to vapor pressure deficit. *Plant Physiol* 167 (3):833-43 doi: 10.1104/pp.114.252940.

McAdam, S. A. M., and T. J. Brodribb. 2018. Mesophyll Cells Are the Main Site of Abscisic Acid Biosynthesis in Water-Stressed Leaves. *Plant Physiol* 177 (3):911-917 doi: 10.1104/pp.17.01829. .

Merilo, E., D. Yarmolinsky, P. Jalakas, H. Parik, I. Tulva, B. Rasulov, K. Kilk, and H. Kollist. 2017. Stomatal VPD Response: There Is More to the Story Than ABA *Plant Physiology* 176 (1):851-864 doi: 10.1104/pp.17.00912.

Mohammed, U., R. S. Caine, J. A. Atkinson, E. L. Harrison, D. Wells, C. C. Chater, J. E. Gray, R. Swarup, and E. H. Murchie. 2019. Rice plants overexpressing OsEPF1 show reduced stomatal density and increased root cortical aerenchyma formation. *Scientific Reports* 9 (1):5584 doi: 10.1038/s41598-019-41922-7.

Momayyezi, M., C. Chu, J. A. Stobbs, R. Y. Soolanayakanahally, R. D. Guy, A. J. McElrone, and T. Knipfer. 2025. Mapping of drought-induced changes in tissue characteristics across the leaf profile of Populus balsamifera. *New Phytologist* 245 (2):534-545 doi: 10.1111/nph.20240.

Mott, K. A. 2007. Leaf hydraulic conductivity and stomatal responses to humidity in amphistomatous leaves. *Plant, Cell & Environment* 30 (11):1444-1449 doi: 10.1111/j.1365-3040.2007.01720.x.

Nagy-Déri, H., E. R. Péli, K. Georgieva, and Z. Tuba. 2011. Changes in chloroplast morphology of different parenchyma cells in leaves of Haberlea rhodopensis Friv. during desiccation and following rehydration. *Photosynthetica* 49 (1):119-126 doi: 10.1007/s11099-011-0020-4.

Negin, B., A. Yaaran, G. Kelly, Y. Zait, and M. Moshelion. 2019. Mesophyll Abscisic Acid Restrains Early Growth and Flowering But Does Not Directly Suppress Photosynthesis. *Plant Physiology* 180 (2):910-925 doi: 10.1104/pp.18.01334.

Nieves-Cordones, M., F. Azeem, Y. Long, M. Boeglin, G. Duby, K. Mouline, E. Hosy, A. Vavasseur, I. Chérel, T. Simonneau, F. Gaymard, J. Leung, I. Gaillard, J. B. Thibaud, A. A. Véry, A. Boudaoud, and H. Sentenac. 2022. Non-autonomous stomatal control by pavement cell turgor via the K+ channel subunit AtKC1. *Plant Cell* 34 (5):2019-2037 doi: 10.1093/plcell/koac038.

Pathare, V. S., N. Koteyeva, and A. B. Cousins. 2020. Increased adaxial stomatal density is associated with greater mesophyll surface area exposed to intercellular air spaces and mesophyll conductance in diverse C4 grasses. *New Phytologist* 225 (1):169-182 doi: 10.1111/nph.16106.

Pichaco, J., A. Manandhar, and S. A. M. McAdam. 2024. Mechanical advantage makes stomatal opening speed a function of evaporative demand. *Plant Physiology* 195 (1):370-377 doi: 10.1093/plphys/kiae023.

Pitaloka, M. K., R. S. Caine, C. Hepworth, E. L. Harrison, J. Sloan, C. Chutteang, C. Phunthong, R. Nongngok, T. Toojinda, S. Ruengphayak, S. Arikit, J. E. Gray, and A. Vanavichit. 2022. Induced Genetic Variations in Stomatal Density and Size of Rice Strongly Affects Water Use Efficiency and Responses to Drought Stresses. *Frontiers in Plant Science* Volume 13 - 2022 doi: 10.3389/fpls.2022.801706.

Ruggenthaler, P., D. Fichtenbauer, J. Krasensky, C. Jonak, and E. Waigmann. 2009. Microtubule-associated protein AtMPB2C plays a role in organization of cortical microtubules,

stomata patterning, and tobamovirus infectivity. *Plant Physiol* 149 (3):1354-65 doi: 10.1104/pp.108.130450.

Rui, M., R. Chen, Y. Jing, F. Wu, Z. H. Chen, D. Tissue, H. Jiang, and Y. Wang. 2024. Guard cell and subsidiary cell sizes are key determinants for stomatal kinetics and drought adaptation in cereal crops. *New Phytol* 242 (6):2479-2494 doi: 10.1111/nph.19757.

Sage, T. L., and R. F. Sage. 2009. The functional anatomy of rice leaves: implications for refixation of photorespiratory CO2 and efforts to engineer C4 photosynthesis into rice. *Plant Cell Physiol* 50 (4):756-72 doi: 10.1093/pcp/pcp033.

Sakoda, K., W. Yamori, T. Shimada, S. S. Sugano, I. Hara-Nishimura, and Y. Tanaka. 2020a. Higher Stomatal Density Improves Photosynthetic Induction and Biomass Production in Arabidopsis Under Fluctuating Light. *Frontiers in Plant Science* 11 doi: 10.3389/fpls.2020.589603.

Sakoda, K., W. Yamori, T. Shimada, S. S. Sugano, I. Hara-Nishimura, and Y. Tanaka. 2020b. Higher Stomatal Density Improves Photosynthetic Induction and Biomass Production in Arabidopsis Under Fluctuating Light. *Frontiers in Plant Science* Volume 11 - 2020 doi: 10.3389/fpls.2020.589603.

Sapala, A., A. Runions, A. L. Routier-Kierzkowska, M. Das Gupta, L. Hong, H. Hofhuis, S. Verger, G. Mosca, C. B. Li, A. Hay, O. Hamant, A. H. Roeder, M. Tsiantis, P. Prusinkiewicz, and R. S. Smith. 2018. Why plants make puzzle cells, and how their shape emerges. *Elife* 7 doi: 10.7554/eLife.32794.

Sapala, A., A. Runions, and R. S. Smith. 2019. Mechanics, geometry and genetics of epidermal cell shape regulation: different pieces of the same puzzle. *Current Opinion in Plant Biology* 47:1-8 doi: 10.1016/j.pbi.2018.07.017.

Schlüter, U., M. Muschak, D. Berger, and T. Altmann. 2003. Photosynthetic performance of an Arabidopsis mutant with elevated stomatal density (sdd1-1) under different light regimes. *Journal of Experimental Botany* 54 (383):867-874 doi: 10.1093/jxb/erg087.

Singh, S. K., and K. Raja Reddy. 2011. Regulation of photosynthesis, fluorescence, stomatal conductance and water-use efficiency of cowpea (Vigna unguiculata [L.] Walp.) under drought. *Journal of Photochemistry and Photobiology B: Biology* 105 (1):40-50 doi: 10.1016/j.jphotobiol.2011.07.001.

Sun, D., Z. Lei, M. Carriquí, Y. Zhang, T. Liu, S. Wang, K. Song, L. Zhu, W. Zhang, and Y. Zhang. 2025. Reductions in mesophyll conductance under drought stress are influenced by increases in cell wall chelator-soluble pectin content and denser microfibril alignment in cotton. *J Exp Bot* 76 (4):1116-1130 doi: 10.1093/jxb/erae467.

Taiz, L., E. Zeiger, I. M. Møller, and A. Murphy. 2015. Plant Physiology and Development.

Tan, N. Y. H., J. V. Armand, J. E. Gray, and A. J. Fleming. 2025. Modelling stomatal mechanics: a critical review. *New Phytologist* n/a (n/a) doi: 10.1111/nph.70826.

Tanaka, Y., S. S. Sugano, T. Shimada, and I. Hara-Nishimura. 2013. Enhancement of leaf photosynthetic capacity through increased stomatal density in Arabidopsis. *New Phytologist* 198 (3):757-764 doi: 10.1111/nph.12186.

Terashima, I., Y. T. Hanba, D. Tholen, and Ü. Niinemets. 2010. Leaf Functional Anatomy in Relation to Photosynthesis. *Plant Physiology* 155 (1):108-116 doi: 10.1104/pp.110.165472.

Théroux-Rancourt, G., A. B. Roddy, J. M. Earles, M. E. Gilbert, M. A. Zwieniecki, C. K. Boyce, D. Tholen, A. J. McElrone, K. A. Simonin, and C. R. Brodersen. 2021a. Maximum CO2 diffusion inside leaves is limited by the scaling of cell size and genome size.

Proceedings of the Royal Society B: Biological Sciences 288 (1945) doi: 10.1098/rspb.2020.3145.

Théroux-Rancourt, G., A. B. Roddy, J. M. Earles, M. E. Gilbert, M. A. Zwieniecki, C. K. Boyce, D. Tholen, A. J. McElrone, K. A. Simonin, and C. R. Brodersen. 2021b. Maximum CO₂ diffusion inside leaves is limited by the scaling of cell size and genome size. *Proceedings of the Royal Society B: Biological Sciences* 288 (1945):20203145 doi: doi:10.1098/rspb.2020.3145.

Tholen, D., C. Boom, K. Noguchi, S. Ueda, T. Katase, and I. Terashima. 2008. The chloroplast avoidance response decreases internal conductance to CO2 diffusion in Arabidopsis thaliana leaves. *Plant Cell Environ* 31 (11):1688-700 doi: 10.1111/j.1365-3040.2008.01875.x.

Tholen, D., C. Boom, and X.-G. Zhu. 2012. Opinion: Prospects for improving photosynthesis by altering leaf anatomy. *Plant Science* 197:92-101 doi: 10.1016/j.plantsci.2012.09.005.

Tomás, M., J. Flexas, L. Copolovici, J. Galmés, L. Hallik, H. Medrano, M. Ribas-Carbó, T. Tosens, V. Vislap, and Ü. Niinemets. 2013. Importance of leaf anatomy in determining mesophyll diffusion conductance to CO2 across species: quantitative limitations and scaling up by models. *Journal of Experimental Botany* 64 (8):2269-2281 doi: 10.1093/jxb/ert086.

Tulva, I., K. Koolmeister, and H. Hõrak. 2024. Low relative air humidity and increased stomatal density independently hamper growth in young Arabidopsis. *Plant J* 119 (6):2718-2736 doi: 10.1111/tpj.16944.

Waadt, R., K. Hitomi, N. Nishimura, C. Hitomi, S. R. Adams, E. D. Getzoff, and J. I. Schroeder. 2014. FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. *Elife* 3:e01739 doi: 10.7554/eLife.01739.

Wang, X., T. Du, J. Huang, S. Peng, and D. Xiong. 2018. Leaf hydraulic vulnerability triggers the decline in stomatal and mesophyll conductance during drought in rice. *Journal of Experimental Botany* 69 (16):4033-4045 doi: 10.1093/jxb/ery188.

Xu, Z., and G. Zhou. 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *Journal of Experimental Botany* 59 (12):3317-3325 doi: 10.1093/jxb/ern185.

Yaaran, A., B. Negin, and M. Moshelion. 2017. Role of guard-cell ABA in determining maximal stomatal aperture and prompt vapor-pressure-deficit response. *bioRxiv*:218719 doi: 10.1101/218719.

Zait, Y., A. Joseph, and S. M. Assmann. 2024. Stomatal responses to VPD utilize guard cell intracellular signaling components. *Frontiers in Plant Science* Volume 15 - 2024 doi: 10.3389/fpls.2024.1351612.

Zekri, M. A., and I. Lang. 2024. Lack of trichomes and variation in stomata properties influence the quantum efficiency of photosynthesis in Arabidopsis. *Environmental and Experimental Botany* 227:105948 doi: 10.1016/j.envexpbot.2024.105948.

Zekri, M. A., C. Leimhofer, N. Drexler, and I. Lang. 2025. A rapid freezing method to determine tissue layer thickness in drought-stressed leaves. *Journal of Microscopy* 297 (3):316-324 doi: 10.1111/jmi.13272.

Zhu, Y., J. Zheng, H. Kang, N. Hui, S. Yin, Z. Chen, B. Du, and C. Liu. 2024. Spatial variations in leaf trichomes and their coordination with stomata in Quercus variabilis across Eastern Asia. *Journal of Plant Ecology* 17 (3) doi: 10.1093/jpe/rtae023.

Zwieniecki, M. A., and C. K. Boyce. 2014. Evolution of a unique anatomical precision in angiosperm leaf venation lifts constraints on vascular plant ecology. *Proceedings of the Royal Society B: Biological Sciences* 281 (1779) doi: 10.1098/rspb.2013.2829.

Zusammenfassung

Seit die grundlegende Arbeit von Gregor Mendel die Genetik durch die Beobachtung von Pflanzenphänotypen begründete, hat die Forschung in der Pflanzenbiologie zunehmend darauf abgezielt, beobachtbare Merkmale mit ihren zugrunde liegenden molekularen und genetischen Mechanismen zu verknüpfen. Ein tieferes Verständnis pflanzlicher anatomischer Merkmale ist ein Weg, molekulare Erkenntnisse in praktische Strategien zur Verbesserung der Widerstandsfähigkeit und Produktivität von Pflanzen unter widrigen Umweltbedingungen zu übertragen. Trotz ihrer Bedeutung sind anatomische Merkmale häufig schwierig zu untersuchen, da technische Einschränkungen eine genaue Quantifizierung struktureller Eigenschaften in dynamischen und schwankenden Umgebungen erschweren. Unter diesen Merkmalen stellen Stomata eine entscheidende anatomische Struktur dar, die das Gleichgewicht zwischen CO_2-Aufnahme für die Photosynthese und Wasserverlust durch Transpiration reguliert. Ein besonders interessantes, jedoch unzureichend verstandenes Phänomen ist die vorübergehende Öffnung der Stomata nach einem plötzlichen Abfall des Blattwasserpotentials. Diese Reaktion, bekannt als Wrong-Way-Response (WWR), beinhaltet eine vorübergehende Öffnung der Stomata, wenn das Wasserpotential abnimmt und eigentlich ein Schließen erwartet würde.

Die Hypothese des epidermalen mechanischen Vorteils besagt, dass diese vorübergehende Öffnung aus mechanischen Wechselwirkungen zwischen Schließzellen und umgebenden Pavementzellen entsteht. Nach dieser Hypothese spielt die mechanische Kopplung innerhalb der Pavement- und Schließzellen eine Schlüsselrolle bei der Bestimmung der Stomatadynamik. Die strukturellen Determinanten, die dieser mechanischen Kopplung zugrunde liegen, sind jedoch noch ungeklärt. Da die Geometrie interagierender Komponenten das Verhalten mechanischer Systeme stark beeinflusst, untersucht diese Arbeit, wie die anatomische Geometrie von Pavementzellen und Stomata zur Dynamik der vorübergehenden stomatären Öffnung beiträgt. Um diese Frage zu adressieren, wurde die Modellpflanze Arabidopsis thaliana verwendet, da sie ein gut charakterisiertes Genom, verfügbare genetische Ressourcen und eine Eignung für kontrollierte Laborexperimente aufweist. Verschiedene Arabidopsis-Linien wurden auf Grundlage von Variationen in der Größe und Dichte der Stomata ausgewählt. Unterschiede in zusätzlichen anatomischen Merkmalen, wie das Fehlen von Trichomen im *tmm1*-Mutanten sowie begrenztes Wissen über die Mesophyllorganisation in den ausgewählten Arabidopsis-Linien, stellten jedoch Herausforderungen für die Entwicklung eines umfassenden Verständnisses des stomatären Verhaltens dar. Daher wurden die physiologischen Reaktionen einer glatten Arabidopsis-Linie mit Kontrollpflanzen verglichen, zusammen mit detaillierten Analysen zentraler stomatärer Merkmale einschließlich Größe und Dichte. Die erste Studie zeigt, dass Trichome die Blatttemperatur beeinflussen, obwohl ihr Beitrag zur Aufrechterhaltung der photosynthetischen Leistung unter Trockenstress begrenzt war. Die zweite Studie führt einen auf Kryofixierung basierenden methodischen Ansatz ein, um die Mesophyllanatomie unter Trockenstress zu untersuchen. Diese Technik ermöglicht die Visualisierung der Mesophyllorganisation und der zellulären Architektur, während Artefakte minimiert werden,

die durch Dehydration während der konventionellen mikroskopischen Präparation entstehen. Mithilfe dieser Methode wurden strukturelle Modifikationen sowohl des Palisaden- als auch des Schwammparenchyms unter kurz- und langfristigem Trockenstress beobachtet. Die dritte Studie konzentriert sich auf die vorübergehende stomatäre Öffnung. Die Ergebnisse liefern empirische Unterstützung für die Hypothese des epidermalen mechanischen Vorteils und zeigen, dass die Größe der Pavementzellen sowohl das Ausmaß als auch die Dauer dieser Reaktion signifikant beeinflusst. Diese Befunde unterstreichen die Bedeutung der epidermalen Geometrie für die Steuerung der Stomatadynamik während schneller Veränderungen des pflanzlichen Wasserstatus.

Zusammen zeigen die in dieser Arbeit präsentierten Ergebnisse, dass pflanzliche Reaktionen auf variable Wasserverfügbarkeit durch anatomische Merkmale über mehrere Blattgewebe hinweg geprägt sind, einschließlich der Epidermis und des Mesophylls. Diese Befunde unterstreichen die Notwendigkeit, über einen ausschließlichen Fokus auf Schließzellen hinauszugehen und stattdessen eine stärker integrierte Perspektive einzunehmen, die berücksichtigt, wie verschiedene Blattstrukturen gemeinsam die Wasserbeziehungen der Pflanze und ihre Stressreaktionen regulieren.