EFFECT OF HEAT AND DROUGHT ON PHENOTYPIC PLASTICITY AND PHYSIOLOGY OF LEAVES OF *ARABIDOPSIS THALIANA*

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Bachelor of Science in Agriculture, Tribhuvan University (Nepal), 2011

A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfilment of the
Requirements for the Degree

MASTER OF SCIENCE

Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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Date of Defence: August 16, 2017

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Abstract

Plants undergo morphological and physiological acclimation when exposed to heat or drought. I exposed wild type *Arabidopsis thaliana* and mutants with open or less dense leaf venation to heat and drought and assessed subsequent changes to vein pattern. To also assess the correlation between the vein pattern and photosynthetic gas exchange, I conducted carbon isotope analysis on leaf tissue. Exposure of wild-type *Arabidopsis thaliana* to heat or drought resulted in a significantly increased vein density together with an increased number of vein meetings and areoles. Exposure of mutants to stress revealed that genes like *FKD1* and *UNH1* are involved in enabling leaf phenotypic plasticity. $^{13}C/^{12}C$ isotopic analysis suggested that, whereas wild type is capable of increasing its photosynthetic capacity under drought, the open vein pattern of the *fkd1* mutant does not allow proper hydration of leaf resulting in reduced stomatal conductance.

Acknowledgements

Dr. Elizabeth Schultz is an amazing person and I thank her for providing me a great opportunity to pursue my education towards betterment. I respect her patience and understanding towards a student like me who was exposed to a different education system before. I am truly and deeply grateful for having you as my supervisor. I am thankful to my committee members Dr. Larry Flanagan and Dr. André Laroche for helping me with my experiments and for providing support and feedback during my graduate studies. I am also grateful to Dr. David Pearce for helping me with several things in the water building and to Dr. Rob Laird for helping with statistics. Many thanks to the department of Biological Sciences, University of Lethbridge for having me as a graduate student.

Thank you, Kurt, for being a nice lab-mate and for helping me out every time. A big thanks to Houlin, Neema, Ryan, Bikash dai, Udaya, Cat, Libby, Aki, Ivie, and everyone else for helping me and giving me valuable suggestions throughout my graduate studies at the University of Lethbridge.

A sincere thanks to SOS Children's Village, Kavre, Nepal, for everything you have done to get me here to this stage.

Finally, thanks a lot to my family for your constant love and support and Dr. Krishna Bhatt for your encouragements and care and for having faith in me.

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List of Abbreviations

ABA = Abscisic Acid
c_a = partial pressure of CO_2 in the atmosphere outside a leaf
c_i = partial pressure of CO_2 within leaf intercellular spaces
Col = Columbia ecotype
FKD1 = FORKED1
FL1 = FORKED LIKE1
PIN = PIN FORMED
Vd = Vein density
A = Net CO_2 assimilation rate
A_{max} = Maximum CO_2 assimilation rate
g_c = stomatal conductance
UNH1 = UNHINGED1
SD = Stomatal density
K_{leaf} = Leaf hydraulic conductivity
$\delta^{13}C$ = carbon isotope ratio ($^{13}C/^{12}C$)

Chapter 1: **Literature Review**

Plants and other photosynthetic organisms are primary producers. The carbon that they fix provides the carbon for humans and other non-photosynthetic organisms. Two components of plant anatomy, the xylem and stomata, are critical to CO_2 and water availability within plants. CO_2 entry into the plant requires stomata to be open, which puts plant tissue at risk of water loss and dehydration, and ultimately to desiccation if water is not replenished in the leaf tissues. To prevent desiccation of vulnerable plant tissues and to provide water for photosynthesis, water is supplied via the xylem. The xylem acts like a straw which, by surface tension, draws water through the narrow vascular xylem strands to the leaves by the process of transpiration. Plants do not expend energy in water transport. The energy is supplied by the sun which drives evaporation and produces negative pressure. These features allow water to be drawn from soil. Thus, veins and stomata are critical to efficient photosynthesis.

1.1 Veins and leaf hydraulic conductivity in plant physiology

1.1.1 Vein structure

Leaf veins are continuous with the vascular system of the plant and enter the leaf through the petiole and spread throughout the leaf. The vascular system, consisting of conducting tissues (phloem and xylem), fibers, parenchyma cells and sclerenchyma cells, extends throughout the entire plant. Phloem transports sugars produced during photosynthesis. Xylem transports water and minerals in the plant and provides mechanical support to the plant in coordination with sclerenchyma fibers and xylem parenchyma. Xylem parenchyma are present within the xylem and do not undergo cell death. Tracheary elements are the conducting cells of xylem which consist of tracheids and vessels elements. The vessel elements have perforation plates at their ends and are joined end to end forming

a vessel. Tracheids are narrow tapering cells with circular bordered pits. They are longer and thinner than the vessels and are found in all vascular plants. Vessel elements are found in some ferns, the Gnetales group in gymnosperms and all angiosperms (Taiz and Zeiger, 2010). The primary xylem is formed from the procambium in all plants whereas the secondary xylem develops from vascular cambium during secondary growth in woody plants only. Primary xylem consists of two types of xylem, protoxylem and metaxylem (Myburg et al., 2013). The earliest formed young xylem, protoxylem, has living cells with all the cell contents, allowing plant growth. Protoxylem differentiates during plant organ elongation compared to mature xylem. It consists of fewer tracheary elements with spiral or ring like lignin deposition, is smaller in diameter, has more parenchyma cells and is less conducting to water. The differentiation of metaxylem, later formed xylem, is completed after the plant organ in which it is located, stops elongation. Metaxylem consists of more tracheary elements, tracheids and vessels elements, which are subjected to programmed cell death upon maturity leaving behind elongated, hollow, thick, lignified secondary cell walls. In metaxylem the lignin deposition is in a reticulate or pitted form. Tracheids have numerous bordered pits and no perforations. The vessel elements have empty lumens with perforation plates at their ends and are joined end to end forming a continuous hollow tube. They also have bordered pits allowing lateral movement of water. Both xylem structures form an interconnected pathway arranged end to end throughout the plant for water transport with low resistance (Myburg et al., 2013). The veins are enclosed within the bundle sheath and are not directly exposed to the mesophyll.

1.1.2 Vein pattern

Angiosperms have the highest variation in vein patterns among the vascular plants (Roth-Nebelsick et al., 2001). A eudicot leaf has veins that emerge in a hierarchical order,

based on their size and the position where later formed veins are joined to previously formed veins (Figure 1.1). A prominent mid-vein, with the thickest vein diameter, enters the leaf through the petiole, followed by secondary, tertiary and quaternary veins where the diameter and xylem size decreases with decreasing hierarchy (Canny, 1990; Turner and Sieburth, 2002). Most dicots have a reticulate venation pattern where the veins are interconnected to each other forming a web like structure. The smallest veins are the minor veins that end as freely ending veins (Nelson and Dengler, 1997) and are embedded in the photosynthetic mesophyll. Minor veins load sugars to phloem and unload water from xylem into the mesophyll. Most monocot species have a parallel venation pattern where veins diverge from the base of the lamina, run parallel across the leaf and converge at the apex (Foster and Gifford Jr., 1974, Figure 1.2 g).

The connection of veins to each other in a plant leaf is called 'vein meeting' (Figure 1.1c) and is proposed to provide alternate transportation routes in case of injury or embolism (Roth-Nebelsick et al., 2001). The smallest area in the leaf that is completely bounded by veins is the areole. Leaf vein density (*Vd*) is the length of veins per unit area in the leaf of which the highest proportion is contributed by minor veins (Canny, 1990; Sack and Tyree, 2005). Leaf vein pattern (density, number of vein meetings, number of areoles) are important parameters of plant physiology and changes in these parameters are known to affect the physiological performance of the plant (Sack and Scoffoni, 2013).

1.1.3 Water flow in the leaf

The physiological activity of the plant is determined in part by leaf hydraulic function (Brodribb, 2009). To fix the carbon necessary to produce a gram of new plant tissue, 500 g of water is absorbed by the root and transported by the xylem to the leaves where it is transpired to the atmosphere via the stomata. Thus, a leaf may exchange up to 100% of its

internal water every hour (Taiz and Zeiger, 2002). The path for this exchange begins when water from the soil enters the xylem either through the apoplast (through cell wall and extracellular space without crossing any membranes), symplast (through the plasmodesmata) or the transmembrane (through a series of cells by entry into the cell from one side and exiting from the other side) pathway depending upon the gradients and resistances (Taiz and Zeiger, 2010). Once in the xylem, xylem tracheids and vessels provide a water transport system with low resistance. Intercellular spaces of leaf mesophyll have a large negative pressure because of water evaporation from the mesophyll and this negative pressure pulls water up from the base, the mechanism of which is famously known as the 'cohesion tension theory' that was proposed by H. H. Dixon in 1894 (Tyree, 1997). The rate of water flow up the xylem in the plant depends upon the diameter of the xylem tubes, the viscosity of water, resistance of bordered pits and the pressure gradient driving the water flow (Sack and Holbrook, 2006; Choat et al., 2008; Scoffoni et al., 2013). Increased diameter favors water conduction in the tracheary elements but sometimes when there is sufficiently strong tension in the water column, the water column can break causing cavitation or creating air bubbles (embolism) (Tyree and Sperry, 1989). Thus, it is a more serious problem in xylem conduits with bigger diameter than smaller diameter.

When water reaches the leaf petiole, it flows through the primary vein into the secondary major veins and veins of lower orders before finally exiting the veins via the freely ending minor veins into the leaf mesophyll (Esau, 1965). After exiting the xylem, water enters the parenchymatous cells of the bundle sheath which surround the vein (Esau, 1965) and thereafter enters into the mesophyll. Once it exits the veins and enters the mesophyll, the water movement through these living cells occurs due to differences in water potential across the leaf (Sack and Holbrook, 2006) before finally evaporating out of

the stomata in the form of water vapor. Water movement through mesophyll varies among species and the mode of water movement (i.e. either apoplastically, symplastically or transcellularly) is not very clear (Sack and Holbrook, 2006).

The highest proportion of total vein length in a given leaf area is contributed by minor veins; thus, most of the water supply to the leaf mesophyll occurs via the minor veins (Canny, 1990; Sack and Tyree, 2005). Water can also exit from major veins into the mesophyll (Canny, 1990) as xylem is leaky. After transpiration, if the water is not replaced by xylem (the hydraulic system), the mesophyll will dehydrate and desiccate which leads to stomatal closure (Sack and Holbrook, 2006). Maintenance of open stomata is determined by a well hydrated leaf with high leaf water potential which is brought about by high plant hydraulic conductivity (Scoffoni et al., 2011). The plant hydraulic system affects the ability of stomata to remain open for photosynthesis without desiccating the leaf (Sack and Holbrook, 2006).

1.1.4 Leaf hydraulic conductivity (K_{leaf})

Leaf hydraulic conductivity (K_{leaf}) represents the water conductance from the petiole to the veins throughout the leaf, out from the bundle sheaths and through or around the mesophyll cells up to the site of transpiration (Tyree and Zimmermann, 2002; Sack and Holbrook, 2006). K_{leaf} measures the efficiency of water transport through the leaf and is given as the water flow rate throughout the leaf divided by the water driving force, the difference in water potential, across the leaf (Sack et al., 2002). Leaf hydraulic conductivity (K_{leaf}) scales with hydraulic conductivity of the plant and therefore, the hydraulic system (veins) does a critical job of letting the stomata open.

When water flows from veins to the stomata through the mesophyll (living cells), there is a resistance to water flow (Figure 1.3). Xylem has low resistance to water flow

while the resistance is much higher in the mesophyll. Increased minor *Vd* decreases the distance from vein endings through the mesophyll to the stomata, resulting in an overall reduction in resistance (increased conductivity) (Brodribb et al., 2007; Boyce et al., 2009). The leaf vein system is highly variable among different plant species in terms of vein architectural arrangement and *Vd* (Roth-Nebelsick et al., 2001) . These parameters play a major role in affecting how water is distributed throughout the leaf (Sack and Holbrook, 2006), thus forming a crucial relationship of the vein system to K_{leaf}.

1.1.4.1 Factors affecting K_{leaf}

K_{leaf} affects the morphology and the physiology of the leaf. Vein anatomy as well as vein pattern is important for K_{leaf} and changes in K_{leaf} can occur through changes in any of these characters. Several studies on diverse plant species have shown the importance of K_{leaf} and its correlation with the rate of photosynthesis (net assimilation (A_{max})) and stomatal conductance (g_c) across diverse plant species. These parameters are in turn positively correlated with leaf *Vd* (Sack and Frole, 2006; Brodribb et al., 2007; Brodribb et al., 2010; McKown et al., 2010; Sack and Scoffoni, 2013). Sack and Frole (2006) tested for correlation of hydraulic resistance with venation in 10 diverse tree species and found the total *Vd* to be negatively correlated with leaf hydraulic resistance. Similarly, Brodribb et al. (2007) found a positive correlation between A_{max} and g_c to K_{leaf} in 43 plant species including mosses, ferns, lycopods, gymnosperms and angiosperms. They also found a positive correlation between A_{max} and *Vd* where photosynthesis increased when veins were closer to the site of evaporation. Boyce et al. (2009) also found *Vd* positively correlated with A_{max}. In another study, Brodribb and Feild (2010) compiled a dataset of 759 vascular plants including extant angiosperms along with living and extinct non-angiosperms and measured and reconstructed their vein density and photosynthetic capacity. They found a

surge in photosynthetic capacity of angiosperms in the Cretaceous era coupled with increased vein density. McKown et al. (2010) tested this correlation using an explicit model of venation system with leaf simulations and found it to be true for diverse species of plants. Thus, according to several researchers, working in diverse species, a higher *Vd* increases the hydraulic conductivity (K_{leaf}) and g_c of the leaf, allowing higher photosynthetic rates. Based on this relationship, plants would benefit if the veins could reach every cell in the leaf, increasing their K_{leaf}, however, veins are expensive to make as they are carbon sinks (Pantin et al., 2012). Therefore, plants trade-off between the energy required for investing in minor veins and increased photosynthesis obtained by increased *Vd* (Brodribb and Feild, 2010).

While the results of the research presented above are obtained from plants of diverse species, there is little research done to find the relationship of K_{leaf} to *Vd* from species of the same genotypes. Nardini et al. (2014) studied the correlation of K_{leaf} to *Vd* in wild type and three cultivated genotypes of *Coffea arabica* and found a positive correlation of K_{leaf} to g_c and A_{max}. They also found a positive correlation between g_c and *Vd*. However, they did not find a positive correlation between K_{leaf} and *Vd*. Another study by Xiong et al. (2015) assessed K_{leaf} and its effects on leaf morphological and physiological characters in four rice cultivars and seven wild cultivars of *Oryza.* They also found a positive correlation between K_{leaf} and A_{max}, but did not find any correlation between *Vd* and K_{leaf}. However, they did find a significant increase in K_{leaf} with respect to increase in proportion of minor vein length. Caringella et al. (2015) worked with wild type *Arabidopsis thaliana* (Col-0) and four mutants (*defectively organized tributaries (dot), dot3-111* and *dot3-134,* and *cotyledon vascular pattern (cvp), cvp1-3* and *cvp2-1*), with different phenotypes and venation pattern than the wild type in *Arabidopsis thaliana*. The cotyledons and juvenile

leaves of *dot3-111* and *dot3-134* had parallel venation like the monocots while *cvp1-3* and *cvp2-1* had open venation pattern with vascular islands. They looked at vein anatomy and K_{leaf} from higher node leaves. They found that K_{leaf} was higher in the wild type despite insignificant differences in *Vd* of wild type and mutants. Their research also showed no correlation between K_{leaf} and *Vd*. However, there was a strong correlation between K_{leaf} and bundle sheath conductance among the genotypes.

1.2 Evolution of angiosperm leaf venation

The land plants evolved from ancestors of the green algae (Charophycean green algae) between 480 and 360 million years ago, according to the phylogenetic record (Kenrick and Crane, 1997; Bateman et al., 1998) (Figure 1.2). The transition from aquatic to terrestrial life required structural and functional changes in the plants. Protection from water loss, absorption of water and minerals from the soil and their efficient distribution throughout the plant called for vascular tissues. Thus, plants evolved vascular tissues to succeed better on the land. By late Devonian, most of the plants having specialized sexual organs, vascular tissues, stomata, sporangia, leaves and roots of various types, seeds and structural tissues had evolved (Kenrick and Crane, 1997). Most of the plants (ferns, progymnosperms and gymnosperms), that arose in the early Devonian and the early Carboniferous period, seemed to have simple and open venation pattern with dichotomous veins, and that formed the basis for evolution of modern venation pattern (Roth-Nebelsick et al., 2001). Non-angiosperms do not have hierarchy of vein order but angiosperms show distinct hierarchy in vein order with the modern plants having more distinct and profound hierarchy, especially the dicots (Roth-Nebelsick et al., 2001). An exception within the gymnosperm is *Gnetum*, which has a hierarchical venation pattern like dicots.

The coordination of the hydraulic system with gas exchange system and leaf structure is likely to have played an important role in early venation evolution. Stomatal density (SD) was low in early land plants (Edwards et al., 1998). Higher stomatal densities evolved later, probably for increased permeability to CO_2 under the condition of low CO_2 availability (Woodward, 1987; Franks and Beerling, 2009). This increase in SD would have led to higher rates of transpiration (Osborne et al., 2004) which could have led to evolution of more efficient hydraulic pathways to support acquisition of sufficient water (Sperry, 2003; Boyce, 2005).

Early plants like ferns have low K_{leaf} along with low *Vd* in comparison to angiosperms (Boyce et al., 2009). The *Vd* of non-angiosperms has remained the same for the last 380 million years whereas angiosperms have developed the highest range in diversity of venation pattern (Boyce et al., 2009). Within angiosperms, K_{leaf} has increased along with *Vd* over time (Brodribb and Feild, 2010). There is no clear explanation as to why angiosperms were the only plant group to develop such a variation in *Vd* and vein pattern. Zodrow and Cleal, (1993), link evolution of a closed venation system in plants to a decrease in water availability. One of the reasons for evolution of multiple vein orders and increased *Vd* could be to protect plants from external and internal damage by creating redundant water transport systems as described above (Nardini, 2001; Hüve et al., 2002; Scoffoni et al., 2013). Brodribb and Feild, (2010), measured the *Vd* and reconstructed photosynthetic capacity from a dataset of 759 vascular plants. They found a threefold increase in *Vd* of plants during the Cretaceous period, which is predicted to have facilitated rapid transpiration brought about by increased SD at that time. Since increased transpiration is correlated with increased rate of photosynthesis (section 1.1) , the huge increase in the *Vd* of angiosperm in the Cretaceous period is predicted to have improved the leaf

physiology with increased transpiration and rate of photosynthesis leading to success of angiosperms over other plants in terms of productivity (Boyce et al., 2009; Brodribb and Feild, 2010).

Leaf venation pattern shows a high variability within and across species in response to external factors. A common trend is that plants in drier regions have higher *Vd* than those in moister areas and leaves found in shade have lower *Vd* than those in sun (Esau, 1965). A higher *Vd* could protect plants from vein damage or cavitation under the condition of drought (Sack and Holbrook, 2006; Nardini et al., 2010). Redundancy of venation pattern in the angiosperm, for example multiple veins with vein meetings and areoles or veins in parallel to each other, is important for tolerance of K_{leaf} against embolism, cavitation or damage in veins by providing alternate transportation routes for water around damaged or blocked veins (Nardini, 2001; Hüve et al., 2002; Scoffoni et al., 2013; Brodribb et al., 2016).

1.3 Response of plants to drought and heat stress

The phenotypic plasticity of plants allows for morphological changes for acclimation following confrontation with stresses, either biotic or abiotic. Living organisms, such as bacteria, fungi, virus, pathogens, insects or other plants are biotic stresses in plants while heat, drought, cold, water-logging and salinity are some examples of abiotic stresses in plants. In 90% of the arable land crops go through some form of stress during their growth period (Murata and Mori, 2013). Plants respond to stress by changing physical parameters and by sending signals to change metabolism.

The morphological responses to abiotic stress are categorized as either escape, avoidance or tolerance mechanisms (Levitt, 1980; Ludlow, 1989; Verslues et al., 2006; Lawlor, 2013). However, the distinction between the avoidance and tolerance mechanism

of stress response is blurred so in this review I am going to consider them together. The escape mechanism is achieved by early maturity of the plant, where they cut short their growth duration. The avoidance mechanism is the ability to reduce the impact of stress and maintain processes by adjusting certain morphological structures or growth rates. It includes balancing water uptake and water loss by conserving water and maintaining high tissue water potential or by increasing water uptake through developing a deep root system. Tolerance mechanism is to protect plant from cellular damage caused by stress (Lawlor, 2013; Verslues et al., 2013).

As described in previous section, plants have shown various morphological changes over the course of evolution because of long-term changes to environmental conditions like water scarcity, change in CO_2 availability and high temperatures (section 1.2). In the short term, many plants show similar responses to drought and heat stress by phenotypic plasticity. Visible acclimations in leaves include alteration in leaf area, leaf *Vd* and SD. Stomatal opening is required for exchange of CO_2 and water vapor (Wong et al., 1979) and veins provide efficient water transport. These changes in leaf morphology result in changes to the rate of photosynthesis and water use efficiency (WUE).

1.3.1 Response of plants to drought

Water limitation or drought results in impaired plant growth (Chaves et al., 2009; Huseynova et al., 2009; Verslues et al., 2013; Zhao et al., 2016). The immediate response of plants to drought and the major mechanism to withstand mild drought is closure of their stomata to reduce transpirational loss and conserve water (Verslues et al., 2013). During early stage of water stress, WUE is high because closing of stomata inhibits transpiration more than CO_2 intake (Murata and Mori, 2013).

Long-term phenotypic response to drought includes processes like faster senescence, increased cuticular wax (Zhao et al., 2016) and mechanisms by which plant copes with reduced water availability such as changing the leaf area, *Vd* and SD (described in following sections). Root growth is enhanced to promote water uptake so that the plant is not dehydrated. Leaf size is reduced through decreased cell division (Poorter et al., 2009). Leaf *Vd* increases (primarily through increase in minor veins) with drought stress to protect plant from vein damage or cavitation and to provide alternate water transport routes in case of embolism contributing to drought tolerance in K_{leaf} (Sack and Holbrook, 2006; Nardini et al., 2010; Scoffoni et al., 2011). Earlier research has shown that air bubbles (embolisms) are formed in the veins, which disrupt water transport in the leaf, decreasing K_{leaf} (Salleo et al., 2000; Brodribb and Holbrook, 2003; Sack et al., 2008) under drought. Recent work by Scoffoni et al. (2017) suggests that decline in K_{leaf} during drought occurs firstly because of vulnerability of outside xylem water pathways including bundle sheath and mesophyll cells and embolism occurs only under the condition of severe drought.

Stomatal closure under drought leads to reduced intercellular carbon content, which is the major cause of reduced photosynthesis under prolonged drought (Flexas et al., 2004; Farooq et al., 2009). Drought also damages photosynthetic pigments and the thylakoid membrane in the green leaf (Huseynova et al., 2009; Anjum et al., 2011) resulting in decreased rate of photosynthesis. Stomatal density shows a high variation among species and within the same species under different drought conditions. Drought leads to a decrease in SD in *Triticum aestivum* (Quarrie and Jones, 1977), *Spondias tuberosa (Silva et al., 2009)* and *Populus balsamifera* (Hamanishi et al., 2012). In a different study, *Triticum aestivum* showed increased SD under drought (Huimin and Genxuan, 2001), whereas no change in SD was seen in *Arachis hypogaea* (Clifford et al., 1995). Stomatal density

increased in perennial grass *Leymus* under moderate drought, but when severe drought was applied, the number decreased (Xu and Zhou, 2008). The precise mechanism of the plasticity of SD with response to drought in plants is unknown (Casson and Hetherington, 2010). Under conditions of low water availability, plants close stomata resulting in low CO_2 availability. Low CO_2 results in increased SD allowing greater permeability to CO_2 (Woodward, 1987). One possibility is that the low CO_2 resulting from drought induced stomatal closure, similarly induces increased SD. Recent research has shown that decreasing SD is useful for drought tolerance in *Arabidopsis thaliana* because of reduced transpiration (Hepworth et al., 2015) obtained from lesser number of stomata.

1.3.2 Response of plants to heat

Heat stress is the rise in temperature above the normal threshold for a certain period in plants life cycle which induces some changes in plant growth. Each plant species has a different range of tolerable minimum and maximum temperature. Some examples of heat damage in the plant are inactivation of enzymes in chloroplasts and mitochondria, inhibition of protein synthesis, protein denaturation and increased fluidity of membrane lipids (Vierling, 1991). Short term avoidance mechanism from heat stress involves opening of stomata for transpirational cooling to keep tissues from overheating and prevent injury from high temperature (Rizhsky et al., 2002). Long-term tolerance to heat is obtained by morphological changes to maximize productivity under the stress condition. The extra transpirational demands caused by increasing temperature are met by increased *Vd* in leaves primarily through increased minor veins (Medek et al., 2011; Hu et al., 2014). Since the density of hydraulic system increases, there is also an increase in K_{leaf} under heat condition (Hu et al., 2014) as mentioned in section 1.1.4.1. Reduced viscosity of water could also contribute to increased K_{leaf} under heat (Sellin and Kupper, 2007). However, under

prolonged heat condition, when the leaves are dehydrated, the K_{leaf} decreases (Sack and Holbrook, 2006). There is also a reduction in cell expansion under prolonged heat resulting in reduced leaf area (Lafta and Lorenzen, 1995; Atwell et al., 1999; Shin et al., 2001). Heat can either increase stomatal densities, as in ryegrass and tobacco (Ferris et al., 1996; Hu et al., 2014)) or decrease them, as in *Arabidopsis thaliana* (Vile et al., 2012).

As the temperature continues to increase above the optimal point, transpiration continues to increase while photosynthesis decreases, thus WUE is expected to decrease with increase in temperature (Allen et al., 2003). The rate of transpiration and photosynthesis of plants control WUE (Farquhar et al., 1989) which are, in turn, affected by varying degree of heat or drought. The rate of photosynthesis increases with heat up to a species specific optimal point, which is different among species (generally between 30^0C to 35^0C), above which photosynthesis decreases with increasing temperature (Schuster and Monson, 1990). Mild heat stress causes deactivation of Rubisco (Sharkey, 2005) and higher temperatures results in less chlorophyll synthesis (Dutta et al., 2009).

While heat and drought are two different abiotic stresses in plants, they often occur simultaneously in the field and result in interrelated plant responses. Reduced transpiration under drought can result in an increase in leaf temperature (Cook et al., 1964). Increased transpiration under heat stress can result in water shortage in the plant, when leaves will lose water more quickly than the roots can intake it thereby dehydrating the leaves. Thus, both stresses can result in the condition of reduced water availability in the plant which reduces the K_{leaf} by long-term stress response via xylem and outside xylem collapse and embolism or cavitation (Nardini et al., 2003; Scoffoni et al., 2011; Scoffoni et al., 2017).

Heat and drought stress causes changes in leaf morphology. The changes are thought to be brought about by stress hormone abscisic acid (ABA) (discussed in section 1.4). *Vd*

and SD seem to change under these conditions to cope with these stresses but how *Vd* corresponds to SD and vice versa is unknown.

Considerable research has been done on the effects of heat and drought stress on plants (Rizhsky et al., 2004; Farooq et al., 2009; Krasensky and Jonak, 2012), but few on the developmental response of *Arabidopsis thaliana* to heat and drought *(Vile et al., 2012*;Wolfe and Tonsor, 2014) and none on the response of venation to heat and drought in *Arabidopsis thaliana*. In an experiment by Vile et al., (2012), 10 different ecotypes of *Arabidopsis thaliana* were subjected to heat, drought and a combination of heat and drought. The different ecotypes are genetically different based on natural selection to a particular environment. In the experiment, those ecotypes had different response to stress treatments (heat and drought) for whole plant and leaf traits along with other plant parameters which suggests that there is a genetic component to the phenotypic plasticity of *Arabidopsis thaliana* leaves under stress conditions. *Arabidopsis thaliana* is very sensitive to heat stress, thus higher temperature can be used only for a short period as it decreases plant viability (Silva-Correia et al., 2014). Variability in heat and drought applied in different studies (Rizhsky et al., 2004; Vile et al., 2012; Wolfe and Tonsor, 2014) makes straightforward comparison between treatments difficult. The phenotypic plasticity of a number of angiosperms to heat and drought have been tested (Section 1.3), but the genetic mechanism that controls phenotypic plasticity is not known. *Arabidopsis thaliana* has been a model plant for dissecting a number of genetic processes (Somerville and Koornneef, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). I will test the hypothesis that *Arabidopsis thaliana* behaves in a similar way as other plants to heat and drought stress by increasing its *Vd* and number of vein meetings and by decreasing its leaf area.

1.4 Abscisic acid (ABA) regulates plant response to heat and drought

Abscisic acid (ABA) is a key stress response hormone produced as soon as the plants undergo water stress. It triggers ABA inducible gene expression causing various response mechanisms which affect leaf and whole plant growth directly or indirectly (Yamaguchi-Shinozaki and Shinozaki, 2006; Tardieu et al., 2010). The gene expression mechanism occurs through a signal transduction pathway which starts with stress signal perception where environmental stress signals are transmitted to transcription factors, and ends with expression of ABA responsive genes and stress response genes (Hirayama and Shinozaki, 2010). Signal transduction pathways control the connection between stress signal and the stress mediated response of gene expression for dehydration tolerance. Dehydration can be perceived by plants through both ABA dependent and ABA independent gene expression pathways (Tuteja, 2007; Finkelstein, 2013).

Changes in gene expression after signal transduction pathways cause changes in plant morphology and physiology to confer tolerance to drought. ABA produced in the root as a stress response is transported to the leaf to regulate stomatal closing (Jones and Mansfield, 1970) to conserve water. ABA activates changes in ion homeostasis in the guard cells and controls the signaling pathways which regulates stomatal closure (Daszkowska-Golec and Szarejko, 2013). Under drought stress, plants close stomata to conserve water. Along with stomatal closure, ABA has been proposed to inhibit cell division and cell expansion (Wang et al., 1998) which leads to reduced leaf area. ABA also inhibits shoot growth but promotes root growth (Sharp et al., 2004) and increases root hydraulic conductivity (Tardieu et al., 2010) to maintain high water uptake capacity, the overall effect of which helps plants cope with water stress (Taiz and Zeiger, 2010).

Application of ABA creates a response similar to the stress response in the plants. Experiments that applied ABA to plants or involved plant lines that overproduce ABA were found to have higher rate of leaf hydration than the control ones (Salah and Tardieu, 1997; Thompson et al., 2007). Studies using *Capsicum annum* seedlings dipped in 1 mM ABA solution (Berkowitz and Rabin, 1988) and 7.6 mM ABA (Goreta et al., 2007) found that ABA was useful in maintaining leaf water potential in the treated seedlings. Plant mutants which are either unable to respond to ABA or unable to synthesize ABA wilted and died easily when stress persisted whereas growth was similar to the wild type plants under normal conditions (Swamy and Smith, 1999). ABA increases in leaf during dehydration but once under favorable condition (after re watering), it decreases (Wan et al., 2009).

ABA affects a number of morphological characteristics that influence hydraulic conductivity including stomatal opening, SD and leaf size. My hypothesis is that it may also affect the pattern of veins within a leaf and hence the *Vd*. I will test this hypothesis by spraying varying concentration of ABA onto the leaves of *Arabidopsis thaliana.*

1.5 Vein pattern mutants of *Arabidopsis thaliana*

Arabidopsis thaliana is used extensively in biological research and has allowed elucidation of the molecular mechanisms behind many complex developmental and environmental response pathways (Møller et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Hotta et al., 2007). It is a model plant (Somerville and Koornneef, 2002) which is easy to grow, small in size, has a lifespan of about 6 weeks and has easy genetic manipulation and transformation, with a large collection of mutants. It has a typical, eudicot simple leaf with secondary veins diverging from the primary midvein, followed by tertiary veins forming a closed reticulum (joined to previously formed vein) and freely ending minor veins (Nelson and Dengler, 1997).

In the past, researchers have used different species to correlate different vein densities to changes in physiological efficiency (Sack et al., 2012; Brodribb and Feild, 2010). More recently, mutations that cause changes to morphology within the same genetic background, have been used to test hypotheses about SD, *Vd* and their effects on physiology (Caringella et al., 2015; Franks et al., 2015; Hepworth et al., 2015). As mentioned previously, Caringella et al. (2015) measured xylem and mesophyll anatomy, K_{leaf} and vein traits for wild type and vein pattern mutants (*dot3-111, dot3-134, cvp13, cvp2*-1). They found that K_{leaf} varies with vein anatomy across wild type and the mutants, with the wild type having higher K_{leaf} in comparison to the mutants. Franks et al. (2015) used altered mutants of *Arabidopsis thaliana* with varied SD and found that reduced SD gives increased WUE without affecting the rate of photosynthesis of the plant. However, the effect of altered *Vd* in mutants on photosynthetic gas exchange has not yet been tested.

I will work on a set of *Arabidopsis thaliana* mutants with defective PIN protein localization which results in altered *Vd* and vein meeting (Figure 1.4). These mutants provide an opportunity to test hypotheses regarding leaf vein pattern parameters and physiological processes in leaves within the same genetic background. The *UNHINGED* gene is a member of the plant GARP complex which is important for targeting the proteins to vacuole. Mutation to *UNHINGED* results in extra PIN proteins on the marginal epidermis, which leads to disconnected secondary veins and fewer higher order veins (Pahari et al., 2014). The *FORKED* gene family consists of nine members in *Arabidopsis thaliana*. *FORKED* is a novel gene that is involved in targeting PIN1 to membranes. Mutation to *FORKED* results failure of distal vein connections resulting in an open vein pattern (Steynen and Schultz, 2003). The quadruple mutant (*fkd1/fl1-2/fl2/fl3*) has a more severe phenotype than *fkd1*, with fewer higher order veins, an open vein pattern and

vascular islands (Prabhakaran Mariyamma et al, manuscript in preparation). Knocking out multiple members of the gene family results in a more extreme mislocalization of PIN proteins than in the single *fkd1* mutant suggesting that the genes act redundantly to localize PIN (Prabhakaran Mariyamma et al, manuscript in preparation).

I will test if mutation in these genes affect the ability of the vein pattern to respond to heat or drought stress. Via stable carbon isotope analysis, I will also test the hypothesis that physiological efficiency, in particular the rate of photosynthesis (*A*) and stomatal conductance (*gc*), is affected by stress induced *Vd* changes in the wild type and the mutants. This will allow us to test the importance of *Vd* and vein meeting to physiological efficiency, both under normal conditions and conditions of abiotic stress.

In summary, the hypotheses I will test in this study are:

1. Wild type *Arabidopsis thaliana* will respond to heat, drought and a combination of heat and drought in a similar way to other plant species by increasing leaf *Vd* and number of vein meetings.
2. The *Vd* response to abiotic stress is mediated by ABA so that I expect the *Vd* to increase following exogenous ABA treatment.
3. The genes that control vein pattern during development also control vein pattern response to drought and heat, thus I expect that mutation to these genes will alter the response.
4. Vein density affects physiological efficiency thus, changes to *Vd* occurring in response to stress will affect physiological efficiency of wild type *Arabidopsis thaliana*.
5. Mutants have varied vein pattern thus, changes to *Vd* and vein meeting occurring through mutation will affect the physiological efficiency in *Arabidopsis thaliana.*

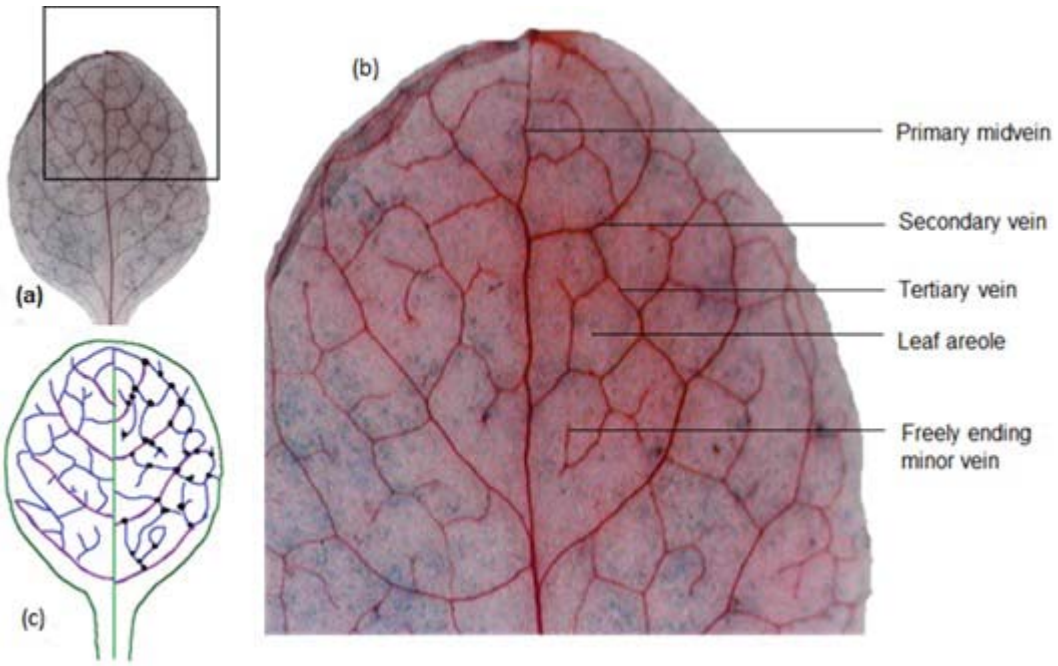


Figure 1.1 Vein pattern of a typical dicot leaf (*Arabidopsis thaliana*). The whole leaf is shown in (a) with the boxed region magnified in (b). Vein hierarchies, areoles and freely ending veins are indicated in (b) and vein meeting in (c).

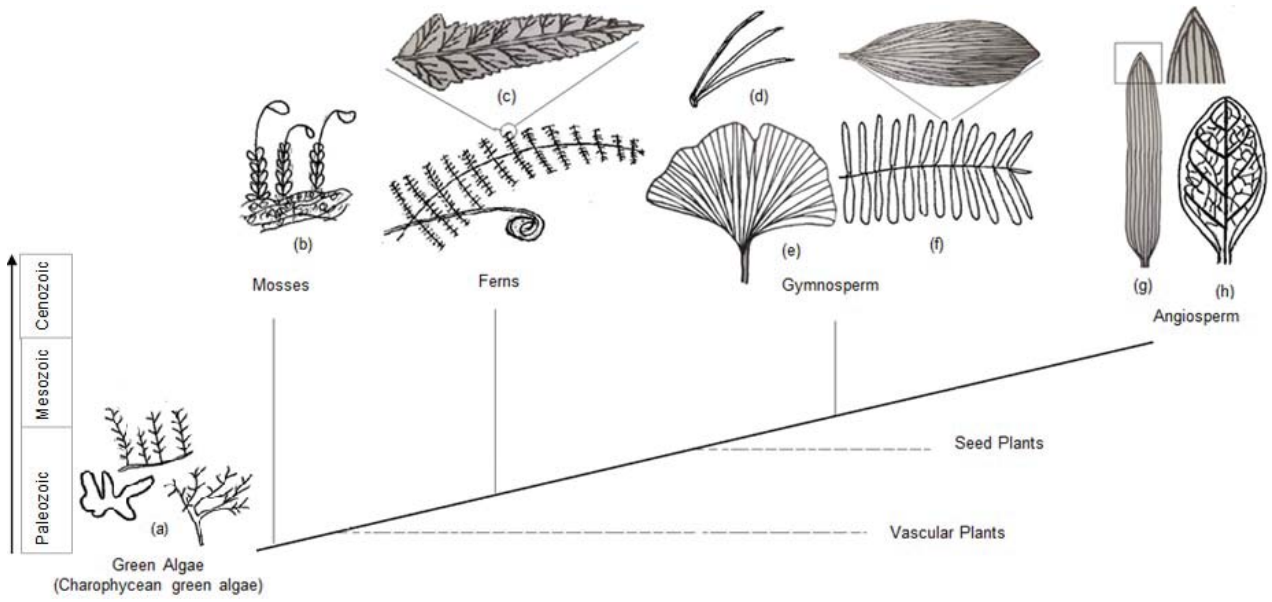


Figure 1.2: Evolution of leaf veins in plants. Algae (a) evolved during early Precambium and non- vascular terrestrial plants such as mosses (b) during early Carboniferous. Vascular non-seed plants, such as fern ((b) *Polystichum acrostichoides*), evolved during early Devonian and have dichotomously branching veins. Seed plants, such as Gymnosperms, arose in the late Devonian. Gingko (e) evolved during early Perminian and has a fan like leaf with open dichotomous vein pattern. The cycads (f) also have a dichotomous vein pattern. Conifers (d) evolved during early carboniferous have needle shaped leaves. Angiosperms evolved during the Cretaceous era. The monocots ((g) *Oryza spp.*) have parallel venation and the dicots ((h) *Arabidopsis thaliana*) have reticulate venation with different vein orders. Figure redrawn from (Foster and Gifford Jr., 1974).

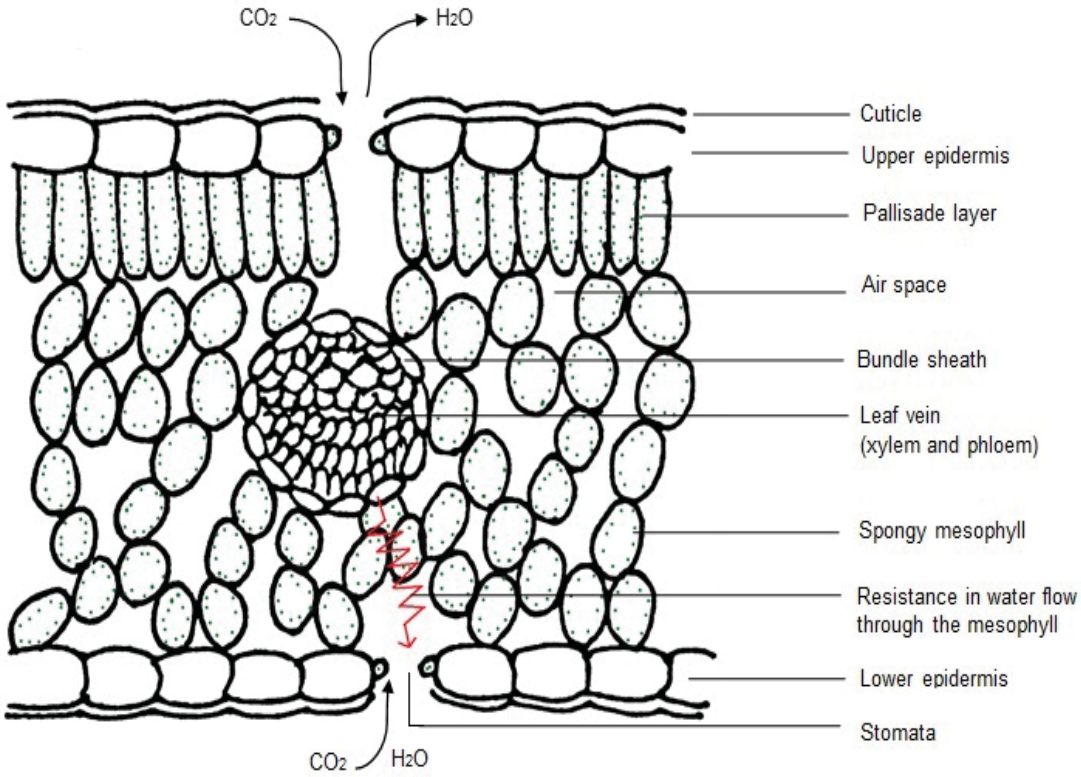


Figure 1.3 Internal structure of a leaf showing features affecting gas exchange and water transport. Water flows out of the vein into the bundle sheath and then passes through the mesophyll cells before finally exiting out of the stomata. The red arrow marks the resistance in water flow when water travels out of conducting veins, through the mesophyll and out through the stomata. Figure redrawn from Brodribb & Feild (2010).

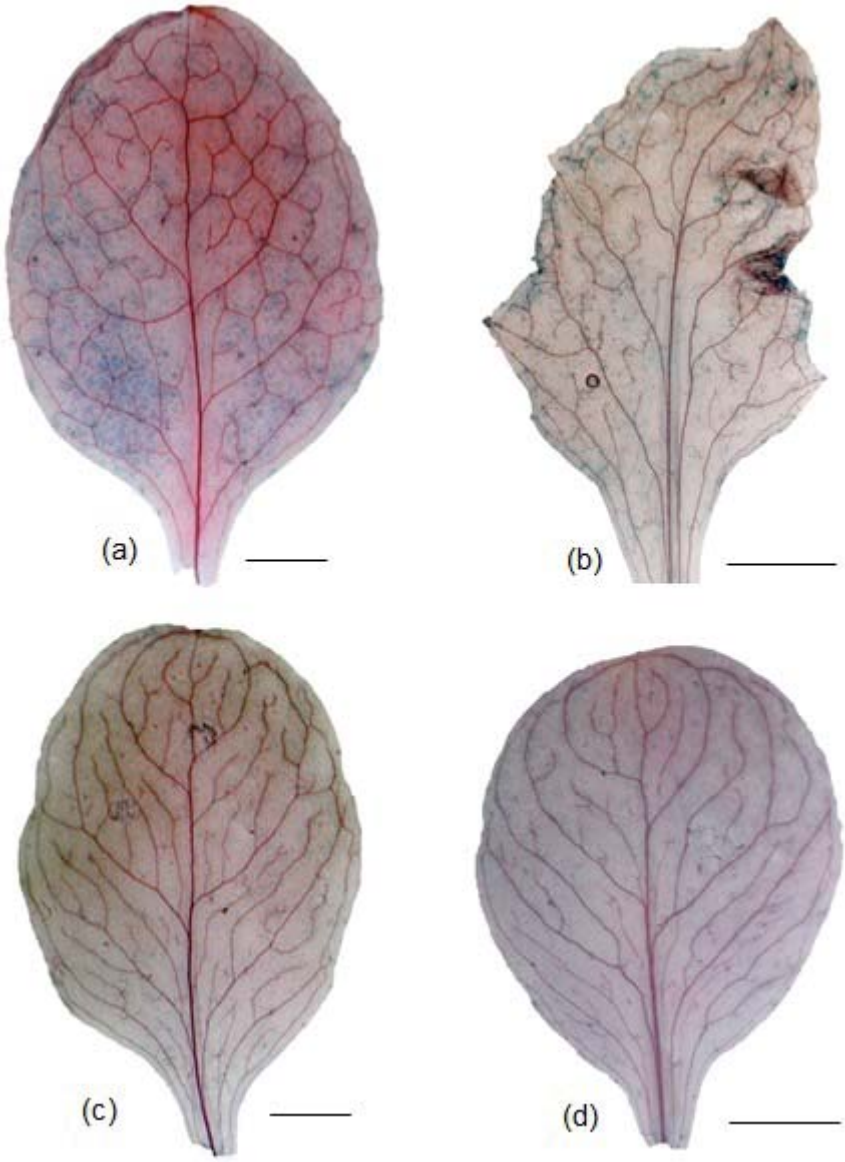


Figure 1.4: Wild type (a) and mutants (b-d) of *Arabidopsis thaliana*. The wild type (a) has a closed reticulum formed by joints between veins. *unh1* (b) has fewer higher order veins with less vein meeting. *fkd1* (c) has an open vein pattern due to decreased vein meeting. The quadruple mutant (*fkd1/fl1-2/fl2/fl3*) (d) has fewer higher order veins with an open vein pattern and vascular islands. Arrow (←) indicates freely ending veins and asterisk (*) indicates vascular islands.

Chapter 2: **Materials and methods**

2.1 Seed stocks

Seeds of wild type, *fkd1 (Steynen and Schultz, 2003), unh1* (Pahari et al., 2014), and the *fkd1/fl1-2/fl2/fl3* quadruple mutant (Prabhakaran Mariyamma et al., manuscript in preparation) were used. For all the experiments, the Columbia ecotype (Col-0) of *Arabidopsis thaliana* was used as wild type.

2.2 Growth condition and stress condition

Flora Compo Plus potting soil (Professional Gardener, Calgary AB) was mixed to homogeneity with vermiculite in the ratio 3:1. Pots of 6 cm x 6 cm x 5.5 cm were used. 50 g of soil mix was put into each pot. 6 pots were placed in a Styrofoam tray and 500ml of deionized water per 6 pots of soil was applied to the Styrofoam tray to moisten the soil. One seed was placed in each pot. A pot represented a unit replication. Thereafter, the pots were covered with Saran Wrap and kept in 4^0C for 4 days for seed stratification and uniform germination of seeds.

After 4 days of cold treatment, the plants were transferred to a growth chamber (Conviron, Model E15, Canada) at 25^0C, 80% relative humidity, and 190-250 $\mu molm^{-2}s^{-1}$ Photosynthetic Photon Flux Density (PPFD). As described in (Flanagan et al., 2017) the air temperature and relative humidity probe (HMP45C (Vaisala Inc.), Campbell Scientific, Inc., Logan, Utah, USA), which was inserted within a naturally ventilated radiation shield (41002, Gill Multi-Plate Radiation Shield, R.M. Young Company, Traverse City, Michigan, USA) was mounted inside the growth chamber. The PPFD was measured using a quantum sensor (LI-190SA, LI-COR Inc., Lincoln, Nebraska, USA). These measurements were scanned at a 5-s interval and were recorded as half-hourly means by a data logger (CR23X) for a period of 7 days. The plants were grown in continuous light with

metal halide (400W/U) and incandescent bulbs. The day the plants were transferred to growth chamber was considered day 0. The Saran Wrap was removed from the pots on day 7. All plants were watered every two days with 15 ml of water per pot starting on day 0.

Control plants were grown at 25^0C and watered every two days. The early-heat treatment transferred plants to a 30^0C chamber from day 7 to day 11 and back to 25^0C for the rest of the experiment. Late-heat treatment transferred plants to a 30^0C chamber from day 10 to day 14 and 25^0C for the rest of the experiment. For both early and late-heat experiments, plants were watered every other day throughout. Under drought treatment, the plants were watered every other day until day 10 and thereafter they were watered only once a week while still being kept at 25^0C. The fourth treatment was a combination of late-heat and drought. There were 24 replications per treatment (14 for *Vd* and 10 for SD). For wild type stress experiment, all the above-mentioned conditions were used. For the experiments comparing wild type and mutants (*unh1*, *fkd1* and *fkd1/fl1-2/fl2/fl3*) only early-heat and drought treatments were used along with the control treatment.

In the mutant experiment, the moisture content of soil was measured using Procheck hand held moisture meter from Decagon Devices, WA, USA along with Echo EC-5 moisture sensor probes. The probes were put into soil of wild type and mutants randomly with each treatment having 4 probes. Raw moisture data were obtained from the hand-held moisture meter and was converted to volumetric moisture content (VWC) in percentage using manufacturer's instructions (https://www.decagon.com/en/support/videos/soil-calibration-video/).

2.3 ABA experiment

For ABA experiment, wild type *Arabidopsis thaliana* plants were grown in 25^0C with water applied every second day. (±)-ABA (Cis Trans Isomer: MP Biomedicals, Santa Ana,

CA, USA) was dissolved in methanol to make 100 mM stock ABA solution. This stock was diluted to 1 mM solution with water and used to make 0.001 mM, 0.01 mM and 0.05 mM ABA and was mixed with 0.02% Tween 20. For control, 0.02% Tween 20 solution was made with water. These solutions were sprayed to drenching point in wild type *Arabidopsis thaliana* from day 10 to 14 or day 10 only. In a second experiment with wild type and *fkd1* mutants, plants were sprayed from 10 to 14 days only.

2.4 Leaf harvesting and clearing

The 3rd and 5th leaves of each plant were harvested at 24th and 27th days respectively. The harvested leaves were cleared and stained in a protocol modified from Scoffoni & Sack, 2013. Leaves were placed in 5% sodium hydroxide (NaOH) for 4 hours and then transferred to 50% bleach solution where they were left for a few seconds. They were then rinsed twice in water and were left aside without water for 1 hour. The next step involved bringing the leaves through an ethyl alcohol dilution series (30%, 50%, 70% and 100% ethyl alcohol) with 5 minutes at each step. After the 100% ethyl alcohol, the leaves were placed in 1% safranin (1mg safranin in 99 ml ethyl alcohol) for 15 minutes after which they were rinsed with 100% ethyl alcohol. 1% fast green (1mg fast green in 99 ml ethyl alcohol) was added to the leaves for 20 s and the leaves were rinsed again with 100% ethyl alcohol. The staining was followed by reverse dilution of the ethyl alcohol series (100%, 70%, 50%, and 30%) after which the leaves were placed in water. Thereafter, leaf samples were mounted in 66% glycerol on slides.

Images of the cleared stained leaves were taken with a digital camera (Nikon Coolpix e990) mounted on Leica MZ8 stereo zoom microscope. ImageJ (Abràmoff et al., 2004; Scoffoni et al., 2013) was used to measure vein length and to count vein meetings and areoles within a central region of each leaf bounded by the midvein, the leaf margin

and adjacent secondary veins for initial wild type stress experiment (Figure 2.1 a). Vein density (*Vd*) was calculated as the total length of all veins within the region divided by the area of the region. Vein meeting density was calculated as number of vein meetings within the region divided by the area of the region. Average areole numbers were calculated by dividing the number of areoles in the region by the area of the region. For all genotypes in the mutant stress experiment, *Vd*, vein meetings and areoles were calculated from half leaf (Figure 2.1 b).

Stomatal density was determined from leaf impressions. For the initial wild type stress experiment, abaxial side of ten freshly harvested leaves were taken. For all genotypes in the mutant stress experiment, dental impressions were taken from both abaxial and adaxial sides of the leaf. A silicon based dental impression material, Coltene® PRESIDENT light body (Sinclair Dental, Vancouver, Canada), was used to make a cast of the base. The base material and the catalyst were mixed in equal proportion and were applied immediately on abaxial and adaxial sides of the freshly harvested leaves. After the impressions hardened, the leaves were removed. Nail polish casts were obtained by applying clear nail polish on those dental impressions (Lawson et al., 1998) and 9 mm^2 sections of the nail polish peel was taken from the middle part of the leaf. Images of those sections were captured in a bright field microscope (Nikon Eclipse E600) using Qcapture. Thereafter, SD was measured using ImageJ from an area of 0.6 mm^2 as number of stomata per unit area (Figures 2.1 c and d).

2.5 Stable carbon isotope analysis of wild type and mutants under stress condition

For isotope analysis, mutants (*fkd1, unh1, fkd1/fl1-2/fl2/fl3)* and wild type were grown under early-heat and drought (growth conditions as described above in Section 2.1). The net assimilation rate in plant physiology given as $A = g_c(C_a-C_i)$, where A is the net

assimilation rate, g_c is the stomatal conductance, C_a is the CO_2 concentration of the air and C_i is the intercellular CO_2 concentration. Carbon isotope composition ($\delta^{13}C$) of leaf tells about changes in A/g_c which affects C_i (Farquhar et al., 1989). An increase in $\delta^{13}C$ can be either due to reduced g_c or increased A. Higher $\delta^{13}C$ value means C_i is lower.

On 27th day, 12 leaf 4, 12 leaf 6 and other remaining plant parts excluding roots and inflorescence were harvested into separate coin envelopes. Tissue was dried in a 60^0C drying oven for 3 days, after which it was stored in a glass vacuum desiccator until sample preparation. Leaf 4 and leaf 6 were too small to obtain minimum sample weight of 6 mg. Thus, other plant parts excluding roots and inflorescence were ground in a centrifuge tube manually. Six mg of the ground tissue was immediately transferred to small tin cups. The tin cup was closed to form a pellet and was introduced into a second tin cup, which was closed and formed into a pellet in the same way as before. All the leaves were kept in the tin cups and were formed into pellets. The pellets were then placed in microtiter plates and were sent to Isotope Science Laboratory in the University of Calgary for $\delta^{13}C$ and %N analysis. The stable carbon isotope ratio, which is expressed using delta notation (δ), in parts per thousand (‰), is given as:

$$\delta^{13}C\left(\frac{Rsample}{Rstandard} - 1\right) X1000$$

where R is the ratio of $^{13}C/^{12}C$, and the standard is the international standard Pee Dee Belemnite (Farquhar et al., 1989).

2.6 Statistical analysis

Data obtained from each experiment were recorded in Microsoft Excel®. The difference in *Vd* and leaf area, leaf vein meeting across wild type and mutants were analyzed by one-way (for wild type stress experiment) and two-way (for rest of the experiments) analysis of variance (ANOVA) using R studio software, version 0.99.484 (RStudio, Inc., http://www.rstudio.com/ 2015). When a significant difference was detected by ANOVA, pairwise comparisons between genotypes within a treatment or between treatments within each genotype were analyzed by using Tukey-Kramer post-hoc test in R. All the statistical tests were conducted at 95% level of confidence.

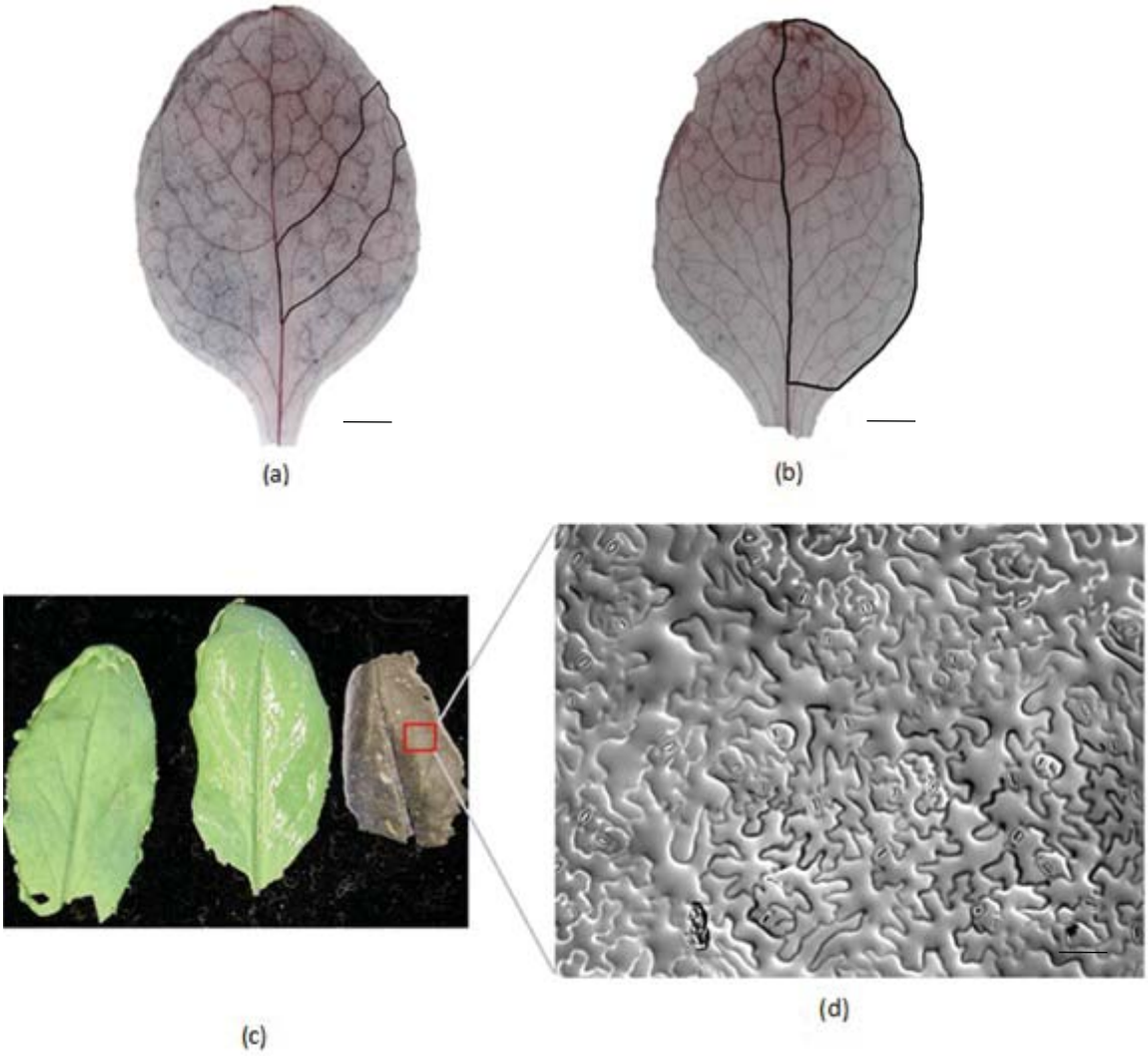


Figure 2.1: Cleared leaves (a, b) and dental peels (c, d) of *Arabidopsis thaliana.* Vein density for wild type stress experiment was taken from the middle section of the leaf (a). For mutant under stress, the vein density was taken from half leaf (b). The dental impression (c) was obtained by using silicon based dental impression material and once it hardened, clear nail polish was applied to it. The nail polish casts obtained were observed under bright field microscope to obtain pictures of stomata (d). Scale = 1mm.

Chapter 3: **Results**

Stress affects plant leaf vein patterns and SD which in turn affects water transport and photosynthetic gas exchange. I applied heat and drought treatments to *Arabidopsis thaliana* wild type and mutants with altered venation to see if these stresses affected the leaf morphology and physiology of both the wild type and the mutants.

3.1 Effect of heat or drought stress on leaves of wild type *Arabidopsis thaliana*

There was a significant effect of all the treatments on leaf *Vd* for leaf 3 and leaf 5 as determined by one-way ANOVA (Table 3.1) ANOVA test was followed by post hoc Tukey-Kramer test. There was a significant and consistent increase in *Vd* under all treatments of heat, drought and heat and drought in both leaf 3 (Figure 3.1 a) and leaf 5 (Figure 3.2 a) with $p < 0.05$. Early-heat had a stronger effect on leaf 3 than late-heat, consistent with the expectation that the early-heat would coincide with the vein pattern developmental period of leaf 3. The effect of drought and late-heat combined was not consistently different from the effect of drought or late-heat alone.

The one-way ANOVA for vein meeting indicated a significant effect of all the treatments on number of vein meetings per unit area for leaf 3 (Table 3.1). Similarly, there was a significant effect of all the treatments on number of areoles per unit area for both leaf 3 and leaf 5 (Table 3.1). The frequency of vein meeting and number of areoles within both leaf 3 (Figures 3.1 b and 3.1 c) and 5 (Figures 3.2 b and 3.2 c) tended to increase with heat and drought treatments. Early-heat resulted in significantly more frequent vein meeting ($p < 0.001$) and areoles ($p < 0.001$) in both leaves. Under the drought condition, vein meeting of both leaves increased significantly ($p < 0.05$). However, there was no significant change in areole number for either leaf 3 or 5 under drought.

Consistent with results from other species, leaf area was reduced when wild type *Arabidopsis* was exposed to heat or drought (Figure 3.3). The stress treatments had a significant effect on leaf area of both leaf 3 and leaf 5 (Table 3.1). Compared to control wild type, areas of both leaves 3 (Figure 3.1 d) and 5 (Figure 3.1 d) were reduced in all 4 treatments. Early-heat and late-heat had no significantly different effect on either leaf 3 or leaf 5 leaf area suggesting that both treatments coincided with the timing of leaf expansion for both leaves. Heat reduced leaf area to a greater extent than drought with early-heat showing stronger effects than drought for leaf 3, and both early and late-heat having a stronger effect than drought in leaf 5. In leaf 3, the combination of late-heat and drought did not result in significantly different area compared to either late-heat or drought alone, whereas for leaf 5 the combination showed significantly reduced area compared to drought but not to heat.

Stomatal density (SD) was taken from the abaxial side of the leaf. Although the treatments had a significant effect on SD for both leaf 3 and leaf 5 (Table 3.1), the change in SD was inconsistent between the two leaves. For leaf 3 it increased in response to early-heat ($p = 0.0011$) and late-heat ($p = 0.017$) only (Figure 3.4 a). In leaf 5, SD (Figure 3.4 b) showed little response to the treatments, with the only significant change being an increase in response to late-heat ($p = 0.007$).

The whole experiment was repeated to check for consistency and similar results were obtained from the repeated experiment (data not shown). Drought and late-heat together infrequently resulted in a significantly different response than each separately. Early-heat seemed to include the development periods of both leaf 3 and 5, thus, I chose only two treatments, early-heat and drought, for further experiments.

3.2 Effect of heat and drought on wild type and mutant genotypes of *Arabidopsis thaliana*

The wild type *Arabidopsis thaliana* forms a closed reticulum of veins formed by several vein orders with the smallest veins completing development as freely- ending veins. *unh1* was described as having reduced *Vd*, vein meetings and areole numbers (Pahari et al., 2014), *fkd1* had an open vein pattern with reduced vein meetings and areoles (Steynen and Schultz, 2003) and the quadruple mutant (*fkd1/fl1-2/fl2/fl3*) also had an open vein pattern with fewer vein meetings and areoles along with vascular islands (Prabhakaran Mariyamma et al., manuscript in preparation). In the mutants, the *Vd* was not evenly distributed through the whole leaf (Figure 1.4). For example, in *unh1* the venation was denser towards the base of the leaf than towards the middle and tip. As a result, the middle section will not be representative of the whole leaf. Thus, vein parameters were measured from the half-leaf.

Early-heat and drought treatments along with control were applied to wild type and *unh1*, *fkd1* and *fkd1/fl1-2/fl2/fl3* mutants of *Arabidopsis thaliana*. Soil moisture data was taken and was converted to volumetric water content from the raw values. The resulting moisture curves are plotted in Figure 3.5. Analysis of vein pattern (density, number of vein meetings, and number of areoles) was done from the half leaf. Two-way ANOVA was used to test the effect of two variables, genotype and treatment, on vein pattern, leaf area and SD. Thereafter, post hoc Tukey-Kramer test was done.

3.2.1 Vein pattern (vein density, vein meetings, and areoles)

In both leaves 3 and 5, treatment and genotype were found to affect *Vd*, vein meetings and areoles with the treatment and genotype showing a significant interaction effect (Tables 3.3 and 3.4) indicating that the genotypes affected the response of vein pattern to heat or drought stress ($p < 0.01$).

Under heat or drought, there was a significant increase in leaf *Vd* in wild type plants for both leaves ($p < 0.001$; Figures 3.6, 3.7 a and 3.8 a). The number of vein meetings and areoles in wild type leaf 3 significantly increased under heat or drought treatments (Figures 3.6, 3.7 b and 3.7 c) whereas in leaf 5 the increase in number of vein meetings and areoles was only significant after heat treatment ($p < 0.01$; Figures 3.6, 3.8 b and 3.8 c)

Under control conditions, *unh1* had a significantly lower *Vd* ($p < 0.05$; Figures 3.7 a and 3.8 a), vein meeting ($p < 0.001$; Figures 3.7 b and 3.8 b) and areole number ($p < 0.001$; Figures 3.7 c and 3.8 c) for both leaves than other genotypes. The *unh1 Vd* increased significantly under heat for leaf 3 ($p < 0.01$; Figure 3.7 a) and under both heat and drought for leaf 5 ($p < 0.001$; Figure 3.8 a). *unh1* showed a significant change in vein meetings and areole number under heat or drought treatments in both leaf 3 or 5 (Figures 3.7 b and c and 3.8 b and c).

The *Vd* of wild type and *fkd1* was similar in leaf 3 (Figure 3.7 a) and leaf 5 (Figure 3.8 a) under control condition whereas the number of areoles and vein meetings was significantly lower in *fkd1* (Figures 3.7 b and c and 3.8 b and c). The *fkd1* did not change *Vd* under either heat or drought in leaf 3 (Figure 3.7 a), or under drought for leaf 5 (Figure 3.8 a), but there was a significant increase in *Vd* under heat for leaf 5 (Figure 3.8 a). *fkd1* did not change its number of vein meetings and areoles under heat or drought in either leaf 3 or 5 (Figures 3.7 b and c and 3.8 b and c).

Both leaves of the quadruple mutant (*fkd1/fl1-2/fl2/fl3*) had a similar *Vd* as the wild type under control condition, but, like *fkd1*, had significantly fewer vein meetings and areoles (Figures 3.7 b and c and 3.8 b and c). Under heat, leaf 3 of the quadruple mutant had a significantly higher *Vd* ($p < 0.001$, Figure 3.7 a) but the vein density did not change in leaf 5 (Figure 3.8 a). The quadruple mutant did not change number of vein meetings or

areole numbers in leaf 5 (Figures 3.6, 3.8 b and c), but in leaf 3, there was a significant increase in the number of vein meetings ($p < 0.01$, Figure 3.7 b) and a trend towards increased number of areoles (statistically non-significant, $p > 0.05$, Figure 3.7 c) under heat.

The mutants showed some statistically significant changes in *Vd* (*unh1* leaf 3 under heat, quadruple mutant leaf 3 under heat, *unh1* leaf 5 under heat and drought, *fkd1* leaf 5 under heat) but the percentage change in *Vd* compared to the control was usually less than in wild type (Table 3.2). In both wild type leaves, the drought or heat-induced *Vd* increases were accompanied by a significant increase in both the number of vein meetings and areole per unit leaf area. However, in all mutants, any drought and heat- induced changes in *Vd* were not accompanied by a significant increase in both the number of vein meetings and areole per unit leaf area, the exception being the *fkd1/fl1-2/fl2/fl3* leaf 3 under heat. Vein meeting and areole number were more correlated with *Vd* in wild type but less correlated in the mutants (Figure 3.9). Thus, in the mutants, when the *Vd* increased, it did so without an increase in number of vein meetings or areoles, presumably through extending the veins.

3.2.2 Leaf area and stomatal density

While both treatments and genotypes affected leaf area, there was no interaction between genotypes and treatments indicating that the genotype did not affect the response of leaf area to heat or drought (Table 3.3 and 3.4). There was a significant decrease in leaf area under heat for wild type in leaf 5. Surprisingly, although there was a trend towards decreased leaf area in wild type under drought for leaf 3 (Figure 3.7 d) and under heat and drought for leaf 5 (Figure 3.8 d), the differences were not statistically significant. *unh1* did not show any significant decrease in leaf area for either leaf (Figures 3.7 d and 3.8 d). *fkd1* showed a significant decrease in leaf area under heat in leaf 5 but not in leaf 3 and decreased leaf area significantly under drought for leaf 3 but not for leaf 5 (Figures 3.7 d and 3.8 d).

fkd1/fl1-2/fl2/fl3 had a significant decrease in leaf area under heat but not drought for both leaves (Figures 3.7 d and 3.8 d).

The stomatal density (SD) was taken from both the abaxial and the adaxial sides of the leaf (Figures 3.10 – 3.13). Genotype affected SD of abaxial and adaxial sides and there was an interaction between genotypes and treatments (Tables 3.3 and 3.4). However, treatments had no effect on SD in leaf 5 while they had an effect in leaf 3.

Generally, the wild type and mutants all showed a trend towards decreased SD under heat and increased SD under drought on both surfaces of both leaves. The wild type *Arabidopsis thaliana* showed a significant increase in SD under drought on both sides of leaf for leaf 3 (Figures 3.14 a and b) while the increase was not significant for leaf 5 (Figures 3.14 c and d). On the adaxial side of wild type leaf 3, the SD decreased significantly under heat, but the decrease was not statistically significant on the abaxial side, nor was it significant for either abaxial or adaxial sides of leaf 5. *unh1* had significantly higher SD under control conditions for both abaxial and adaxial sides in both leaves (Figure 3.14). While *unh1* did not significantly change SD under heat or drought on the abaxial leaf side for both leaves (Figures 3.14 b and d) or on the adaxial side for leaf 5 (Figure 3.14 c), there was a significant decrease in SD under heat on the adaxial side of leaf 3 (Figure 3.14 a). The SD of *fkd1* was increased significantly under drought for both sides of both leaves, and was decreased significantly under heat for the abaxial side of leaf 3 (Figure 3.14 b). Finally, the *fkd1/fl1-2/fl2/fl3* mutant did not change SD in leaf 3 (Figures 3.14 a and b) but there was a significant decrease in SD under drought on both surfaces of leaf 5 (Figures 3.14 c and d).

3.3 Response of wild type and *fkd1 Arabidopsis thaliana* to ABA

I was interested to know if ABA changed vein patterns as it affects other phenotypic characters that respond to stress. Because the effect of ABA on *Arabidopsis thaliana* leaf morphology has not been assessed, I chose ABA concentrations based on previous experiments described by Xing and Rajashekar (2001) and Finkelstein (2013). Two methodologies of ABA application had been used, one spraying directly on plants and the other adding ABA to the plates. To most closely mimic the growth conditions in the heat and drought treatment, I chose to spray ABA on soil grown plants. I chose the concentrations (5 mM and 1 mM ABA) where the ABA had been sprayed directly on older plants (Xing and Rajashekar, 2001; Finkelstein, 2013) and began by spraying plants for the growth period of day 7 to day 18. Under this treatment, the plants stopped growing shortly after ABA was applied (Figure 3.15 b), and I was unable to take any measurements of leaf 3 or 5 characteristics. Therefore, in the next experiment, I decreased the ABA concentration to 1 mM and reduced the spraying to only on day 10 or to a period similar to late-heat treatment (day 10 to 14). For the day 10 to 14 treatment, while an increase in *Vd* was observed, leaf area was more reduced than what was observed under heat or drought experiments (data not shown). The single application of ABA had no effect (data not shown).

I next applied ABA to wild type and *fkd1* plants at lower concentrations (0.001 mM, 0.01 mM and 0.05 mM) each day from day 10 to 14. Leaves were harvested at day 24 and day 27 and analyzed as for heat and drought treatments. A two-way ANOVA revealed a significant effect of ABA on *Vd* with a significant interaction between genotype and treatment suggesting that *fkd1* affects the response to ABA (Table 3.5).

Among the ABA concentrations, 0.05 mM ABA resulted in a significant decrease in leaf area ($p < 0.01$) for both genotypes in both leaves (Figures 3.16 d and 3.17 d) and showed a significant increase in leaf *Vd* ($p < 0.01$) in both genotypes on leaf 5 (Figure 3.17 a). Increase in *Vd* in leaf 5 of wild type was accompanied by an increase in number of vein meetings per unit area ($p < 0.001$, Figure 3.17 b) and an increase in areole number per unit area ($p < 0.001$, Figure 3.17 c). Similarly, increase in *Vd* in *fkd1* was accompanied by an increase in number of vein meetings per unit area ($p < 0.05$, Figure 3.17 b) but there was no change in number of areoles per unit area (Figure 3.17 b).

3.4 Effect of heat or drought treatments on photosynthetic gas exchange characteristic

To assess photosynthetic gas exchange from different genotypes under heat or drought treatments, $\delta^{13}C$ (‰) and nitrogen (%) values were obtained. Unfortunately, the $\delta^{13}C$ and N% for samples of *unh1* and *fkd1/fl1-2/fl2/fl3* subjected to heat or drought were lost. A two-way ANOVA of drought and heat treatments on wild type and *fkd1* for $\delta^{13}C$ indicated a significant effect of treatments on the $\delta^{13}C$ (‰) value (Table 3.6). *fkd1* showed statistically higher $\delta^{13}C$ content compared to wild type when all treatments were included in the comparison (Table 3.6). However, there was no interaction effect between the treatments and the genotypes (Table 3.6). There was a significant increase in $\delta^{13}C$ value under drought for both wild type and *fkd1* ($p < 0.05$), whereas the change was non-significant for heat treatment in both genotypes (Table 3.7).

The mean %N is 60% higher in wild type under drought than in *fkd1* under drought but the difference was not statistically significant, perhaps because of the small sample size. Although the %N was increased in both genotypes, because the total biomass was decreased the %N may not reflect the total amount of N assimilated at the whole plant level.

To calculate the total plant nitrogen, I multiplied the total dry weight (mg) of plant shoot by %N (mg N / 100 mg dry weight) to obtain mg N per plant shoot. Total plant shoot nitrogen content was not significantly different between wild type and the *fkd1* (Table 3.6). There was a significant effect of treatment and also a significant interaction effect between the treatments and the genotypes (Table 3.6), indicating that total plant shoot nitrogen of wild type and *fkd1* were responding differently to the treatments. There was a significant increase in plant nitrogen content under drought for wild type ($p < 0.05$) but there was no change in nitrogen content under drought for *fkd1* ($p = 0.97$). The change in total plant shoot nitrogen content was non-significant for heat treatment in both the wild type and *fkd1*.

Table 3.1: One-way Analysis of Variance (ANOVA) between leaf parameters and stress (heat or drought) treatments in leaf 3 and leaf 5 of wild type *Arabidopsis thaliana.*

Leaf parameter	Source of variation	Degree of freedom (Df)	Sum of squares (SS)	Mean sum of squares (MSS)	F value	*p* value
Leaf 3						
Vein density (mm/mm^2)	Treatment	4	7.354	1.838	18.54	0.000
	Residuals	65	6.445	0.099		
Number of vein meeting per mm^2	Treatment	4	33.73	8.431	18.17	0.000
	Residuals	65	30.15	0.464		
Areole number per mm^2	Treatment	4	10.22	2.554	11.68	0.000
	Residuals	65	14.21	0.219		
Leaf area (mm^2)	Treatment	4	37005	9251	27.91	0.000
	Residuals	65	21547	331		
Stomatal density (no. per mm^2)	Treatment	4	19755	4939	8.336	0.000
	Residuals	45	26661	592		
Leaf 5						
Vein density (mm/mm^2)	Treatment	4	10.333	2.583	18.82	0.000
	Residuals	65	8.933	0.1374		
Number of vein meeting per mm^2	Treatment	4	47.59	11.898	13.3	0.000
	Residuals	65	58.14	0.894		
Areole number per mm^2	Treatment	4	14.72	3.681	10.72	0.000
	Residuals	65	22.32	0.343		
Leaf area (mm^2)	Treatment	4	172715	43479	72.63	0.000
	Residuals	65	38644	595		
Stomatal density (no. per mm^2)	Treatment	4	55231	13808	6.861	0.0002
	Residuals	45	90569	2013		

Table 3.2: Vein density and leaf area (n = 15, Mean ± Standard Error) and percentage change in *Vd* and leaf area with respect to control treatment for wild type and mutants of *Arabidopsis thaliana*. Asterisk (*) symbol marks significant difference within the same genotype under heat or drought where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ (Tukey- Kramer test).

Genotype	Treatment	*Vd* ± SE (mm/mm^2)	Change in *Vd* with respect to control (%)	Leaf area ± SE (mm^2)	Change in leaf area with respect to control (%)
Leaf 3					
Col-0	Control	2.3 ± 0.06		81.3 ± 7.82	
	Heat	2.8 ± 0.07***	21.7	61.6 ± 6.93	-24.23
	Drought	2.8 ± 0.07***	21.7	80.5 ± 6.81	-0.98
unh1	Control	1.8 ± 0.05		34.3 ± 3.53	
	Heat	2.3 ± 0.11**	27.8	16.5 ± 1.45	-51.90
	Drought	2.1 ± 0.06	16.7	22.8 ± 1.95	-33.53
fkd1	Control	2.2 ± 0.06		83.8 ± 8.20	
	Heat	2.3 ± 0.05	4.5	58.8 ± 3.35	-29.83
	Drought	2.2 ± 0.06	0.0	55.2 ± 3.24*	-34.13
fkd1/fl1-2/	Control	2.3 ± 0.04		74.2 ± 7.31	
fl2/fl3	Heat	3.1 ± 0.09***	34.8	42.6 ± 3.44**	-42.51
	Drought	2.4 ± 0.05	4.3	69.0 ± 6.20	-7.01
Leaf 5					
Col-0	Control	2.5 ± 0.07		166.8 ±13.80	
	Heat	3.5 ± 0.13***	40.0	87.9 ± 7.01***	-47.30
	Drought	3.1 ± 0.08***	24.0	133.8 ± 7.36	-19.78
unh1	Control	2.2 ± 0.05		66.8 ± 7.43	
	Heat	2.7 ± 0.06***	22.7	36.9 ± 7.76	-44.76
	Drought	2.7 ± 0.06***	22.7	44.8 ± 3.05	-32.93
fkd1	Control	2.6 ± 0.05		146.7 ± 11.86	
	Heat	3.0 ± 0.07**	15.4	88.3 ± 6.06***	-39.81
	Drought	2.9 ± 0.04	11.5	109.2 ± 6.34	-25.63
fkd1/fl1-2/	Control	2.7 ± 0.06		109.7 ± 11.80	
fl2/fl3	Heat	3.0 ± 0.11	11.1	63.3 ± 5.18**	-42.30
	Drought	3.1 ± 0.06	14.8	88.9 ± 6.50	-18.96

Table 3.3: Two-way Analysis of Variance (ANOVA) between leaf parameters and stress (heat or drought) treatments in leaf 3 of wild type and mutant genotype of *Arabidopsis thaliana*.

Leaf parameter	Source of variation	Degree of freedom (Df)	Sum of squares (SS)	Mean sum of squares (MSS)	F value	*p* value
Leaf 3						
Vein density (mm/mm^2)	Treatment	2	7.107	3.553	51.899	0.000
	Genotype	3	10.313	3.438	50.211	0.000
	Treatment*Genotype	6	3.268	0.545	7.955	0.000
	Residuals	168				
Number of vein meeting per mm^2	Treatment	2	3.7	1.849	23.725	0.000
	Genotype	3	40.96	13.652	175.17	0.000
	Treatment*Genotype	6	2.86	0.477	6.118	0.000
	Residuals	168	13.09	0.078		
Areole number per mm^2	Treatment	2	0.437	0.219	13.939	0.000
	Genotype	3	19.419	6.471	412.428	0.000
	Treatment*Genotype	6	0.669	0.111	7.105	0.000
	Residuals	168	2.636	0.016		
Leaf area (mm^2)	Treatment	2	16568	8284	18.084	0.000
	Genotype	3	65820	21940	47.896	0.000
	Treatment*Genotype	6	5434	906	1.977	0.0716
	Residuals	168	76957	458		
Adaxial stomatal density (no. per mm^2)	Treatment	2	93635	46817	92.279	0.000
	Genotype	3	125907	41969	82.722	0.000
	Treatment*Genotype	6	14166	2361	4.654	0.0002
	Residuals	132	66970	507		
Abaxial stomatal density (no. per mm^2)	Treatment	2	62621	31311	47.851	0.000
	Genotype	3	105178	35059	53.58	0.000
	Treatment*Genotype	6	14664	2444	3.735	0.0018
	Residuals	132	86372	654		

Table 3.4: Two-way Analysis of Variance (ANOVA) between leaf parameters and stress (heat or drought) treatments in leaf 5 of wild type and mutant genotype of *Arabidopsis thaliana*.

Leaf parameter	Source of variation	Degree of freedom (Df)	Sum of squares (SS)	Mean sum of squares (MSS)	F value	*p* value
Leaf 5						
Vein density (mm/mm^2)	Treatment	2	11.078	5.539	67.298	0.000
	Genotype	3	7.062	2.354	28.602	0.000
	Treatment*Genotype	6	1.915	0.319	3.878	0.0012
	Residuals	168	13.827	0.082		
Number of vein meeting per mm^2	Treatment	2	8.91	4.453	24.807	0.000
	Genotype	3	68.76	22.919	127.665	0.000
	Treatment*Genotype	6	7.27	1.212	6.753	0.000
	Residuals	168	30.16	0.18		
Areole number per mm^2	Treatment	2	1.37	0.684	15.101	0.000
	Genotype	3	32.81	10.936	241.529	0.000
	Treatment*Genotype	6	1.12	0.186	4.108	0.000
	Residuals	168	7.61	0.045		
Leaf area (mm^2)	Treatment	2	85560	42780	40.539	0.000
	Genotype	3	166900	55633	52.719	0.000
	Treatment*Genotype	6	11120	1853	1.756	0.111
	Residuals	168	177288	1055		
Adaxial stomatal density (no. per mm^2)	Treatment	2	881	4405	2.832	0.0625
	Genotype	3	263128	87709	56.380	0.000
	Treatment*Genotype	6	103862	17310	11.127	0.000
	Residuals	132	205349	1556		
Abaxial stomatal density (no. per mm^2)	Treatment	2	49569	24784	15.79	0.000
	Genotype	3	52963	17654	11.25	0.000
	Treatment*Genotype	6	136418	22736	14.49	0.000
	Residuals	132	207140	1569		

Table 3.5: Two-way Analysis of Variance (ANOVA) between leaf parameters and ABA treatments (0.001mM, 0.01mM and 0.05mM concentration of ABA) in leaf 3 and leaf 5 of wild type and *fkd1 Arabidopsis thaliana*.

Leaf parameter	Source of variation	Degree of freedom (Df)	Sum of squares (SS)	Mean sum of squares (MSS)	F value	*p* value
Leaf 3						
Vein density (mm/mm^2)	Treatment	3	0.893	0.2977	2.866	0.041
	Genotype	1	0.243	0.2433	2.342	0.129
	Treatment*Genotype	3	1.403	0.4677	4.503	0.0054
	Residuals	88	9.139	0.1039		
Number of vein meeting per mm^2	Treatment	3	1.91	0.64	2.21	0.0925
	Genotype	1	32.49	32.49	113	0.000
	Treatment*Genotype	3	1.24	0.41	1.442	0.2361
	Residuals	88	25.3	0.29		
Areole number per mm^2	Treatment	3	0.39	0.13	2.34	0.0788
	Genotype	1	15.549	15.549	279.744	0.000
	Treatment*Genotype	3	0.036	0.012	0.217	0.885
	Residuals	88	4.891	0.056		
Leaf area (mm^2)	Treatment	3	79762	26587	35.22	0.000
	Genotype	1	1233	1233	1.634	0.2046
	Treatment*Genotype	3	7454	2485	3.291	0.0243
	Residuals	88	66430	755		
Leaf 5						
Vein density (mm/mm^2)	Treatment	3	16.809	5.603	33.87	0.000
	Genotype	1	0.749	0.749	4.527	0.036
	Treatment*Genotype	3	5.221	1.74	10.521	0.000
	Residuals	88	14.558	0.165		
Number of vein meeting per mm^2	Treatment	3	95.01	31.67	60.33	0.000
	Genotype	1	63.91	63.91	121.75	0.000
	Treatment*Genotype	3	43.62	14.54	27.7	0.000
	Residuals	88	46.2	0.52		
Areole number per mm^2	Treatment	3	26.26	8.75	34.36	0.000
	Genotype	1	37.8	37.8	148.390	0.000
	Treatment*Genotype	3	21.67	7.22	28.36	0.000
	Residuals	88	22.42	0.25		
Leaf area (mm^2)	Treatment	3	347291	115764	47.704	0.000
	Genotype	1	6020	6020	2.221	0.14
	Treatment*Genotype	3	27418	9139	3.371	0.022
	Residuals	88	238552	2711		

Table 3.6: Two-way Analysis of Variance (ANOVA) between $\delta^{13}C$ ‰) and mg of nitrogen and stress (heat or drought) treatments in wild type and *fkd1* mutant of *Arabidopsis thaliana***.**

Parameters	Source of variation	Degree of freedom (Df)	Sum of squares (SS)	Mean sum of squares (MSS)	F value	*p* value
$\delta^{13}C$ (‰)	Treatment	2	6.492	3.246	14.243	0.000
	Genotype	1	1.993	1.993	8.743	0.006
	Treatment*Genotype	2	0.199	0.099	0.436	0.65
	Residuals	30	6.838	0.228		
mg N per plant	Treatment	2	271878	135939	11.841	0.000
	Genotype	1	34844	34844	3.035	0.092
	Treatment*Genotype	2	166775	83387	7.264	0.0027
	Residuals	30	344408	11480		

Table 3.7: Carbon isotope ratio ($\delta^{13}C$ ‰), dry weight of plants in milligram (mg), percentage nitrogen (N) content and mg of nitrogen per plant (n = 6, Mean ± SE) of wild type and *fkd1* mutant of *Arabidopsis thaliana*. Asterisk (*) symbol indicates significant difference within the same genotype between the control and heat or drought where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. The diamond (◊) symbol marks significant difference between the wild type and the *fkd1* under control, heat or drought where ◊ indicates $p < 0.05$, ◊◊ indicates $p < 0.01$ and ◊◊◊ indicates $p < 0.001$ (Tukey-Kramer test).

Genotype	Treatment	$\delta^{13}C$ (‰)	shoot dry weight (mg)	% N	mg N per shoot
Col-0	Control	-31.2 ± 0.24	30.7 ± 3.53	0.9 ± 0.12	0.3 ± 0.04
	Heat	-31.0 ± 0.10	14 ± 1.22***	0.7 ± 0.03	0.1 ± 0.01
	Drought	-30.3 ± 0.18*	22.1 ± 1.94	2.1 ± 0.17**	0.5 ± 0.07*
fkd1	Control	-30.9 ± 0.21	31.9 ± 3.58	0.9 ± 0.16	0.3 ± 0.03
	Heat	-30.4 ± 0.12	14.7 ± 1.63***	1.1 ± 0.13	0.2 ± 0.03
	Drought	-29.7 ± 0.27**	13.2 ± 1.33***	1.6 ± 0.41	0.2 ± 0.06 ◊◊

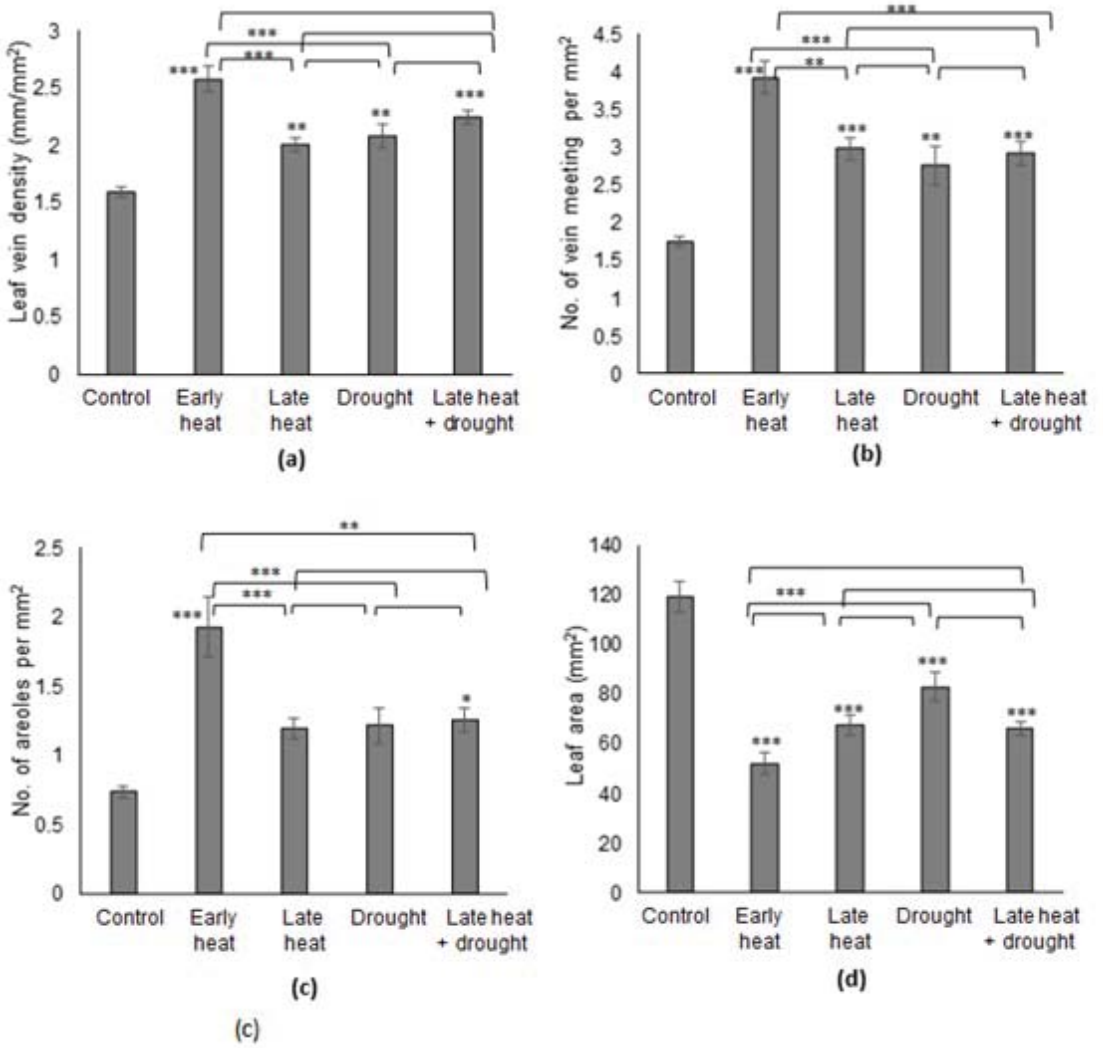


Figure 3.1: Response of leaf 3 characteristics of wild type *Arabidopsis thaliana* to heat or drought treatments. Leaf vein density (a), number of vein meeting per mm^2 (b), areole number per mm^2 (c) were measured from the mid-section of cleared leaves harvested on day 24 (Mean ± SE, n = 14). Area (d) of whole cleared leaves were measured using ImageJ (Mean ± SE, n = 14. Asterisk above error bars indicates significant difference from control. Asterisk above bracket indicates significant differences between treatments. Level of significance are denoted by the asterisk (*) symbol where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ (Tukey- Kramer test).

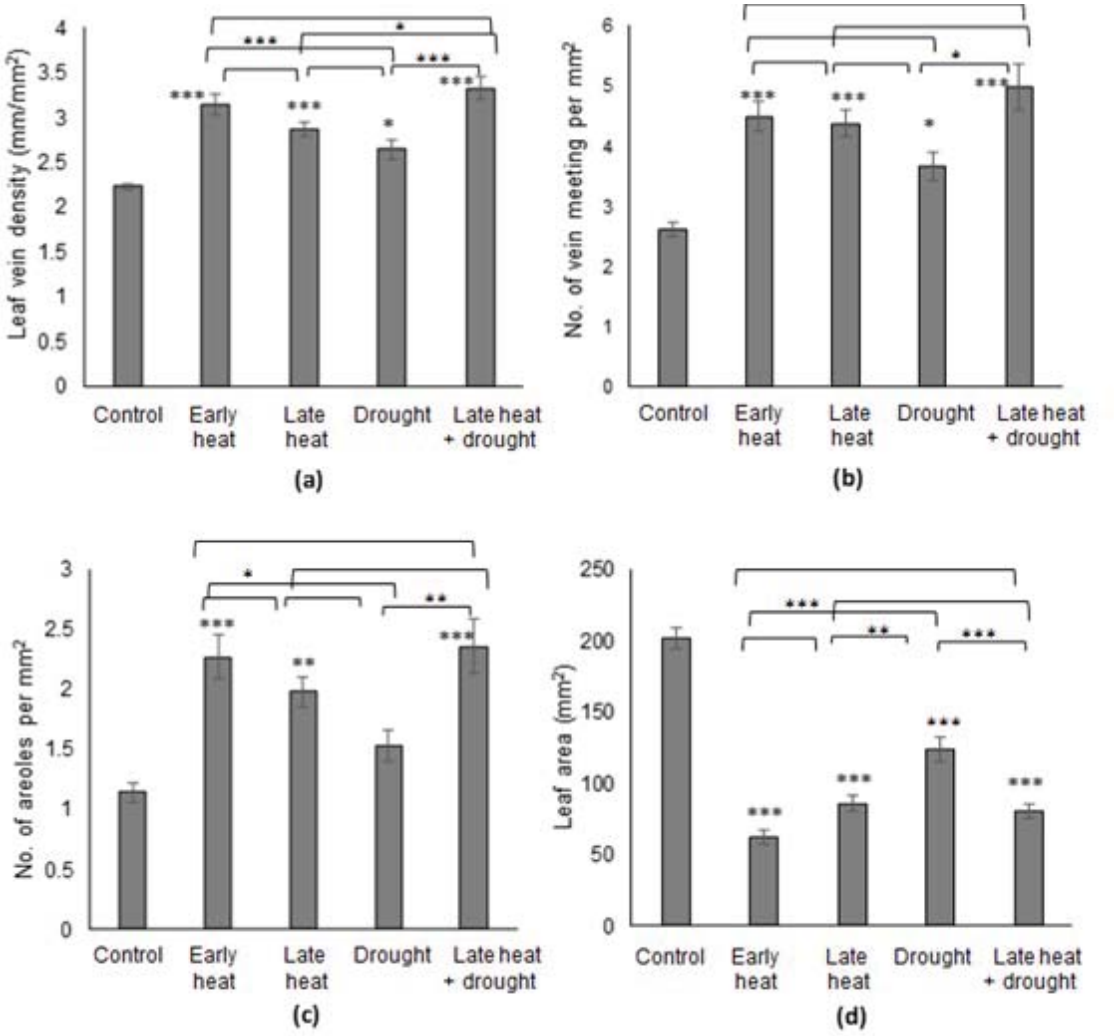


Figure 3.2: Response of leaf 5 characteristics of wild type *Arabidopsis thaliana* to heat or drought treatments. Leaf vein density (a), number of vein meeting per mm^2 (b), areole number per mm^2 (c) were measured from the mid-section of cleared leaves harvested on day 27 (Mean ± SE, n = 14). Area (d) of whole cleared leaves were measured using ImageJ (Mean ± SE, n = 14. Asterisk above error bars indicates significant difference from control. Asterisk above bracket indicates significant differences between treatments. Level of significance are denoted by the asterisk (*) symbol where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ (Tukey- Kramer test).

Figure 3.3: Day 18 plants of *Arabidopsis thaliana* grown under different conditions. (a) is the control with 25^{0}C and watered every other day. (b) is the early-heat treatment with 30^{0}C from day 7 to11 and watered every other day. (c) is late-heat with 30^{0}C from day 10 to 14 and watered every other day. (d) is the drought treatment which was kept at 25^{0}C and watered every day until day 10 after which watering was done once a week. (e) is the combination of late-heat and drought treatment.

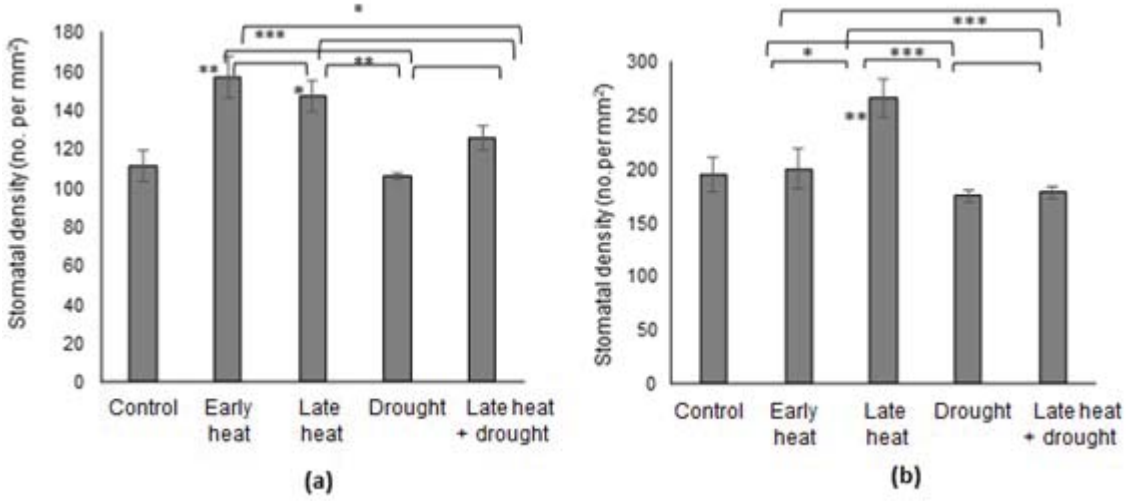


Figure 3.4: Response of stomatal density of leaf 3 (a) and leaf 5 (b) of wild type *Arabidopsis thaliana* to heat or drought treatments. Stomatal density was measured from an area of 0.6 mm^2 cast of abaxial surface of leaves (Mean ± SE, n = 10). Asterisk above error bars indicates significant difference from control. Asterisk above bracket indicates significant differences between treatments. Level of significance are denoted by the asterisk (*) symbol where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ (Tukey-Kramer test).

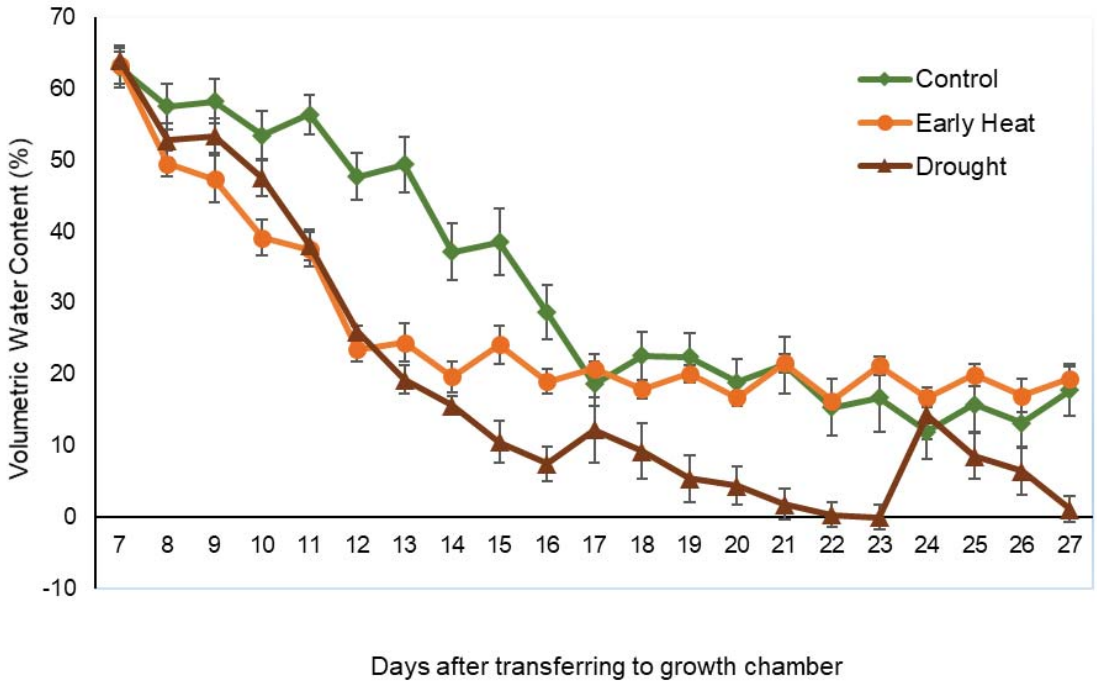


Figure 3.5: Volumetric moisture content of the soil for mutants and wild type exposed to early-heat and drought (Mean ± SE, n = 4). EC-5 moisture sensor probes were put into soil of wild type and mutants randomly and raw soil moisture data reading was taken from Decagon hand held moisture on a daily basis. These raw values were converted to volumetric moisture content (%) according to manufacturers' instruction for calibration method from https://www.decagon.com/en/support/videos/soil-calibration-video/ .

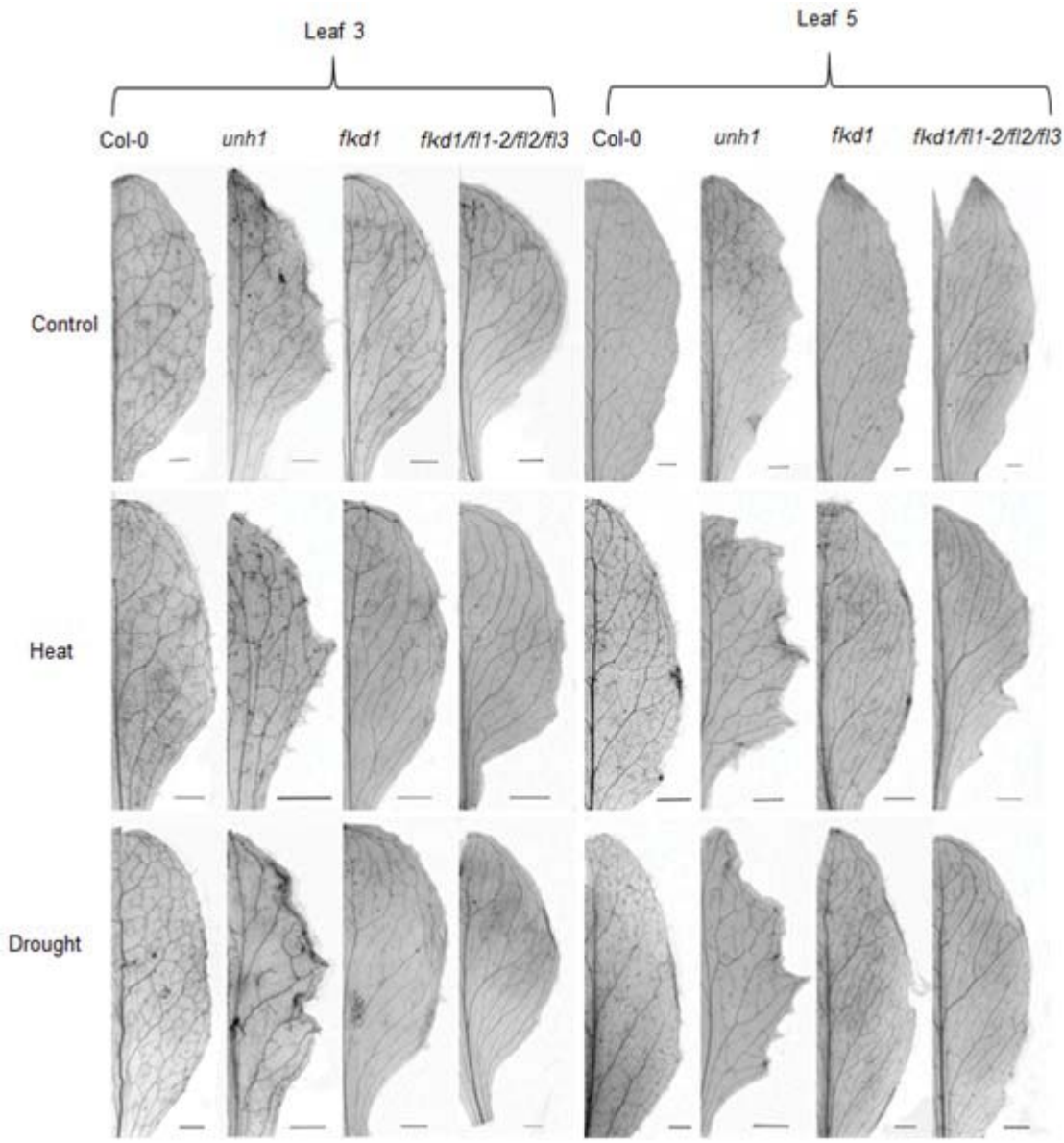


Figure 3.6: Third and fifth leaves of different genotypes of *Arabidopsis thaliana* grown under control, heat or drought. The first four columns of leaves are leaf 3 followed by four columns of leaf 5. First column of each of leaf 3 and 5 is the wild type (Col-0), second column is the *unh1* mutant, third column is the *fkd1* mutant and the fourth column is the quadruple mutant (*fkd1/fl1-2/fl2/fl3*). The leaves on the first row are the controls, second row is heat treated and third row is drought treated. Leaf 3 was harvested at day 24 and leaf 5 at day 27. Scale = 1mm.

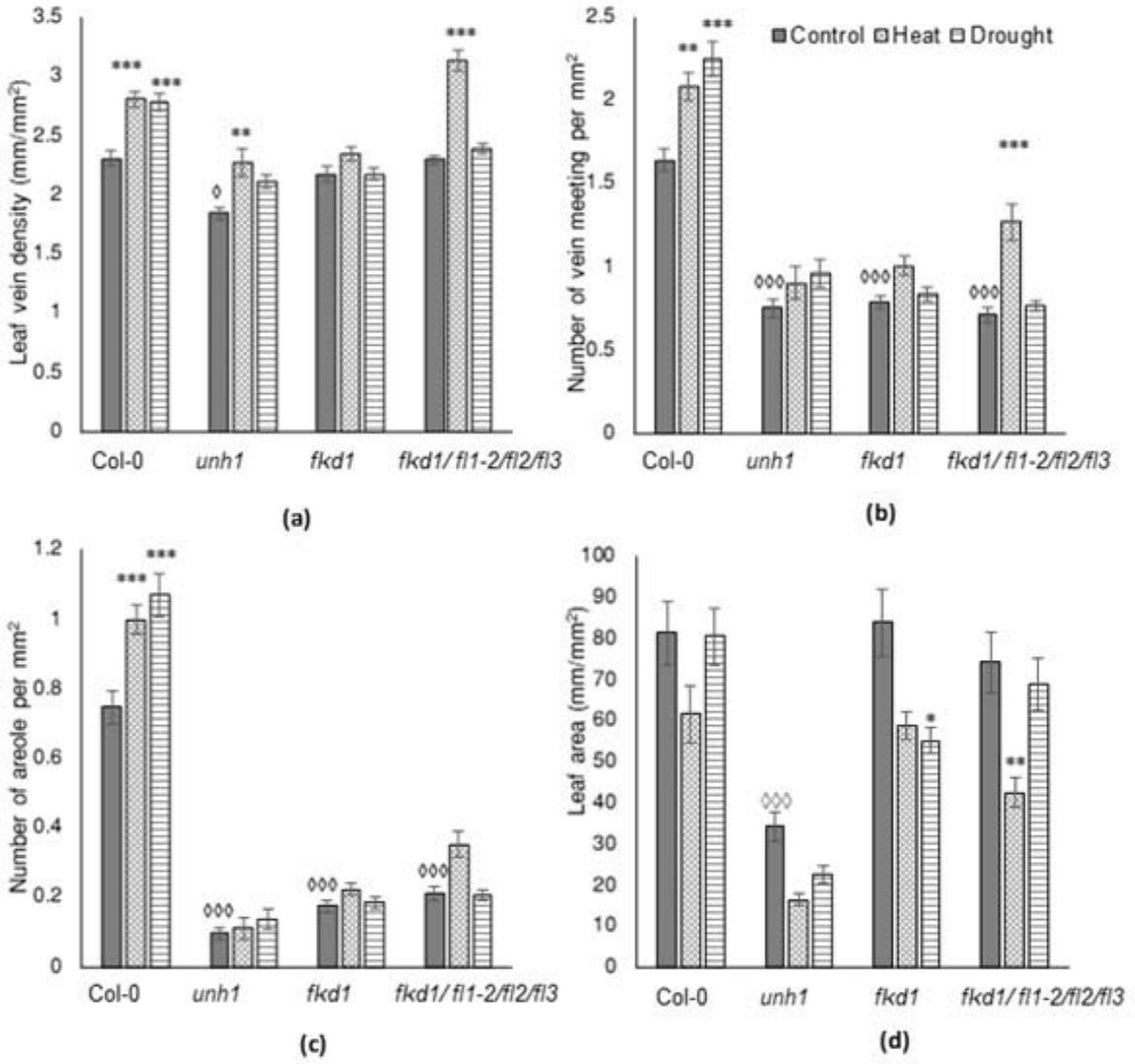


Figure 3.7: Response of characteristics of leaf 3 of wild type and mutant *Arabidopsis thaliana* to early-heat or drought treatments. Leaf vein density (a), number of vein meeting per mm^2 (b), areole number per mm^2 (c) were measured from the cleared half leaves harvested on day 25 (Mean ± SE, n = 15). Area (d) of whole cleared leaves were measured using ImageJ (n = 15, Mean ± SE). Asterisk (*) symbol indicates significant difference within the same genotype between the control and heat or drought where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. The diamond (◊) symbol marks significant difference between the wild type and the mutants under control condition where ◊ indicates $p < 0.05$, ◊◊ indicates $p < 0.01$ and ◊◊◊ indicates $p < 0.001$ (Tukey-Kramer test).

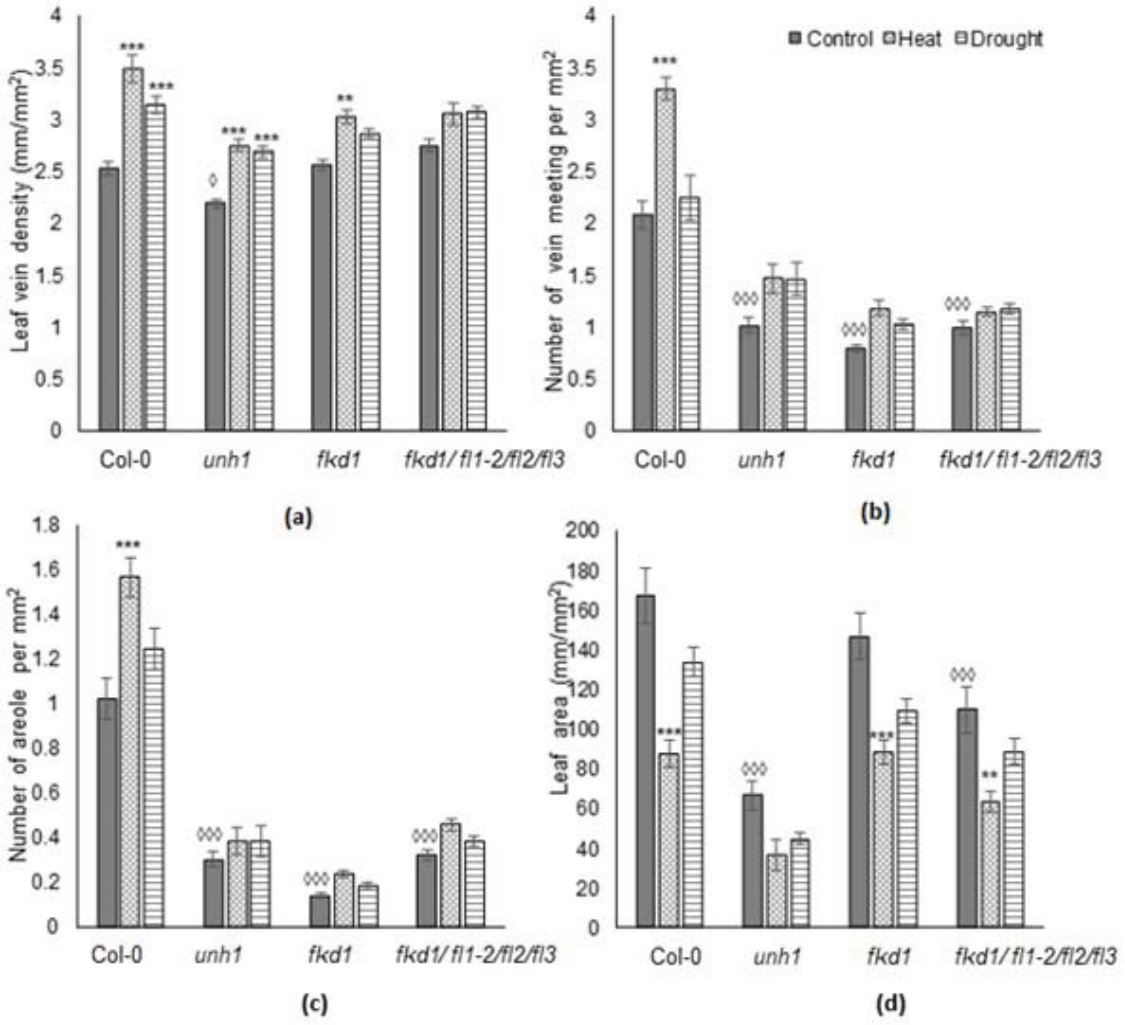


Figure 3.8: Response of characteristics of leaf 5 of wild type and mutant *Arabidopsis thaliana* to early-heat or drought treatments. Leaf vein density (a), number of vein meeting per mm^2 (b), areole number per mm^2 (c) were measured from the cleared half leaves harvested on day 27 (Mean ± SE, n = 15). Area (d) of whole cleared leaves were measured using ImageJ (n = 15, Mean ± SE). Asterisk (*) symbol indicates significant difference within the same genotype between the control and heat or drought where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. The diamond (◊) symbol marks significant difference between the wild type and the mutants under control condition where ◊ indicates $p < 0.05$, ◊◊ indicates $p < 0.01$ and ◊◊◊ indicates $p < 0.001$ (Tukey-Kramer test).

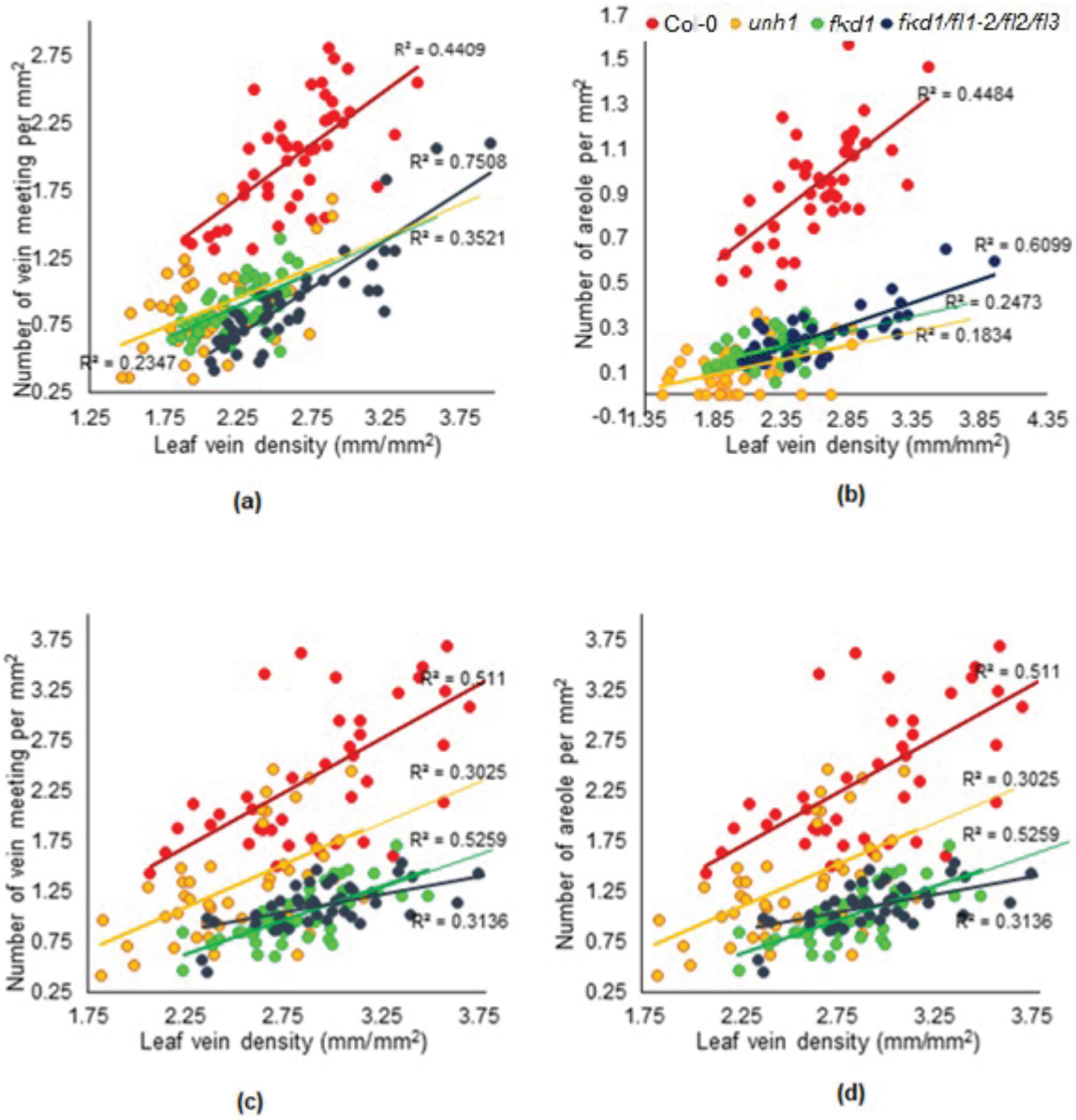


Figure 3.9: Correlation between *Vd* and vein meetings and *Vd* and areole numbers for leaf 3 (a, b) and leaf 5 (c, d). The graphs were made in Microsoft Excel© and line of best fit was put with R^2 values obtained from Microsoft Excel©.

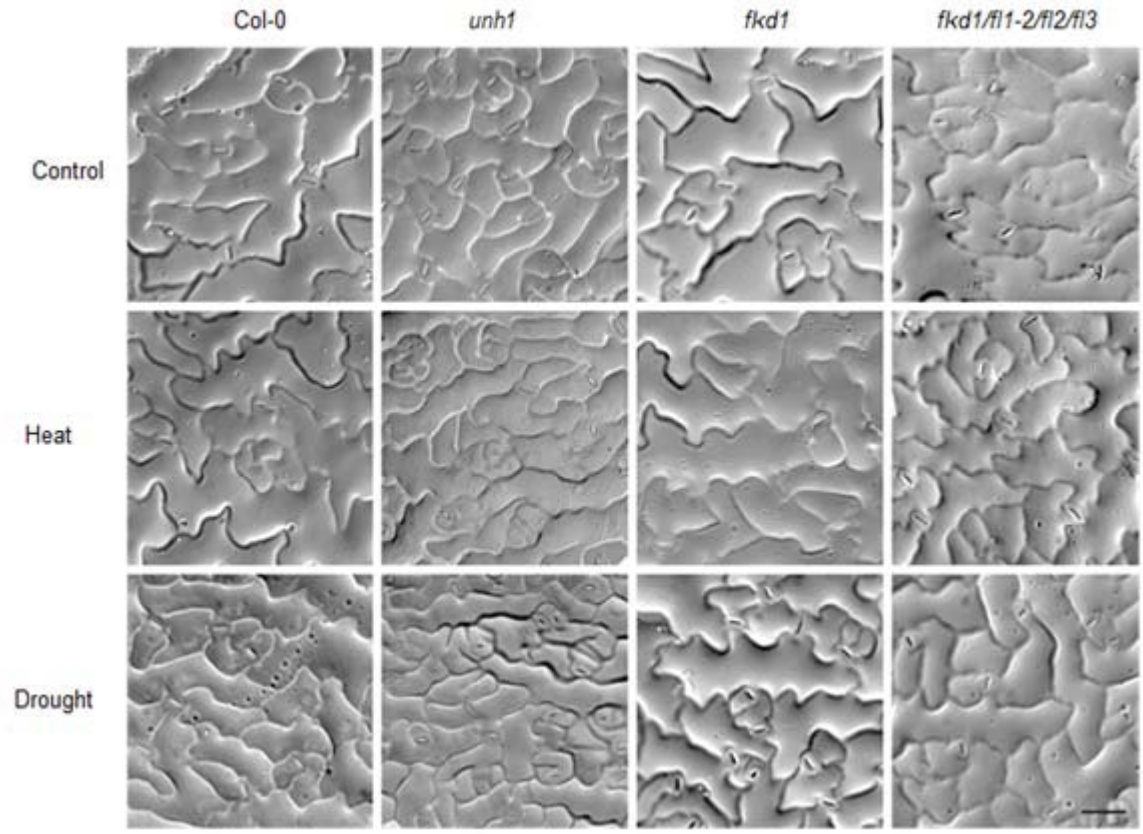


Figure 3.10: Stomata from the adaxial leaf side of leaf 3 from different genotypes grown under control, early-heat and drought. The first column is the wild type, the second column *unh1*, the third column of cells are the *fkd1* mutant and the last column is the quadruple mutant. The first row is the control, second row is the heat and the third row is the drought treatment. Casts were taken from the leaves and nail polish peels of casts were viewed under a compound microscope. Scale = 0.05 mm

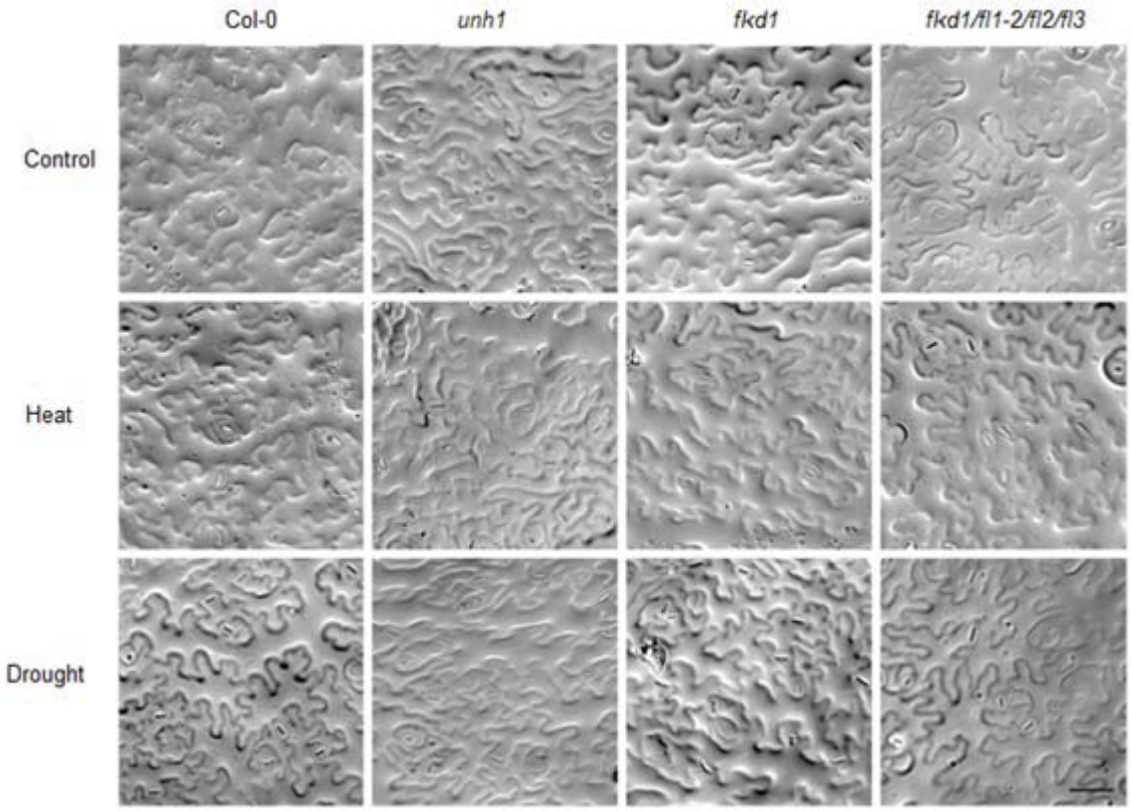


Figure 3.11: Stomata from the abaxial leaf side of leaf 3 from different genotypes grown under control, early-heat and drought. The first column is the wild type, the second column *unh1*, the third column of cells are the *fkd1* mutant and the last column is the quadruple mutant. The first row is the control, second row is the heat and the third row is the drought treatment. Casts were taken from the leaves and nail polish peels of casts were viewed under a compound microscope. Scale = 0.05 mm.

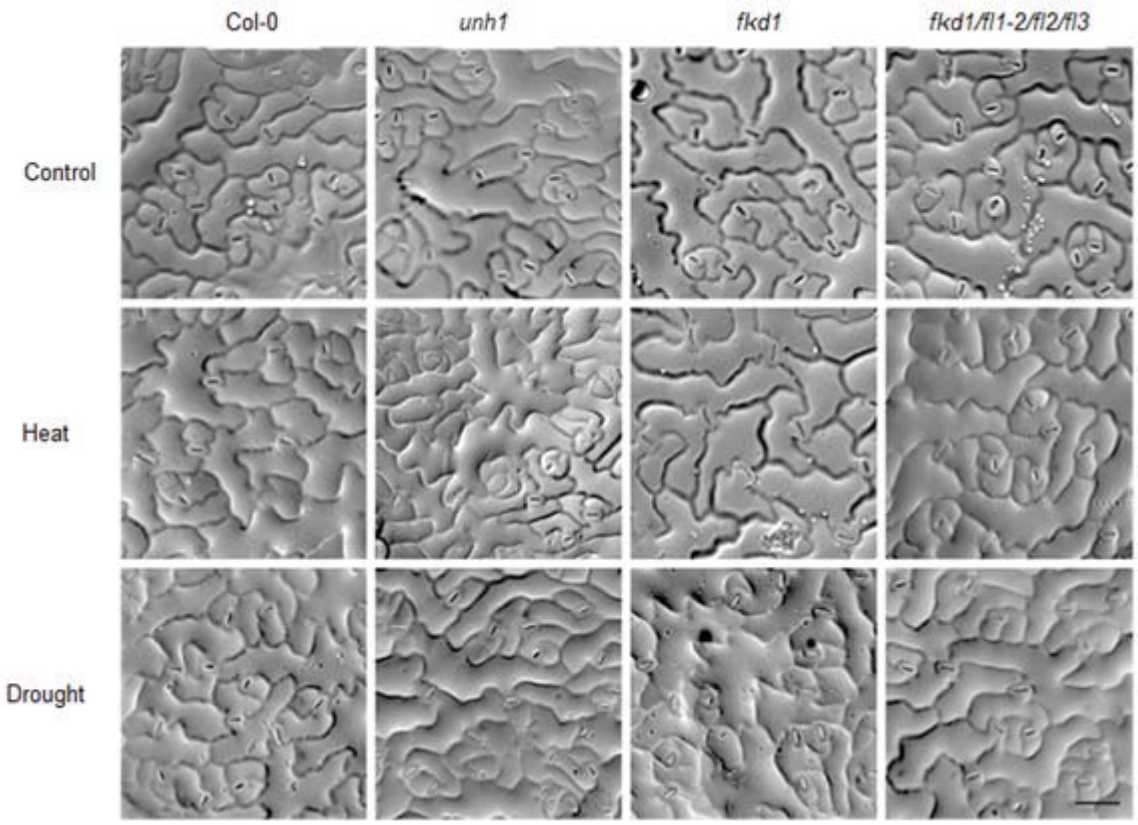


Figure 3.12: Stomata from the adaxial leaf side of leaf 5 from different genotypes grown under control, early-heat and drought. The first column is the wild type, the second column *unh1*, the third column of cells are the *fkd1* mutant and the last column is the quadruple mutant. The first row is the control, second row is the heat and the third row is the drought treatment. Casts were taken from the leaves and nail polish peels of casts were viewed under a compound microscope. Scale = 0.05 mm.

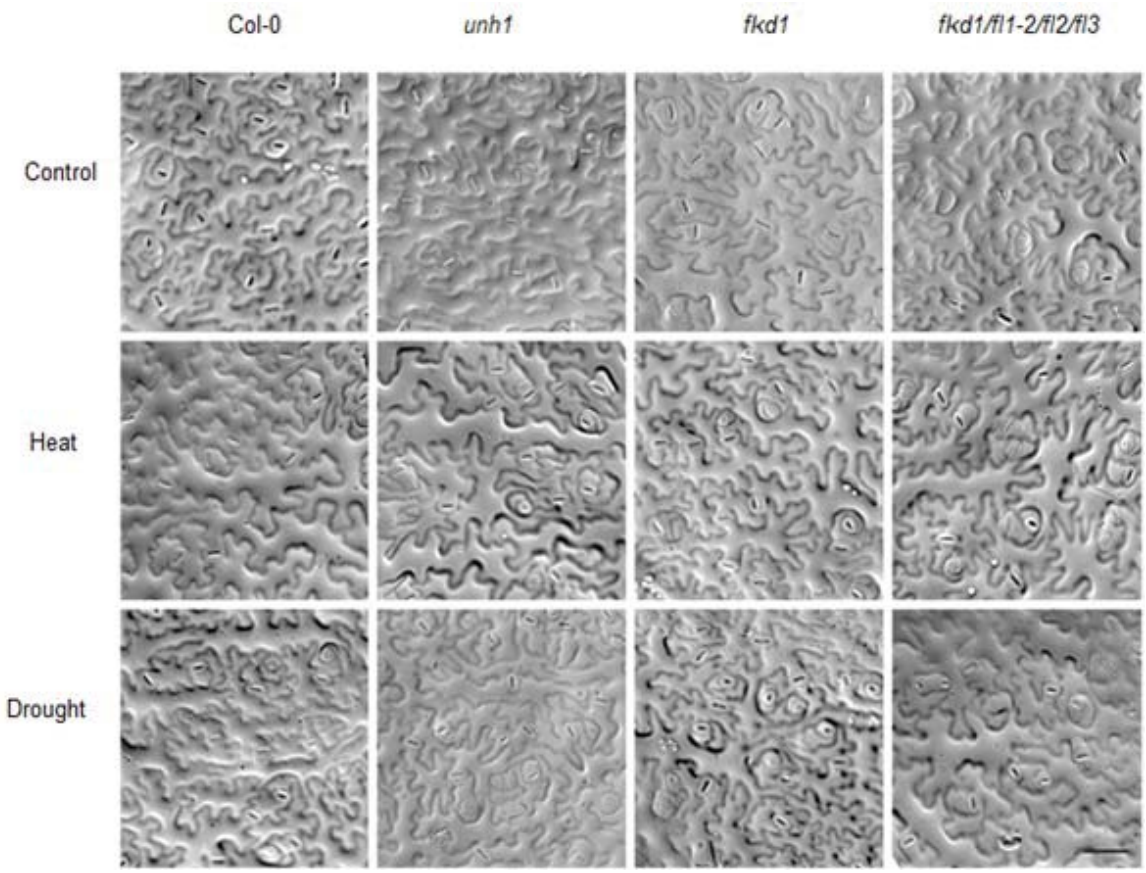


Figure 3.13: Stomata from the abaxial leaf side of leaf 5 from different genotypes grown under control, early-heat and drought. The first column is the wild type, the second column *unh1*, the third column of cells are the *fkd1* mutant and the last column is the quadruple mutant. The first row is the control, second row is the heat and the third row is the drought treatment. Casts were taken from the leaves and nail polish peels of casts were viewed under a compound microscope. Scale = 0.05 mm.

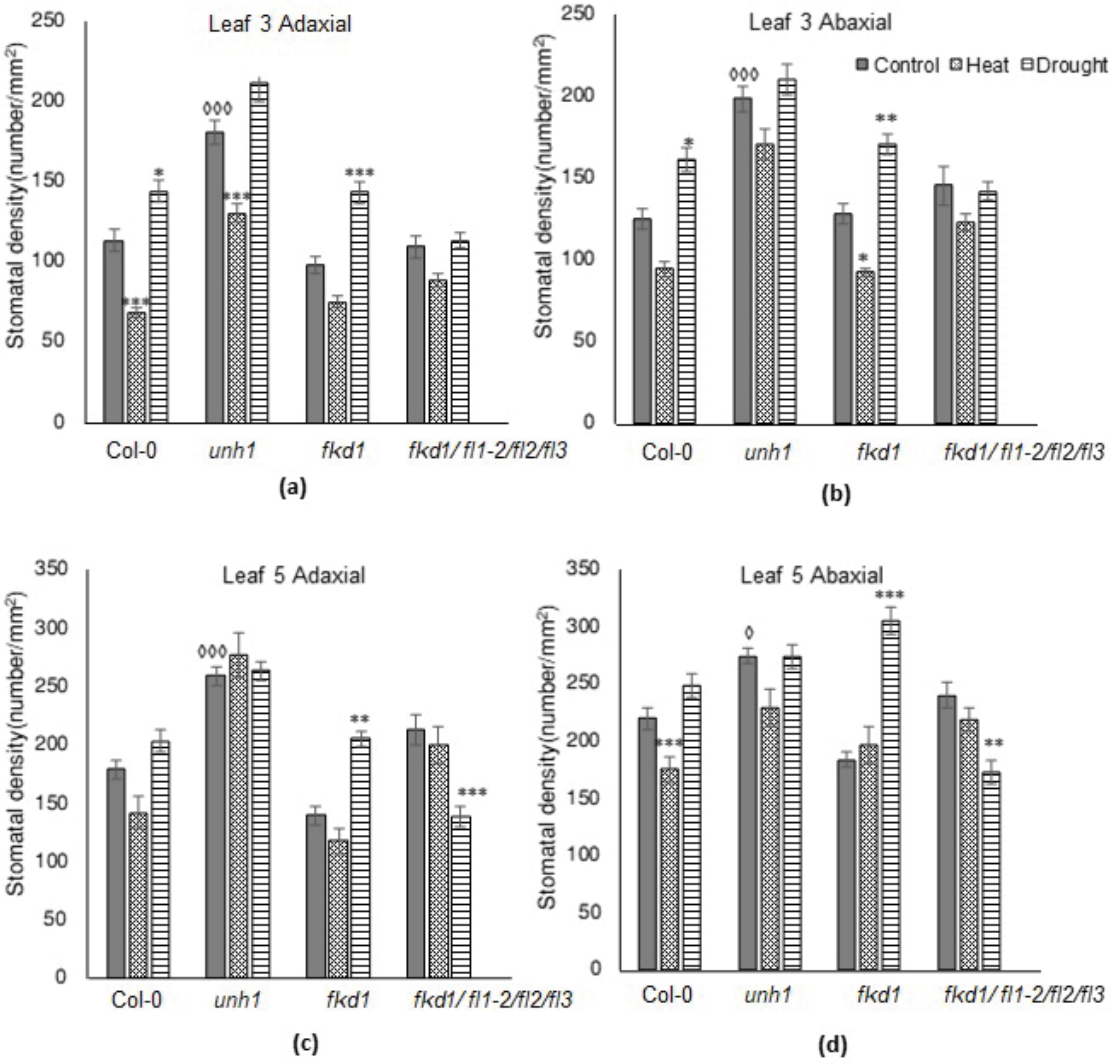
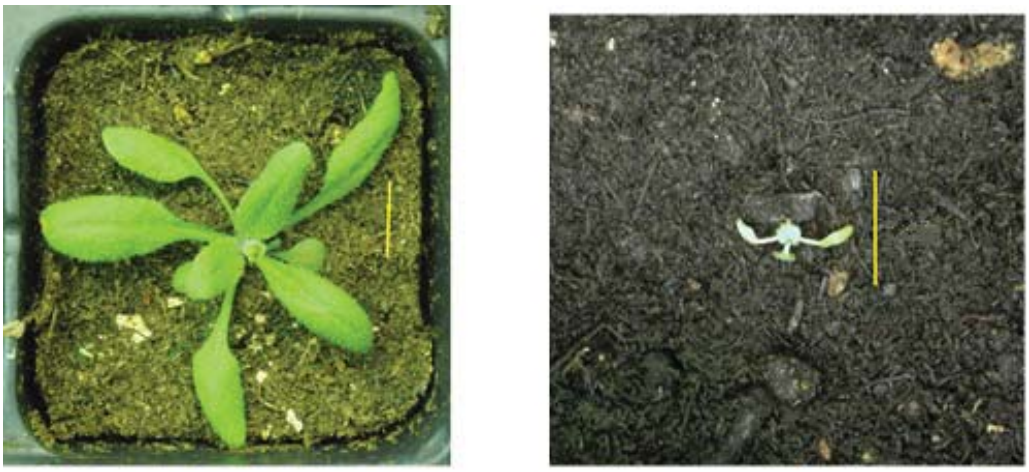


Figure 3.14: Response of stomatal density of the adaxial (a, c) and abaxial (b, d) side of leaf 3 (a, b) and leaf 5 (b, d) of wild type and mutant *Arabidopsis thaliana* to early-heat and drought treatments. Stomatal density was measured from an area of 0.6 mm^2 cast of abaxial surface of leaves (Mean ± SE, n = 12). Asterisk (*) symbol indicates significant difference within the same genotype between the control and heat or drought where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. The diamond (◊) symbol marks significant difference between the wild type and the mutants under control condition where ◊ indicates $p < 0.05$, ◊◊ indicates $p < 0.01$ and ◊◊◊ indicates $p < 0.001$ (Tukey Kramer test).



(a) (b)

Figure 3.15: Day 18 *Arabidopsis thaliana* with no ABA (a) and sprayed with 5 mM of ABA (b). ABA or control solution was sprayed directly onto plants from day 7 to day 18. Scale = 1 cm.

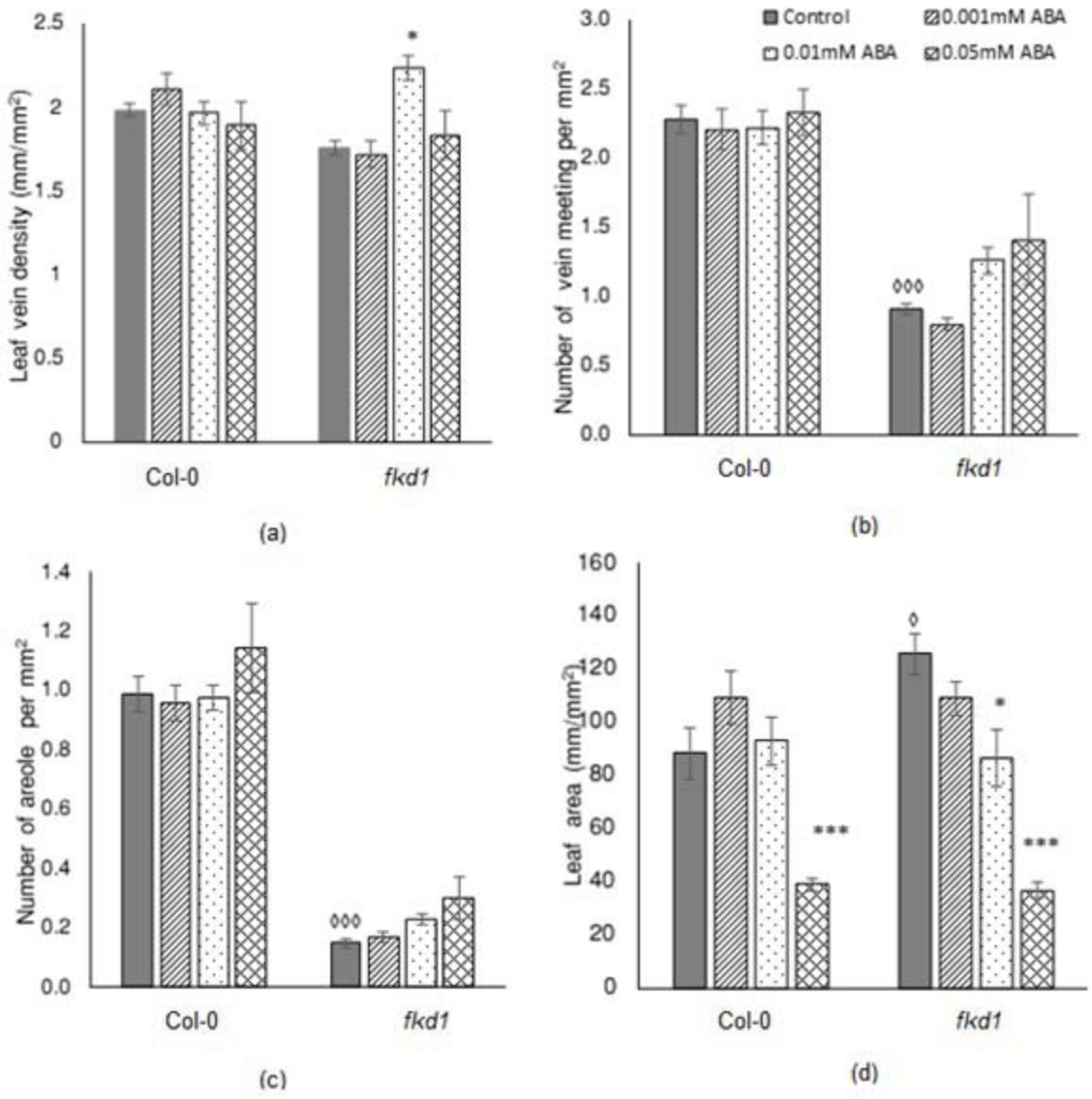


Figure 3.16: Response of characteristics of leaf 3 of wild type and *fkd1* mutant of *Arabidopsis thaliana* to different ABA concentrations (0.001 mM, 0.01 mM and 0.05 mM). Leaf vein density (a), number of vein meeting per mm^2 (b), areole number per mm^2 (c) were measured the mid-section of cleared leaf harvested on day 25 (Mean ± SE, n = 15). Area (d) of whole cleared leaf was measured using ImageJ (n = 15, Mean ± SE). Asterisk (*) symbol indicates significant difference within the same genotype under different ABA concentrations where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. The diamond (◊) symbol marks significant difference between the wild type and the *fkd1* under control condition where ◊ indicates $p < 0.05$, ◊◊ indicates $p < 0.01$ and ◊◊◊ indicates $p < 0.001$ (Tukey-Kramer test).

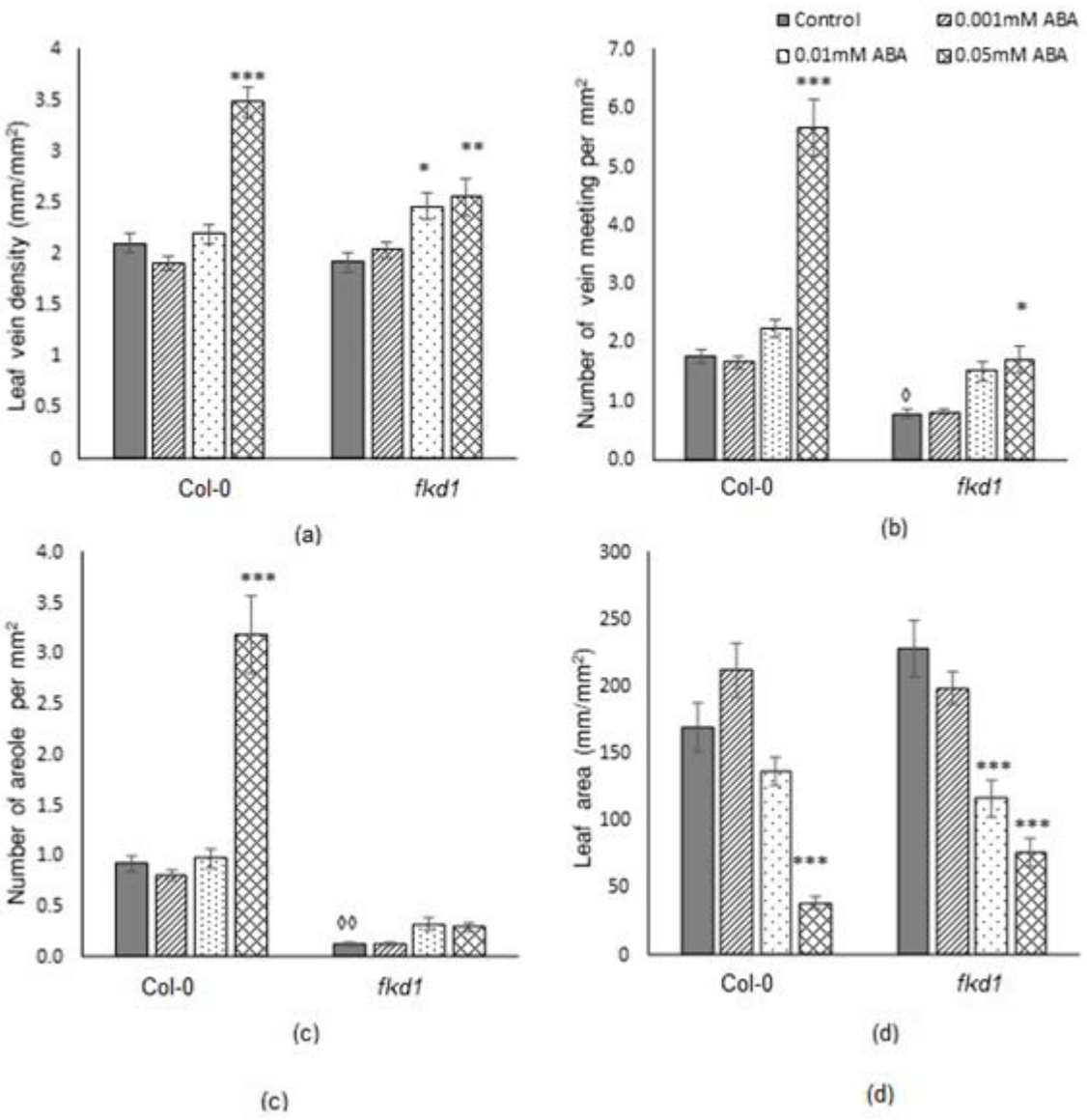


Figure 3.17: Response of characteristics of leaf 5 of wild type and *fkd1* mutant of *Arabidopsis thaliana* to different ABA concentrations (0.001 mM, 0.01 mM and 0.05 mM). Leaf vein density (a), number of vein meeting per mm^2 (b), areole number per mm^2 (c) were measured the mid-section of cleared leaf harvested on day 27 (Mean ± SE, n = 15). Area (d) of whole cleared leaf was measured using ImageJ (n = 15, Mean ± SE). Asterisk (*) symbol indicates significant difference within the same genotype under different ABA concentrations where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. The diamond (◊) symbol marks significant difference between the wild type and the *fkd1* under control condition where ◊ indicates $p < 0.05$, ◊◊ indicates $p < 0.01$ and ◊◊◊ indicates $p < 0.001$ (Tukey Kramer test).

Chapter 4: **Discussion**

4.1 Response of wild type *Arabidopsis thaliana* vein patterns to heat and drought

I found that wild type *Arabidopsis thaliana* showed an increased *Vd* under both drought and heat conditions. This was true for both leaf 3 (early leaf with less *Vd*) and leaf 5 (later leaf with higher *Vd*). I expected late-heat but not early-heat to coincide with the vein developmental period of leaf 5, but, surprisingly, early-heat and late-heat had a similar effect on leaf 5. This suggested that the early and late-heat included some of the developmental window of leaf 5. The short heat treatment from day 7 to 11, it seemed, not only caused a change in leaf vein patterns but also had an affect on soil moisture (Figure 3.5). In fact, the short-term heat was accompanied by a longer term moderate drought. This combination of early-heat and subsequent drought on the young plants could be the reason that the wild type *Arabidopsis thaliana* showed a stronger response to the heat treatments than to the drought treatment alone. Compared to drought, heat had a similar effect on leaf 5 but the drought effect was reduced in leaf 3 which suggested that drought, which was not imposed until day 10, did not significantly overlap with the window of leaf 3 vein development.

The increase in *Vd* was associated with increased number of vein meetings and areole number per unit area in wild type *Arabidopsis thaliana* under heat. Drought, however, resulted in no change in number of areoles per unit area although the number of vein meetings per unit area increased. Increase in vein meeting could be due to formation of extra vein branches or due to formation of areoles which requires a minimum of two vein junctions. Thus, increase in vein meeting but not areole number suggests that the plants formed additional freely ending veins but did not form closed loops under drought. According to Scarpella et al., (2004), veins develop from procambial cells and are initially

formed as freely ending pre-procambial domains that later fuse to become interconnected. Once the mesophyll starts differentiating from the ground meristem, vein differentiation cannot occur. It might be that the drought occurs sufficiently late during leaf vein formation that new veins could branch but were unable to complete the loop.

It was interesting to note that the wild type *Arabidopsis thaliana* showed a response to these changes even though it is a short-lived annual plant. Higher numbers of areoles are proposed to correlate with total *Vd* and may provide the advantage of higher *Vd* in plants (Sack and Scoffoni, 2013). Thus, *Arabidopsis thaliana*, a short lived annual, responded in a similar way as other plant species, including woody perennial plants, by increasing *Vd* under heat or drought (Sack and Holbrook, 2006; Nardini et al., 2010; Medek et al., 2011; Scoffoni et al., 2011; Hu et al., 2014). Increased *Vd* under heat or drought is expected to allow water delivery closer to the sites of evaporation improving the water transport capacity (Brodribb et al., 2007) thus optimizing photosynthetic gas exchange under stress. Vein meetings are proposed to provide alternate transport route in the plants in case of injury or embolism (Sack and Scoffoni, 2013). While we might have expected the benefit of a change in vein pattern to be less than the cost in a plant with a short life cycle, the change in *Vd* under stress indicated that *Vd* is important for *Arabidopsis thaliana* acclimation to stress. Secondly, the results revealed that *Arabidopsis thaliana* can be used as a model system for dissecting the molecular processes behind the change.

4.2 Response of wild type *Arabidopsis thaliana* leaf size and stomatal density to heat and drought

The wild type *Arabidopsis thaliana* decreased its leaf area under all the stress treatments for both leaf 3 (Figure 3.1 d) and leaf 5 (Figure 3.2 d) in the first experiment, whereas in the second experiment only heat caused a decrease in leaf area (Figures 3.7d

and 3.8 d). Early-heat and late-heat had no significant effect on leaf area suggesting that both treatments coincide with the timing of leaf expansion. In my earlier experiment (wild type stress experiment), there was a greater reduction in leaf area in leaf 3 and leaf 5 ($p < 0.001$, Figures 3.1 d and 3.2 d) under early-heat whereas the reduction in area was not as much in the later experiment (mutant stress experiment) in leaf 3 ($p > 0.05$) and leaf 5 ($p < 0.001$, Figures 3.7 d and 3.8 d). Decreased leaf area has been found to be due to decreased cell division or cell expansion in several plant species (Poorter et al., 2009) which are induced by ABA in the *Arabidopsis* leaf under stress (Wang et al., 1998).

Stomata close to reduce transpiration and to conserve water under drought resulting in low CO_2 availability. Low CO_2 results in increased SD allowing greater permeability to CO_2 (Woodward, 1987). It is possible that the low CO_2 resulting from drought induced stomatal closure, caused increased SD. Wild type *Arabidopsis thaliana* changed SD consistently on both sides of the leaf (Figure 3.14). The SD, however, varied across experiments. In the experiment done only for wild type abaxial side, the SD increased under heat and was constant for other treatments (Figures 3.4 a and b). Unfortunately, I do not have soil moisture data for this experiment so I cannot relate the different responses to possible differences in soil moisture. In the second experiment, SD decreased under heat and increased under drought. While an increase in number of stomata per unit area under heat has been proposed to meet the rapid transpirational demand required to cool the plant (Ferris et al., 1996; Hu et al., 2014) SD must also depend on the balance amongst the rate of transpiration, the water supply and the relative humidity of the environment. In fact, I found that the short-term heat treatment was followed by a sharp decrease in soil moisture (Figure 3.5) which could cause plants to produce fewer stomata to conserve water as has been suggested for poplar and *Arabidopsis thaliana* (Hamanishi et al., 2012; Vile et al.,

2012). The relative humidity was the same in all the experiments (80%). Alternatively, the increased leaf area in the later experiment relative to the earlier experiment could have diluted the number of stomata per unit leaf area resulting in lower SD under early-heat as has been suggested by Vile et al., 2012 for different genotypes of *Arabidopsis thaliana*.

4.3 Effect of vein pattern mutants on response of vein pattern to heat or drought

Mutants of *Arabidopsis thaliana* (*unh1*, *fkd1* and *fkd1/fl1-2/fl2/fl3*) having alteration in vein pattern were subjected to early-heat and the drought treatment along with the wild type *Arabidopsis thaliana*. Thereafter I assessed the vein patterns, leaf area and SD of leaves 3 and 5 to find the consequences of vein pattern mutation to plant's ability to alter vein pattern.

4.3.1 Response of mutant leaf area and stomatal density (SD) to heat and drought

The wild type and the mutants showed the same trend for change in leaf area and SD under stress. The mutants showed a trend of decreased leaf area under both treatments but the decrease in leaf area was not significant in some treatments (Figures 3.7 d and 3.8 d). This could be due to insufficient sample size as even a 50% decrease in leaf area was deemed non-significant by statistical analysis (Table 3.2). The mutants (except the quadruple mutant leaf 5 under drought) also showed a similar trend as the wild type for SD, although changes were often insignificant under drought. Following heat treatment, plants of all genotypes showed a trend of decreased SD although it was not significantly different in most cases. This decrease in SD could be associated with a sharp decrease in soil moisture from day 8 to 11 (Figure 3.5). The similar trends in response of leaf area and SD suggested that these genes (*UNH1, FKD1, FL1, FL2, FL3*) were not affecting the response of leaf area and SD to heat or drought.

4.3.2 Consequences of vein pattern mutation to phenotypic plasticity of vein pattern

In previous experiments, cotyledons and first leaves of *unh1, fkd1* and *fkd1/fl1-2/fl2/fl3* had lower *Vd*, number of vein meetings and areoles per unit area than the wild type (Steynen and Schultz, 2003; Pahari et al., 2014; Prabhakaran Mariyamma et al, manuscript in preparation) but older leaves had not been assessed. I found that, as in the cotyledons and first leaves, *unh1* had lower *Vd*, vein meetings and areoles than the wild type for both leaf 3 and 5. However, the *Vd* of leaves 3 and leaf 5 for *fkd1* and *fkd1/fl1-2/fl2/fl3* was similar to wild type. A similar result was found by Caringella et al. 2015 where the true leaves (leaves from higher nodes) of mutants had a greater *Vd* than that described for cotyledons and juvenile leaves. Despite the similarity in *Vd* among wild type, *fkd1* and *fkd1/fl1-2/fl2/fl3* in leaves 3 and 5, these mutants did have lower numbers of vein meetings and areoles compared to the wild type as described for the cotyledons and the first leaf.

If the genes that were responsible for vein pattern formation also acted during response to heat or drought, we would expect the mutant vein pattern to respond to heat or drought differently or to a lesser extent than the wild type. Two-way ANOVA analysis for response of wild type and mutant *Arabidopsis thaliana* to heat or drought treatment showed a strong interaction effect between the genotype and the heat or drought treatments ($p < 0.01$) suggesting that treatments affect the genotypes differently. In fact, my results showed that while the mutants of *Arabidopsis thaliana* increased their *Vd* under heat or drought stress, their response, particularly that of *fkd1* and *fkd1/fl1-2/fl2/fl3,* was less than that of the wild type (Table 3.2).

Whereas *unh1* increased its *Vd* significantly under heat in both leaves and under drought in leaf 5, and *fkd1* increased its *Vd* significantly under heat in leaf 3, the percentage change of *Vd* in *fkd1* leaves was less compared to *unh1* (Table 3.2). This suggested that

FKD1 was more involved than *UNH1* in enabling leaf phenotypic plasticity. Generally, the *fkd1/fl1-2/fl2/fl3* quadruple mutants showed a similar response as the *fkd1*. This was quite surprising since the quadruple mutant is missing the *FKD1* gene as well as three other genes with similar sequence (*FL1/FL2/FL3)* which have been shown to act redundantly with *FKD1* under control conditions in cotyledon and leaf 1 (Prabhakaran Mariyamma et al, manuscript in preparation). The generally similar response of the quadruple mutant to heat and drought compared to *fkd1* suggested that *FL1, FL2,* and *FL3* did not have a major role in processes affecting changes in the density of leaf veins in response to heat or drought.

An exception to the similar responses of *fkd1* and quadruple mutant was a sharp increase in *Vd* under heat in leaf 3 for the quadruple mutant (Figure 3.7 a) accompanied by increase in number of vein meetings per unit area (Figure 3.7 b). Several possibilities explain this anomaly, 1) misscoring leaf 4 for leaf 3 - the cotyledons and first leaf in the quadruple mutant were very small and were smaller under drought and heat and they may have been missed in leaf counting. 2) *FL1, FL2,* and *FL3* act differently in leaf 3 under heat than under drought or control condition. 3) Slow growth of quadruple mutant (Prabhakaran Mariyamma et al, manuscript in preparation) resulted in exposure of leaf 3 heat at a more sensitive developmental stage.

Under control condition, the number of vein meetings and areoles per unit leaf area was significantly lower in *unh1*, *fkd1* and *fkd1/fl1-2/fl2/fl3* relative to the wild type. Unlike wild type, when these mutants were under heat or drought, they did not significantly change their numbers of vein meetings or areoles per unit leaf area which strongly indicated that the genes were involved in the pathway that altered the number of vein meeting and areole per unit leaf area response to drought. As for *Vd,* leaf 3 of quadruple mutant under heat was an exception in that it significantly increased number of vein meetings per unit area. The

reduced change in mutant genotypes suggested that at least a subset of genes involved in initial development of veins were also involved in developmental pathways altering leaf phenotypic plasticity.

4.4 Response of *Arabidopsis thaliana* leaf area and leaf vein density to ABA

ABA had been previously described as affecting leaf size and stomatal closure in plants under stress (Wang et al., 1998). ABA is a primary stress response hormone that, under stress conditions such as heat or drought, is transported to the leaf where it results in differential gene expression leading to stomatal closure and reduction of leaf area. I wanted to test the hypothesis that like leaf area, vein pattern might be an ABA induced change. When sprayed with 0.05 mM of ABA, both leaf 3 and leaf 5 of wild type showed a decrease in leaf area indicating that our timing of ABA application was appropriate to influence leaf development. Further, I found that wild type leaf 5 (Figure 3.17 a) changed *Vd* when sprayed with ABA but not leaf 3 (Figure 3.16 a) suggesting that spraying may have been too late for leaf 3. The *Vd* change was accompanied by a strong increase in number of vein meetings and areoles (Figures 3.17 b and c).

Since *fkd1* altered the response of vein pattern to heat and drought, I asked if it might also affect leaf vein pattern response to ABA. The area of both *fkd1* leaf 3 (Figure 3.16 d) and 5 (Figure 3.17 d) responded to 0.05 mM ABA in a similar fashion to wild type. However, the vein density of *fkd1* leaf 5 (Figures 3.17 a, b and c) increased less than wild type in response to 0.05 mM ABA. This indicated that while the *fkd1* is not involved in ABA response to leaf size, it is partially involved in the vein pattern response to ABA hormone. It is possible that ABA acts through genes like *FKD1* to alter vein pattern in response to heat and drought condition. *fkd1* has an ABA response element (ABRE) in its promoter region and pFKD1: GUS (the *FKD1* promoter fused to the reporter gene B-

glucuronidase) shows altered expression when sprayed with ABA (Veenendaal, J., unpublished results).

4.5 Physiological impact of stress on *Arabidopsis thaliana* by carbon isotope analysis

During the process of photosynthesis, there is fractionation of carbon isotopes i.e. plants will preferentially take in the lighter carbon isotope (^{12}C) rather than the heavier isotope (^{13}C). This discrimination against heavier carbon isotopes results in plant biomass with an abundance of ^{12}C (Farquhar et al., 1989). Enrichment of $\delta^{13}C$ in the plants could be either due to reduced stomatal conductance (*gc*) or due to increased assimilation rate (*A*) either of which reduces intercellular CO_2 levels resulting in less discrimination against ^{13}C (Marshall and Zhang, 1994). Conversely, reduced $\delta^{13}C$ could be due to increased g_c or lower *A* which results in greater discrimination against the heavier isotope.

Wild type *Arabidopsis thaliana* under drought was more enriched ($\delta^{13}C$ = -30.3‰) in ^{13}C compared to the control ($\delta^{13}C$ = -31.2‰). Drought has been found to increase $\delta^{13}C$ values in many crop plants (Kano-Nakata et al., 2014). This enrichment of $\delta^{13}C$ could be either due to increased *A* or reduced g_c leading to less intercellular CO_2. *A* can be inferred from nitrogen (N) content which is a proxy for photosynthesis because approximately 50% of plant nitrogen is involved in the photosynthetic apparatus (Lambers et al., 1998). Usually, greater $\delta^{13}C$ values are associated with lower g_c (Carey et al., 1998). In wild type, the total N content was significantly higher under drought compared to the control which suggested an increased *A* relative to g_c under drought. The increased *Vd* under drought could have also contributed to increased *A* in wild type *Arabidopsis thaliana* as described previously in section 1.1.4.1. The higher mg N per plant under drought in wild type could contribute to the less negative $\delta^{13}C$ values. Carey et al., (1998) found an increase in *A* for pine trees under drought condition by carbon isotope analysis where increased $\delta^{13}C$

correlated with N increased concentration in the leaves. Under drought, there is more biomass allocation to the roots (Wilson, 1988) as plants tend to increase their root length during water shortage. Thus, it has been suggested that trees under drought, may allocate more carbon to the roots which leads to concentration of a increase in N percentage within the leaves (Waring and Schlesinger, 1985). The same thing could be happening with *Arabidopsis thaliana*. Unfortunately, I did not take the root data.

Although drought had an effect on $\delta^{13}C$ of wild type, heat did not. Heat was applied for a short time only (day 7 to 11), but the isotope data were taken from plant parts, excluding root, stalk, leaves 3, 4, 5, and 6, that were 27 days old. It is possible that *Arabidopsis thaliana* recovered from heat after day 13 - 17 as the moisture curve went back to being similar as the control treatment (Figure 3.5). The leaves formed after that time might not have increased *Vd*. The low N assimilation would suggest a reduced *A* under heat in wild type. Despite the reduced *A* under heat, the $\delta^{13}C$ value was similar to the control condition suggesting that g_c must be reduced to a similar extent as *A*.

Thus, my results suggested that both drought and heat affected *Arabidopsis thaliana* physiology with drought treated plants showing an increased *A* in comparison to g_c which resulted in a less discrimination against ^{13}C. In contrast, heat treated plants showed a reduced *A* but no change in $\delta^{13}C$ indicating a similarly reduced g_c.

Two-way ANOVA showed a significant genotype effect of *fkd1* and wild type on $\delta^{13}C$ value with the *fkd1* genotype resulting in a reduced discrimination against ^{13}C compared to the wild type which suggested an overall trend towards either increased *A* or reduced *gc*. As for wild type under heat, *fkd1* $\delta^{13}C$ changed little compared to control. Like wild type, *fkd1* under drought was more enriched in $\delta^{13}C$ (1.2‰) in ^{13}C than the control. However, in contrast to wild type, there was no significant difference in mg N per plant in

fkd1 under drought. This suggested that *A* was not changing and therefore that the increase in $\delta^{13}C$ value were due to reduced *gc*. The SD in *fkd1* increased under drought suggesting that the decreased g_c is due to reduced stomatal conductivity. The resulting reduced intercellular CO_2 levels would cause less discrimination against ^{13}C (Marshall and Zhang, 1994) which would lead to enrichment of $\delta^{13}C$ in the plants. Thus, my result suggests that variation in $\delta^{13}C$ value of wild type is linked primarily to differences in rate of *A* while for *fkd1* it is linked to differences in *gc*.

The plant hydraulic system affects the ability of stomata to remain open for photosynthesis without desiccating the leaf (Sack and Holbrook, 2006). According to Caringella et al. (2015) mutants with open or less dense vein pattern have lower K_{leaf} than the wild type because of abnormalities in vein patterning. I would have expected *fkd1,* which had a similar phenotype to *cvp2,* to have had a lower K_{leaf} with less efficient water transport even though it had the same *Vd* as the wild type. The major difference in vein pattern between *fkd1* and wild type under drought conditions is not the *Vd* but rather the number of vein meetings and areole number. Moreover, my results suggest that wild type can increase its photosynthesis under drought suggesting that it can maintain open stomata and CO_2 entry. In contrast, *fkd1* cannot increase *A,* implying that under drought conditions *fkd1* has closed its stomata. One explanation is that the closed vein pattern of wild type allows proper hydration of mesophyll even under conditions of low water availability. In contrast, under the same conditions, the open vein pattern of *fkd1* causes the mesophyll to be improperly hydrated, resulting in stomatal closure.

Chapter 5: **Conclusion**

Arabidopsis thaliana responds in a similar way to other plant species by changing its leaf vein pattern, leaf area and SD so as to acclimate to drought and heat stress and maximise the physiological parameters such as photosynthetic gas exchange. Moreover, the results of this study suggest that the vein pattern changes, like other morphological responses to abiotic stress, are induced by ABA. This study has also shown that *FKD1*, which is involved in vein development, has a role in leaf phenotypic plasticity induced in response to abiotic stresses like drought and heat. My results also suggest that *FKD1* may be involved in vein pattern response to the ABA stress hormone.

Stable carbon isotope ratio analysis in this experiment indicates that the change in $\delta^{13}C$ values under drought for wild type (which has an increased *Vd*) is due to changing rate of photosynthesis (*A*), while it is due to change in stomatal conductance for *fkd1*. This result suggests that *fkd1,* which has an open venation pattern, is not able to hydrate mesophyll as well as wild type which results in closing of stomata to prevent mesophyll desiccation. Further experiments testing $\delta^{13}C$ values of other vein pattern mutants is necessary to determine the effect of drought and heat stress on photosynthetic gas exchange. The reduced stomatal conductance suggests that the open vein pattern reduced K_{leaf}, and whereas wild type can increase *A, fkd1* can not. It would be interesting to determine if *unh1*, which changes *Vd* but not vein meeting and areole number, has similar effects on $\delta^{13}C$ and leaf nitrogen content.

This research improves our understanding of the importance of leaf vein pattern to drought and heat tolerance. In the long-term, we may be able to find the genes responsible for these modifications that will form the basis of establishing the molecular mechanism behind the leaf response to drought and heat. *Arabidopsis thaliana* is representative of

higher crop plants like canola, thus it may be possible to engineer crop plants with optimal number of veins or stomata to maximize stress tolerance.

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