Disentangling drought-responsive traits with focus on Arabidopsis

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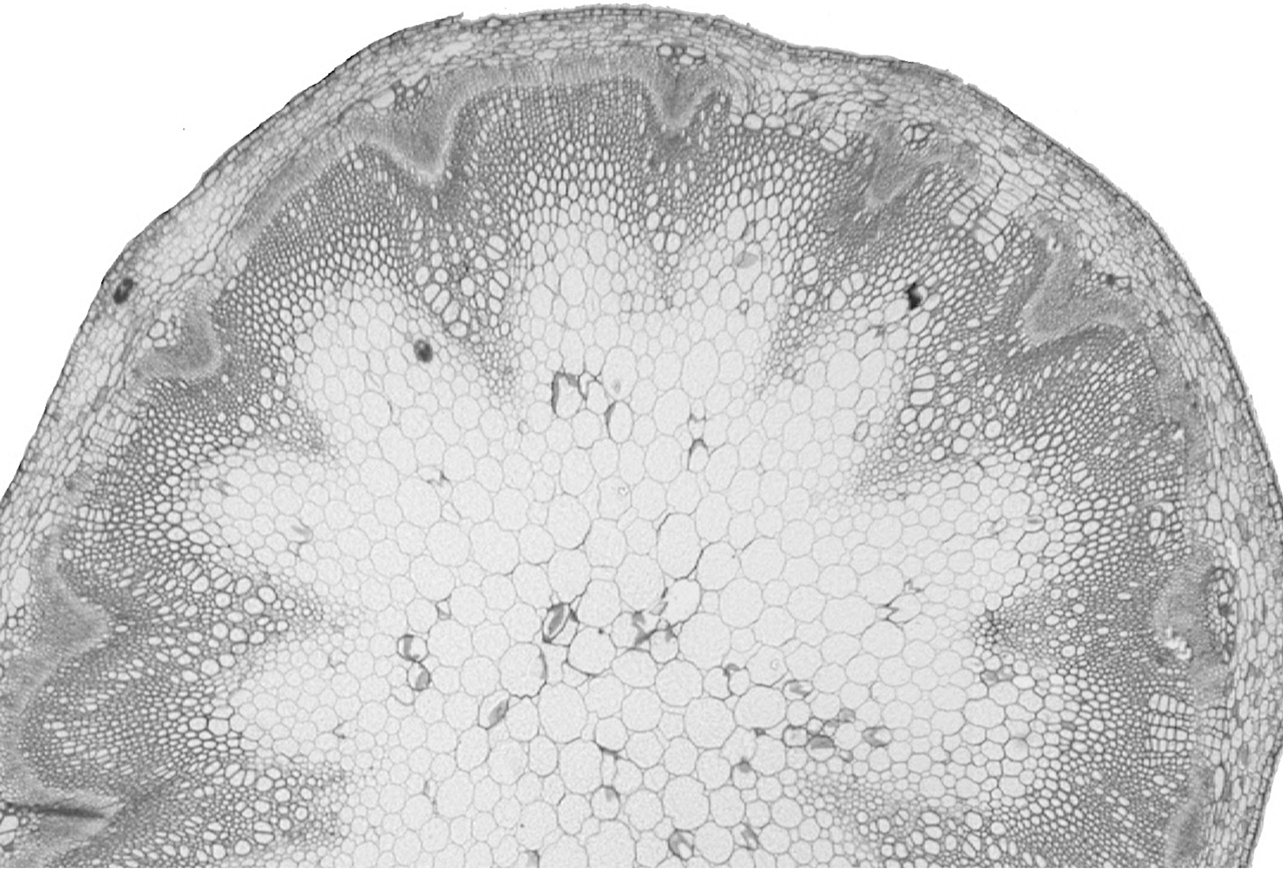
Citation

Thonglim, A. (2023, November 9). *Disentangling drought-responsive traits with focus on Arabidopsis*. Retrieved from https://hdl.handle.net/1887/3656528

Chapter 5

GENERAL DISCUSSION AND FUTURE PERSPECTIVES



General discussion

The increasing frequency and severity of drought events resulting from climate change already have significant implications on global forest mortality and agricultural productivity (Allen *et al.*, 2009, 2015; Brás *et al.*, 2021; Gleason *et al.*, 2022; Hammond *et al.*, 2022). As plants heavily rely on an adequate water supply in the soil, the limitations imposed by reduced water availability have far-reaching effects on their overall development and ability to thrive (Choat *et al.*, 2018; Martinez-Vilalta *et al.*, 2019; Sapes *et al.*, 2019; Brodribb *et al.*, 2020). Therefore, identifying the key traits that underlie drought response mechanisms and a comprehensive understanding of the diverse range of plant adaptive strategies to drought is essential for accurately estimating the risk of forest die-off and crop yield loss and improving plant resilience and productivity in water-limited environments.

The role of ecophysiological traits on drought response

Plant desiccation and mortality are tightly linked to the failure of the water transport system, primarily due to the accumulation of embolism caused by drought within xylem conduits that surpasses the threshold beyond where water transport becomes irrecoverable (Sperry and Tyree, 1988; Venturas *et al.*, 2017; McDowell *et al.*, 2022; Johnson *et al.*, 2022). Consequently, the ability to avoid and/or resist embolism formation under the highly negative pressure induced by soil water deficit holds great significance in plant survival. Two sets of key ecophysiological traits are thought to be associated with plant hydraulic failure: (1) stomatal regulation that maintains the water potential (Ψ) and optimizes water use efficiency at the leaf level (Klein, 2014; Buckley, 2019; Papastefanou *et al.*, 2020; Joshi *et al.*, 2022) (discussed in chapter 2 and 3), and (2) embolism resistance in the xylem conduits that ensures the integrity of the water transport system under water deficit conditions (Choat *et al.*, 2012; Tng *et al.*, 2018) (discussed in chapter 1 and 2). Consequently, attaining a harmonious synergy between these two types of traits is imperative for an effective response to drought, enabling optimal plant growth without encountering hydraulic failure.

Stomatal regulation plays a crucial role in enabling plants to effectively balance water conservation and photosynthetic activity when faced with limited water availability (Assmann and Wang, 2001; Buckley, 2005; Messinger *et al.*, 2006; Ainsworth and Rogers, 2007). The closure of stomata is one of the earliest drought responses, considerably reduces water loss due to transpiration, and delays the decrease of leaf water potential (Ψ_l) (Bartlett *et al.*, 2016; Choat *et al.*, 2018). Importantly, stomatal closure precedes substantial embolism formation, thereby effectively preventing/delaying detrimental levels of embolism that can result in hydraulic failure (Brodribb *et al.*, 2003; Mencuccini *et al.*, 2015; Martin-StPaul *et al.*, 2017; Scoffoni *et al.*, 2017; Choat *et al.*, 2018; Creek *et al.*, 2020). However, stomata closure also limits photosynthetic rates due to reduced gas exchange, potentially impairing carbon metabolic function, especially during long drought episodes (McDowell *et al.*, 2008; Brodribb *et al.*, 2017*b*; Martin-StPaul *et al.*, 2017; Martínez-Vilalta and Garcia-Forner, 2017; Knipfer *et al.*, 2020). The findings presented in chapters 2 and 3 highlight the distinct stomatal regulation behaviors in response to drought observed among the Arabidopsis and tomato genotypes studied. However, regardless of the specific regulatory mechanisms, the results underscore the importance of maintaining a high and stable leaf water potential (Ψ_l) during drought stress. This can be achieved through strict stomatal regulation, as observed in Arabidopsis genotypes, or through alternative mechanisms, as seen in the case of tomato and Arabidopsis *JUB1OX* (discussed further in the last paragraph). In Arabidopsis, most genotypes exhibiting higher drought resistance have low stomatal conductance under well-watered conditions (g_s; chapter 2). This stomatal regulation limits excessive decreases in Ψ_l, ensuring that the water demand from the leaves remains within the capacity of the hydraulic system (Li *et al.*, 2017; Dayer *et al.*, 2020; Lemaire *et al.*, 2021*a*). Additionally, these genotypes can keep their stomata open for a longer duration during drought, maximizing carbon assimilation, meaning that their stomatal control is rather relaxed. Conversely, drought-sensitive genotypes exhibit a stricter stomatal control, characterized by higher initial g_s and more rapid complete closure of stomata at the onset of drought. Interestingly, our dataset reveals that the rate of g_s under well-watered conditions is more crucial in responding to drought than the speed of stomatal closure. This is evident in the sensitive genotypes (Cvi, Col-0, Kel-4, and *jub1kd*), which experience higher transpiration rates and hence lose more water, despite closing their stomata earlier than the more drought-tolerant genotypes that have lower initial g_s. These findings suggest that genotypes with higher drought resilience allow for a more effective balance between water loss and gas exchange.

The maintenance of the root-to-shoot hydraulic system depends on a species' ability to withstand embolism formation under the negative pressure caused by soil water deficit. Embolism resistance is often quantified as the xylem water potential inducing a 50% loss of maximum conductivity (P_{50}), which is often used as a proxy for drought resilience: species exhibiting more negative P_{50} are thought to be more drought tolerant, while those with less negative P_{50} values tend to be more sensitive to drought (Choat *et al.*, 2012; Anderegg *et al.*, 2016; Brodribb, 2017). Embolism resistance can vary considerably between and within species, with species inhabiting drier habitats generally displaying greater resistance to embolism compared to those from wetter areas (Larter *et al.*, 2015; Lens *et al.*, 2016; Trueba *et al.*, 2017; Dória *et al.*, 2019). P_{50} has recently been considered a mechanistic ‘super-trait’ due to its strong predictive power of plant performance and distribution across environmental gradients (Brodribb, 2017; Larter *et al.*, 2017). The results presented in this thesis also show a similar trend: more drought-tolerant Arabidopsis genotypes demonstrate more negative P_{50} values, while the most sensitive genotype, Cvi, exhibits the least negative P_{50} (chapter 1-2). This suggests that resistant genotypes likely enhanced their intrinsic embolism resistance, allowing them to close stomata later during drought, thereby maximizing plant productivity (Klein, 2014; Skelton *et al.*, 2015; Anderegg *et al.*, 2016). However, there is a concern that assessing embolism resistance solely based on P_{50} does not fully capture its physiological relevance, especially under non-extreme conditions where water status is primarily regulated by the stomatal control (Meinzer *et al.*, 2009). In this regard, the stomatal safety margin (SSM), defined as the difference between the water potential at stomatal closure (Ψ_{gs90}) and P_{50}, carries more physiological significance and reflects the strength of the hydraulic system in estimating a plant's ability to tolerate drought (Sperry and Tyree, 1988; Meinzer et al., 2009; Anderegg et al., 2016; Martin-StPaul et al., 2017; Creek et al., 2020; Dayer et al., 2020; Skelton et al., 2021). Numerous studies have shown that across angiosperms (mainly woody), a large positive safety margin is generally associated with a lower risk of lethal levels of drought-induced embolism compared to species with narrower (or even negative) safety margins (Choat *et al.*, 2012;

Anderegg *et al.*, 2016; Martin-StPaul *et al.*, 2017; Eller *et al.*, 2018; Creek *et al.*, 2020; Skelton *et al.*, 2021; Nolan *et al.*, 2021; Oliveira *et al.*, 2021). The findings presented in chapters 2 support that the more resilient genotypes display wider SSMs, whereas the susceptible ones show narrower SSMs and even a negative SSM in the case of Cvi. However, the drought-resistant genotype *p35S:AHL15*, despite its resilience, surprisingly exhibits a relatively narrow SSM, suggesting that a narrow SSM can be compensated by other drought-responsive traits to improve the drought resilience of an individual. For instance, the width of the SSM does not account for all aspects of stomatal regulation (i.e., rate of g_s) and the dynamics of leaf water potential during drought (Martínez-Vilalta and Garcia-Forner, 2017; Knipfer *et al.*, 2020). Interestingly, among Arabidopsis genotypes studied with comparable levels of drought tolerance, there are substantial variations in stomatal regulation, while P_{50} values consistently align with the whole plant's drought tolerance, except for *JUB1* overexpression line that employs a totally different drought response (Figure 1). This observation suggests that P_{50} outperforms SSM in explaining the responses to drought among the genotypes studied.

The role of xylem anatomical traits on embolism resistance

The resistance to embolism in plants could be determined by various xylem anatomical features such as wood density, fiber wall thickness, vessel wall thickness and conduit diameter, and intervessel pit membrane. These traits provide mechanical support to plants, which in turn might help mitigate the potential damage caused by high tensions and enable plants to maintain water transport during drought. In recent years, the significant role of intervessel pit membranes in embolism resistance has been increasingly put forward in structure-function studies (Meyra *et al.*, 2007; Choat *et al.*, 2008; Jansen *et al.*, 2009; Lens *et al.*, 2013; Li *et al.*, 2016; Dória *et al.*, 2018; Simioni *et al.*, 2023). In angiosperms, thicker membranes, characterized by longer multiconstriction paths, exhibit smaller pore constriction sizes that act as bottlenecks for fluid (and gas) transport. In addition, the increased thickness of pit membranes corresponds to a higher number of constrictions, which further promotes the occurrence of gas bubble snap-offs that are assumed to be coated with a stabilizing layer of surfactants (Berg *et al.*, 2013; Schenk *et al.*, 2015; Park *et al.*, 2019; Lens *et al.*, 2022). This functionally explains why species with thicker intervessel pit membranes are more drought-tolerant compared to species with thinner pit membranes (Kaack *et al.*, 2019, 2021; Yang *et al.*, 2020; Zhang *et al.*, 2020). However, most of these observations have been made at the interspecific level (Jansen *et al.,* 2009; Lens *et al.,* 2011; Plavcová and Hacke, 2012; Plavcová *et al.,* 2013; Scholz *et al.,* 2013; Li *et al.,* 2016; Dória *et al.,* 2018; Trueba *et al.,* 2019; Guan *et al.,* 2022), with limited focus on intraspecific variation (Schuldt *et al.*, 2016). Therefore, in this thesis, I emphasized the correlation between T_{PM} and embolism resistance among different genotypes of Arabidopsis. The findings from chapters 1 and 2 consistently demonstrate that T_{PM} is the key trait that best explains the variation of embolism resistance, with the more drought-resilient genotypes (more negative P_{50}) possessing thicker intervessel pit membranes, while the *JUB1* overexpression genotypes increasing drought tolerance using another set of traits (Figure 1).

In addition to the thickness of intervessel pit membranes, the degree of lignification or woodiness also plays a crucial role in determining the embolism resistance in both woody (Greenwood *et al.*, 2017; Liang *et al.*, 2021) and predominantly herbaceous angiosperm lineages (Tixier *et al.*, 2013; Lens *et al.*, 2016; Dória *et al.*, 2018). Species with woodier or more lignified stems generally exhibit better resistance to drought-induced embolism (Hacke *et al.*, 2001; Jacobsen *et al.*, 2007*a*; Willson *et al.*, 2008; Hoffmann *et al.*, 2011; Lens *et al.*, 2013, 2016; Dória *et al.*, 2018, 2019). Accordingly, our findings indicate a strong correlation between the proportion of lignified area per total stem area (P_{LIG}) and embolism resistance. Although P_{LIG} is not considered a key functional trait contributing to vulnerability to embolism in stems of the Arabidopsis genotypes studied, as it is not included in the most parsimonious multiple regression P_{50} model (chapters 1, 2, and 3), it still possesses significant predictive value due to its association with other traits that are thought to be more relevant such as T_{PM}. Plants with higher levels of embolism resistance are also believed to develop thicker vessel walls (Jansen *et al.*, 2009; Bouche *et al.*, 2014; Li *et al.*, 2016) or an increased fiber matrix (more and thicker fiber wall) (Jacobsen *et al.*, 2005, 2007*b*; Pratt and Jacobsen, 2017; Dória *et al.*, 2018), as is supported by our Arabidopsis dataset (chapter 1-2): the more resistant genotypes exhibit thicker vessel walls and a higher proportion of fiber wall area per fiber cell area (PF_WF_A), suggesting the functional roles of these traits in explaining the variation of embolism resistance. It is noteworthy that the majority of studies investigating functional-structure traits have primarily focused on trees, with limited attention given to herbaceous plants. Nevertheless, our study reveals that the herbaceous Arabidopsis exhibits a similar pattern of functional traits as those observed in trees. This finding implies that irrespective of being trees or herbs, both types of plants show the need to regulate stomata to balance water conservation and photosynthetic efficiency, alongside the development of resistance against embolism to ensure survival during drought conditions.

Unraveling the diversity of drought response strategies in Arabidopsis thaliana

Drought resilience in plants requires a complex interaction of traits influenced by a range of functional attributes (Violle *et al.*, 2007). As a result, the effectiveness and degree of drought tolerance can vary across species, depending on the synergistic interactions among these traits. This thesis has revealed remarkable findings, demonstrating that even in species with a short life cycle, like Arabidopsis, multiple strategies can be employed to achieve their respective levels of drought tolerance (Figure 1). These strategies primarily involve two key approaches: the development of xylem resistance through specific anatomical traits (Levionnois *et al.*, 2021), and the maintenance of high and stable leaf water potential to prevent sap pressure from reaching critical thresholds through precise stomatal regulation (Martin-StPaul *et al.*, 2017). Among the Arabidopsis genotypes studied, the *soc1ful* knockout mutant demonstrates the greatest tolerance to drought. *Soc1ful* shows a unique combination of traits, including the most negative P_{50}, widest stomatal safety margin (SSM), thickest intervessel pit membranes, and highest stem lignification, along low initial stomatal conductance that gradually decreases during drought, ensuring a stable leaf water potential (Ψ_l) (Figure 1; chapter 2; Thonglim *et al.*, 2021, 2023). The other two genotypes, Sha and *p35S:AHL15,* demonstrate a comparably high level of drought tolerance (Figure 1) despite exhibiting some variation in the drought-responsive traits. For instance, Sha has a relatively high initial stomatal conductance, but its g_s decreases rapidly during drought. *p35S:AHL15* has considerably lower g_s, enabling it to sustain a relatively high and stable Ψ_l, but its stem P_{50} value is less negative, resulting in a smaller SSM compared to Sha. The three most resilient Arabidopsis genotypes also showed a similar expression of four drought-responsive genes (*RD29A,* *DREB2A, ABI2,* and *AREB1*) compared to the more sensitive genotypes during our drought experiment (chapter 2). This pattern was also supported by an analysis of the chlorophyll content in the leaves at the end of the drought experiment (chapter 2).

Unlike the other drought-resistant genotypes, the resilient Arabidopsis *JUB1OX* does not possess hydraulic and stem anatomical traits that have been associated with increased drought resilience (observed in chapter 3). Instead, A-*JUB1OX* plants have less negative P_{50}, thin intervessel pit membranes, and a less lignified stem (Figure 1). A-*JUB1OX* (but not tomato *JUB1OX*) compensates for these characteristics by displaying a lower initial stomatal conductance (g_s) that gradually decreases during drought. Interestingly, *JUB1OX* in tomato exhibit similar g_s to the wild-type, indicating that overexpression of *JUB1* does not lead to lowered stomatal conductance. This implies that the high Ψ_l during drought stress is driven by another mechanism that has not been investigated in this PhD (see next section). With respect to the sensitive Arabidopsis genotypes, including Col-0, Cvi, Kel-4, and *jub1kd*, we have observed traits that are typically associated with low resilience to drought. These traits encompass a less negative P_{50}, thin intervessel pit membranes, narrow SSMs, low levels of stem lignification, high initial g_s, and stomatal closure occurring later during drought (Figure 1; chapters 1-2). Altogether, these traits contribute to increased water loss through transpiration, a rapid decline in Ψ_l during drought, resulting in immediate wilting, and a significant reduction in chlorophyll content.

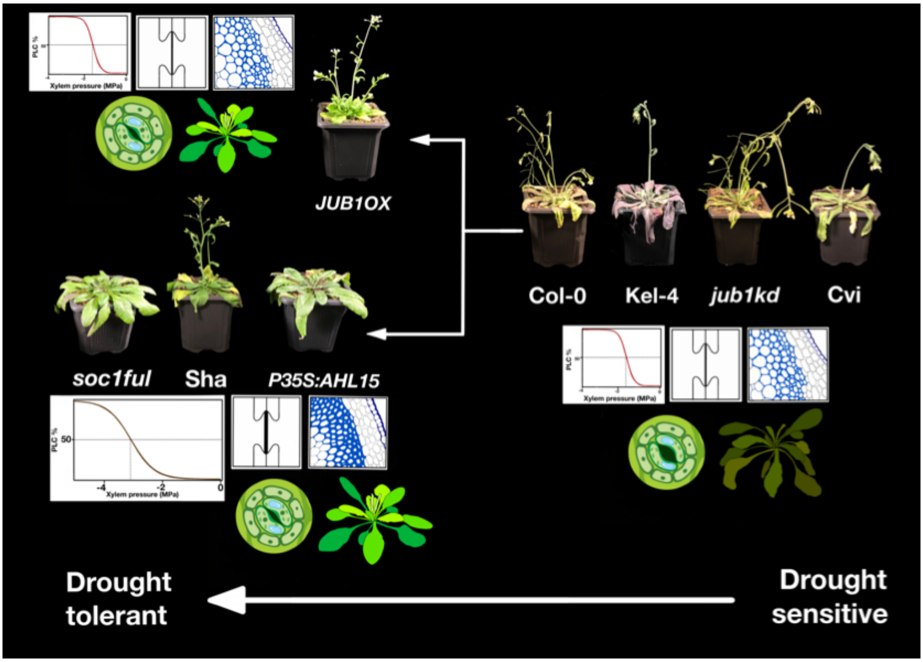


Figure 1 Diverse drought response strategies observed in Arabidopsis genotypes studied. The figure illustrates the phenotype of each Arabidopsis genotype during the same drought period highlighting a set of key traits used by each genotype in response to drought, with two distinct drought strategies in *JUB1OX* compared to *soc1ful*, Sha, and *p35S:AHL15*. Genotypes that are highly susceptible to drought are depicted on the right side, while the more resistant genotypes are depicted on the left side.

JUB1 mediates drought response in Arabidopsis slightly differs from that in tomato

Although the drought response strategy of *JUB1OX* in Arabidopsis is clearly different from the other drought-resilient genotype (Figure 1), there are some differences when comparing Arabidopsis and tomato *JUB1OX* with their wild-types, highlighting the species-specific effects of these mechanisms. Unlike the A-*JUB1OX*, the tomato genotype displays a high initial stomatal conductance and keeps its stomata fully open for extended periods, resembling the drought-sensitive genotypes of Arabidopsis. However, despite these traits, tomato *JUB1OX* demonstrates a greater tolerance to drought by maintaining a high and stable Ψ_l compared to the Money Maker wild type. It is intriguing to observe that the increased drought tolerance in tomato *JUB1OX* does not rely on either stomatal regulation or anatomical and hydraulic traits. According to literature, the observed elevation in Ψ_l during drought periods in tomato *JUB1OX* can potentially be attributed to the accumulation of osmoprotectants, such as proline (Pro), in its leaves (Wu *et al.*, 2012; Shahnejat-Bushehri *et al.*, 2017; Tak *et al.*, 2017; Alshareef *et al.*, 2019; Welsch, 2022). Proline functions as an osmolyte, enabling the uptake of additional water and minimizing the negative impact of water deficit by adjusting cellular osmotic potential and stabilizing cellular structures (Heuer, 2010; Blum, 2017; Hasanuzzaman *et al.*, 2019; Ahmad *et al.*, 2020; Ozturk *et al.*, 2021). Additionally, the overexpression of *JUB1* may hamper the accumulation of reactive oxygen species (ROS) during drought stress (Shahnejat-Bushehri *et al.*, 2012, 2016; Wu *et al.*, 2012; Ebrahimian-Motlagh *et al.*, 2017; Thirumalaikumar *et al.*, 2018). This helps prevent oxidative damage in plant cells, thereby promoting drought tolerance in *JUB1OX* plants. Although the concentration of osmoprotectants and ROS was not analyzed in this thesis, the potential role of *JUB1* as discussed in the literature provides a promising starting point for a following up study to investigate the precise mechanisms by which *JUB1* influences osmoprotectants accumulation and ROS mitigation at the molecular level.

In conclusion, this study has shed light on the complex mechanisms of drought resilience in plants. Two different mechanisms have been identified in Arabidopsis: (1) a synergistic interplay among hydraulic (P_{50} and SSM) and anatomical traits (T_{PM} and stem lignification), along with low stomatal conductance and high leaf water potential Ψ_l, and (2) high Ψ_l likely driven by osmoprotectants accumulation in leaves. These two distinct drought response strategies within a single annual species highlight the remarkable adaptive capabilities of plants to respond to challenging environmental conditions. It is therefore crucial to do in-depth drought experiments based on multiple accessions within a single species to understand the full breadth of drought responses in for instance crops. This in-depth screening at the population-ecotype level will benefit the development of more resilient crops in an era characterized by global warming where the human population is still exponentially growing.

Future perspectives

The presence of diverse drought strategies observed in this thesis showcases the remarkable adaptive capabilities of plants in coping with drought stress. However, it is important to acknowledge that the majority of research in the field of ecophysiology has predominantly focused on trees (Poorter *et al.*, 2010; Choat *et al.*, 2012; Mitchell *et al.*, 2013; Binks *et al.*, 2016*a*, *b*; Inoue *et al.*, 2017; Domingues *et al.*, 2018; Levionnois *et al.*, 2021; McDowell *et al.*, 2022; Johnson and Brodribb, 2023), while herbaceous plants have received far less attention (Lens *et al.*, 2016; Scoffoni *et al.*, 2018; Brodribb *et al.*, 2021; Fletcher *et al.*, 2022). Furthermore, the existing observations have primarily centered around individual species or generalized findings to the level of genera (Jansen *et al.*, 2009; Lens *et al.*, 2011; Plavcová and Hacke, 2012; Plavcová *et al.*, 2013; Scholz *et al.*, 2013*b*; Li *et al.*, 2016; Dória *et al.*, 2018; Trueba *et al.*, 2019; Guan *et al.*, 2022), disregarding the significance of intraspecific variation. This underestimation of intraspecific variability has hindered the comprehensive understanding of how plants respond to drought. To bridge this critical knowledge gap, it is crucial to prioritize and conduct more drought experiments that encompass a wide range of genotypes and species. By doing so, we can gain a broader perspective and develop a more comprehensive understanding of the diverse range of plant drought-responsive strategies. However, conducting drought experiments across numerous genotypes and species is a time-consuming task, particularly when precise measurements of key physiological traits are required. Advancements in technology have started to revolutionize this process, enabling efficient experimentation with hundreds of plants at once. One such innovative technology is PlantArray (Plant-Ditech, Rehobot, Israel) (Halperin *et al.*, 2017), a fully automated, multi-sensor gravimetric-based platform that streamlines the analysis of whole-plant performance. It facilitates the simultaneous measurements of various physiological traits, including plant transpiration, biomass accumulation, water, and nutrient use efficiency. This system provides accurate in-depth information on plant-environment interactions and the impact of environmental stresses on plant development and behavior under specific growth conditions, allowing for the efficient selection of optimal plant varieties and growing conditions that contribute to enhanced yields and stress response.

Given the significant findings regarding the integration of information from leaves and stems highlighted in chapter 2, it becomes evident that also the investigation of additional plant organs and associated traits would greatly contribute to our understanding of plant drought response. An often overlooked but fundamental aspect of the soil–plant–atmosphere continuum is the below-ground component. Roots play an important role in supplying water to compensate for transpiration losses during the process of gas exchange (Passioura, 1982; McCormack *et al.*, 2015; Cuneo *et al.*, 2021). Both woody and herbaceous species have been found to exhibit sensitivity to drought-induced declines in root hydraulic conductance (K_r), as demonstrated by Bourbia *et al.* (2021). Additionally, studies have shown that K_r is a key factor in driving stomatal closure in olive plants (Rodriguez-Dominguez and Brodribb, 2020). Thus, investigating the below-ground dynamics and understanding the implications of root hydraulic conductance on plant responses to drought is crucial for a comprehensive assessment of drought tolerance and resistance at the whole-plant level. In addition, considering the crucial role of the intervessel pit membrane in enhancing embolism resistance, it becomes imperative to conduct detailed investigations of this structure across all organs and at a fine scale. While some studies have dived into the three-dimensional structures of pit membranes, our knowledge regarding their fine-scale structures and chemical composition remains limited (Schenk *et al.*, 2015, 2017, 2018, 2021; Kaack *et al.*, 2019, 2021; Zhang *et al.*, 2020). The same applies to the composition of the xylem sap, which is much more than only water (Lens *et al.*, 2022), but how changes in xylem sap composition in time and space affect embolism resistance is not known.

Another black hole in our knowledge of mechanisms leading to plants' drought responses is how they manage to coordinate gene regulation leading to key traits, and how different gene regulatory pathways in different organs are intertwined to obtain a certain level of drought resilience. A promising approach to identifying key drought genes is to investigate the causal relationship between gene regulation and physiological responses, such as stomatal closure and osmoregulation, drought-associated traits like P_{50}. To achieve this, ecophysiologists and molecular biologists should team up and perform a high-resolution time-course gene expression analysis during drought and during the recovery phase after rewatering across different organs and plant developmental stages.

In conclusion, our current understanding of plant responses to drought remains limited. By embracing the future perspectives mentioned above, we can propel our knowledge of plant drought tolerance forward. This progress will not only enable us to accurately predict plant mortality on both crop and large forest scales but also facilitate the development of more resilient crop varieties capable of withstanding water scarcity. Through continued research and implementation of effective strategies, we can ultimately ensure a sustainable and secure agricultural future, even in the face of climate change.