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**Search for missing components in high VPD-
induced stomatal closure pathway**

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Search for missing components in high VPD-induced stomatal closure pathway

Abstract

Climate warming is associated with rising atmospheric Vapour Pressure Deficit (VPD), which affects plant physiology and production. Some proteins in the high VPD-induced stomatal closure pathway are known (e.g., protein kinase OST1), but there are still missing components. To address this knowledge gap, the study aimed to investigate *Arabidopsis* mutants defective in selected genes in order to reveal new elements that might be involved in VPD-induced stomata closure. Mutants were selected based on Wang et al. (2020) table of OST1 putative substrates and the expression levels of these genes in guard cells versus mesophyll cells. Experiments were conducted using a gas exchange measurement device to study plants' stomatal conductance in response to high VPD and a plant stress hormone - abscisic acid (ABA). The study results showed no statistically significant differences in the steady-state stomata conductance and closure responses among mutants and wild-type, suggesting that these genes are not involved in VPD-induced stomata closure. Therefore, more studies are needed to reveal the missing components in the stomatal high VPD-induced closure pathway above OST1. Current results still add knowledge about stomatal behavior in future climatic conditions.

Keywords:

Climate change, VPD, ABA, stomatal conductance, OST1

CERCS: B310 Physiology of vascular plants

Otsides puuduvaid komponente õhulõhede kõrge VPD-toimelises sulgumises

Lühikokkuvõte

Kliima soojenemine toob kaasa atmosfääri niiskusdefitsiidi (VPD) tõusu, teisisõnu, vahe õhu küllastunud ja tegeliku veeauru kontsentratsioonivahel kasvab. VPD tõus mõjutab taimi negatiivselt, põhjustades suuremat aurustumist, mis omakorda viib õhulõhede sulgumiseni, fotosünteesi vähenemiseni ja lõpuks saagikus langeb. Kuigi VPD-st põhjustatud õhulõhede sulgumise molekulaarset rada on uuritud ja mitmed olulised komponendid selles protsessis on teada (nt kinaas OST1), on teadmised õhulõhede VPD-toimelise sulgumise rajast siiski lünklikud. Käesoleva töö eesmärk oli uurida valitud geenide osas defektseid Arabidopsis'e mutante, et paljastada uusi elemente, mis võivad olla seotud VPD-st põhjustatud õhulõhede sulgumisega. Geenid valiti välja Wang jt (2020) OST1 oletatavate substraatide tabeli põhjal, tehes kindlaks, et nende geenide ekspressioonitase õhulõhede sulgrakkudes on kõrgem kui lehe mesofüllirakkudes. Valitud geenide osas defektsed mutantsed taimed telliti Arabidopsis' keskest. Katsed viidi läbi gaasivahetusaparatuuriga, mis võimaldab mõõta taimede õhulõhede juhtivust stabiilses olekus, samuti õhulõhede vastuseid kõrgele VPD-le ja taime stressihormoonabstsiinshappele (ABA). Katsetulemused näitasid, et statistiliselt olulisi erinevusi õhulõhede juhtivuses ega õhulõhede sulgumises kõrge VPD ja ABA toimel mutantide vahel polnud. Seega võib öelda, et need geenid ei osale VPD-st ega ABA-st põhjustatud õhulõhede sulgumise protsessis. Otsing õhulõhede VPD-toimelise sulgumise komponentide osas peab seega jätkuma, ent saadud tulemused on olulised õhulõhede tulevikukäitumise aspekti silmas pidades.

Võtmesõnad:

Kliimamuutused, VPD, ABA, õhulõhede juhtivus, OST1

CERCS: B310 Vaskulaarsete taimede füsioloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

ABA	Abscisic acid; a plant hormone
Ea	Actual water vapour pressure
Es	Saturated water vapour pressure
ET	Evapotranspiration
GCs	Set of guard cells
GMC	Guard mother cell
Gs	Stomatal conductance
MMC	Meristemoid cell
OST1	OPEN STOMATA 1
PCR	Polymerase Chain Reaction
PP2C	PROTEIN PHOSPHATASE 2C
PYR/PYL/RCAR	PYRABACTIN RESISTANCE/PYR-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR
RH	Relative humidity
SLAC1	SLOW ANION CHANNEL1
SLGC	Stomata lineage ground cell
SnRK2	SNF1-RELATED PROTEIN KINASE 2
SPCH	SPEECHLESS; master transcription factor regulating stomata development
VPD	Vapour pressure deficit

INTRODUCTION

Earth's climate has experienced a rise in temperatures over the past several decades, leading to increased atmospheric vapour pressure deficit (VPD). VPD is the difference between the actual amount of moisture in the air (E_a) and the amount of moisture that air can hold at saturation (E_s) based on air temperature. A high VPD means that the air is relatively drier, which negatively affects plants. Under high VPD, the small pores on the surface of the plant leaves, called stomata, close to conserve water. Stomata consist of two guard cells and a pore between them, and they regulate the movements of carbon dioxide and water between the leaf and the atmosphere. Growing in high VPD conditions, plant stomata close, leading to reduced photosynthesis and production. Additionally, several other changes happen in plants under high VPD. For instance, changes in stomatal density, size, internal leaf anatomy, root growth, and biochemical composition have been detected in plants growing in high VPD conditions (López et al., 2021). Studying the stomatal closing and opening pathways in response to future climate conditions is essential. Stomatal closure can be passive or active. The hydropassive response occurs by passively adjusting the water potential of the plant, including leaf and guard cells, to the surrounding environment. The hydroactive pathway, on the other hand, involves the activation of signaling cascades, which eventually lead to guard cell anion channel activation, ion efflux and stomatal closure. In the case of high VPD, the protein kinase OPEN STOMATA1 (OST1) is involved in guard cell signaling to initiate stomatal closure. Abscisic acid (ABA) is an important plant hormone in the VPD-mediated stomatal closure pathway. However, it is still unclear which components are involved in VPD-induced stomatal closure above the activation of OST1, which is mediated by RAF-like kinases. This study aims to uncover new components in the VPD-induced stomatal closure pathway using a reverse genetics approach. Arabidopsis mutants with disrupted single genes were selected based on Wang et al. (2020) table of OST1 putative substrates and the expression levels of these genes in guard cells. VPD sensitivity of mutants defective in selected genes was measured in gas exchange experiments. Additionally, the ABA responsiveness of these mutants was also determined. This study addresses a gap in our understanding of stomatal closure in response to rising VPD and thus provides information for breeding plants that are more suitable for future environments. Stomatal closure under high VPD increases plant water use efficiency, reduces water consumption, and maintains plant water content, eventually contributing to sustainable agriculture.

1 LITERATURE REVIEW

1.1 Climate change and the associated increase in VPD

Atmospheric Vapour Pressure Deficit (VPD) has a direct impact on ecosystems and plants, and it increases during climate change, leading to a number of consequences. VPD is defined as the difference between saturated water vapour pressure (E_s) and actual water vapour pressure (E_a) for a given temperature.

$$VPD = E_s - E_a = E_s - RH * E_s / 100,$$

where E_s represents saturated vapour pressure, E_a - actual water pressure, and RH is a relative air humidity.

VPD increases during climate change due to an inevitable rise in E_s and a regional decrease in E_a (Ficklin & Novick, 2017). E_s rises due to higher air temperature (Campbell & Norman, 1998). Importantly, global air temperature has increased by 0.2 degrees per decade over the last 30 years (Hartmann et al., 2014). Meanwhile, E_a , or actual vapor pressure, is a product of relative humidity (RH) and saturation vapour pressure (Novick et al., 2024). The trends in relative humidity are locally different. On the one hand, the relative humidity declined dramatically in southwestern North America, the Amazonian, southern South America, and the Sahel region from 1979 to 2014 (Vicente-Serrano et al., 2018). On the other hand, it is suggested that the air's relative humidity will rise due to the local increase in the amount and frequency of rainfalls (Betts et al., 2014). For instance, RH has already increased in the Nordic-Baltic region (Xiao et al., 2020). High VPD leads to higher evapotranspiration (ET), which, in turn, has an effect on soil moisture and precipitation dynamics (Ficklin & Novick, 2017).

Globally, 53% to 64% of land areas have experienced higher VPD levels since the late 1990s (Yuan et al., 2019). Notably, VPD has risen by 0.25 kPa from 1979 to 2013 in the USA. Moreover, the general circulation model predicted a 51% increase in VPD for summers in the future, up to 2099 (Ficklin & Novick, 2017).

1.2 Impact of rising VPD on plants

Rising VPD can have a series of consequences for plants' gas exchange, stomatal conductance, growth, and survival (Schönbeck et al., 2022) (Figure 1: Adapted from López et al., 2021). López et al. (2021) found that among 112 species, 84% showed a significant response to increased VPD. Under high VPD exposure, plants experience an immediate rise

in transpiration, leading to high water loss counteracted by stomatal closure and a decline in stomatal conductance (G_s), which is accompanied by reductions in photosynthesis (Buckley, 2016; López et al., 2021). The future projections suggest that stomatal conductance will decrease by 9-51% due to high VPD (Ficklin & Novick, 2017). High VPD not only slows down the growth rate but can also lead to plant mortality. High VPD treatment resulted in a decline in plant height, leaf number, stem diameter, and flower number (López et al., 2021).

The increase in VPD promotes a rise in water tensions in the xylem, resulting in loss of hydraulic conductance followed by hydraulic failure and cellular dehydration (Choat et al., 2018; Novick et al., 2024). Hydraulic conductance dynamics are affected more by atmospheric evaporative demand than soil moisture, and a decrease in hydraulic conductance reduces stomatal conductance and CO₂ uptake (Brodribb & Holbrook, 2004).

The decreased growth rate is associated with hormonal and chemical composition changes. As mentioned above, increased transpiration under high VPD leads to low leaf water potential, causing nutrient and hormone accumulation as part of the acclimation strategy to water stress (López et al., 2021). For instance, the accumulation of the stress hormone abscisic acid leads to stomatal closure to minimize transpiration and promote water conservation (Ackerson, 1982). As a result, the leaf area declines to adjust its evaporative surface by reducing stomatal size, epidermal cell size, the air space fraction inside the leaf, and cell expansion rate (López et al., 2021).

These changes have a significant impact on crop yields. A study conducted in the United States between 1995 and 2012 concluded that rising VPD affected maize drought sensitivity and yield (Lobell et al., 2014). In China, it has been reported that wheat, maize and soybean yields decreased by more than 10% during the period from 1980-2008 (Zhang et al., 2017).

Furthermore, high VPD accelerates soil drying, leading to an increased risk of wildfires. In the western United States, there is a clear and positive correlation between VPD and fire occurrence, size, severity, and burned area (Williams et al., 2014; Zhuang et al., 2021).

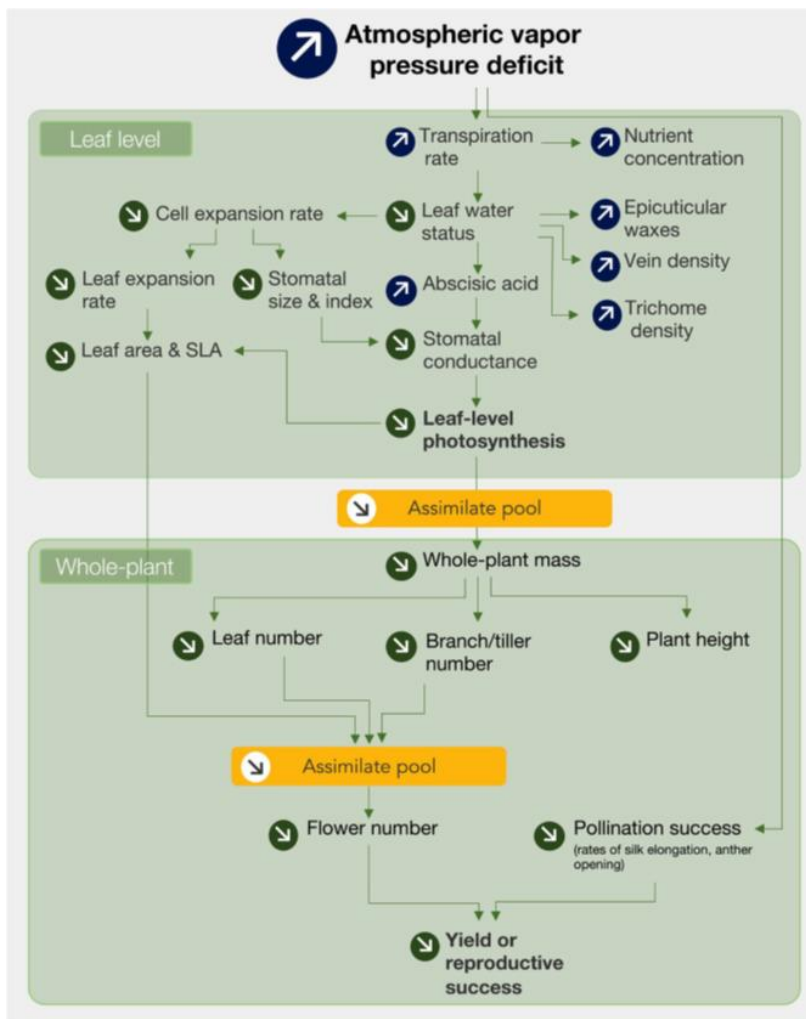


Figure 1. General effects of rising atmospheric pressure deficit (VPD) on plant physiology, productivity and yield. The arrows in the circles represent decrease or increase of specific traits in response to elevated level of VPD. The two main scales, leaf and whole plant, are placed in separate green boxes. Adapted from López et al. (2021).

1.3 Stomata

Stomata are small pores surrounded by two guard cells located on the surfaces of leaves and stems of plants. Stomata primarily control the gas exchange, particularly fluxes of water vapour and carbon dioxide, between the inside of the leaf and the external atmosphere by opening and closing. The movement of gases depends on the number of stomata present on the epidermis of the leaf (i.e., stomatal density) and their aperture widths, both of which are regulated by environmental factors (Hetherington & Woodward, 2003; Driesen et al., 2020). The stomatal density is determined during leaf development and stays the same, while the stomatal aperture width can change in minutes (Kim et al., 2010). Stomatal

conductance describes the amount of water lost through stomata per leaf area and time; it depends on stomatal density and aperture width.

There are two types of guard cells: dumb-bell-shaped guard cells typical for grasses and kidney-shaped forms found in other plant species, including *Arabidopsis* (Hetherington & Woodward, 2003) (Figure 2). Depending on the species and the environmental conditions, stomata range in size from about 10 to 80 μm in length and at densities between 5 and 1,000 mm^{-2} of epidermis (Willmer & Fricker, 1996).

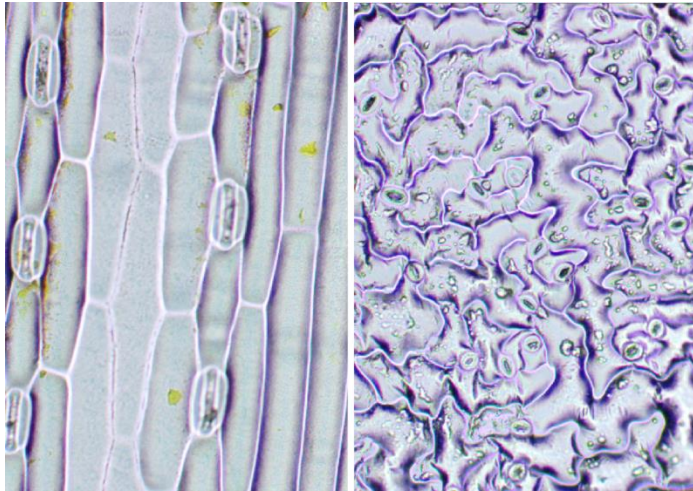


Figure 2. Two types of guard cells: dumb-bell-shaped guard cells typical for monocots and kidney shaped- for dicots. On the left, dumb-bell-shaped guard cells of barley, which are arranged in parallel along the leaf veins, are shown. Right side presents kidney-shaped guard cells in *Arabidopsis*, where the stomata are placed randomly. Photographed by Daana Morozova.

1.3.1 Stomatal development

Stomatal development starts with young meristematic cells in their early embryonic stage called promeristem cells, undergoing symmetric and asymmetric divisions to form the leaf epidermis. Protodermal cells, which are derived from promeristem cells, differentiate into three main cell types: leaf hairs, pavement cells, and stomatal guard cells. The development of mature stomata consists of many steps called the stomatal lineage. The first step of stomatal lineage is a protodermal cell becoming a meristemoid cell (MMC). Then, MMC undergoes a symmetric division to form a stomata lineage ground cell (SLGC) and a meristemoid (Zoulas et al., 2018). Next, these cells develop in three ways. SLGCs can undergo amplifying division to form pavement cells. Also, it is possible to subject to spacing division to create a new meristemoid (Pillitteri & Torii, 2012). Lastly, SLGC and

meristemoid cells can form a guard mother cell (GMC) and later a set of guard cells (GCs) through symmetrical divisions (Zoulias et al., 2018) (Figure 3).

Master transcription factors regulate stomata formation: SPEECHLESS (SPCH), MUTE, and FAMA (Chen et al., 2020).

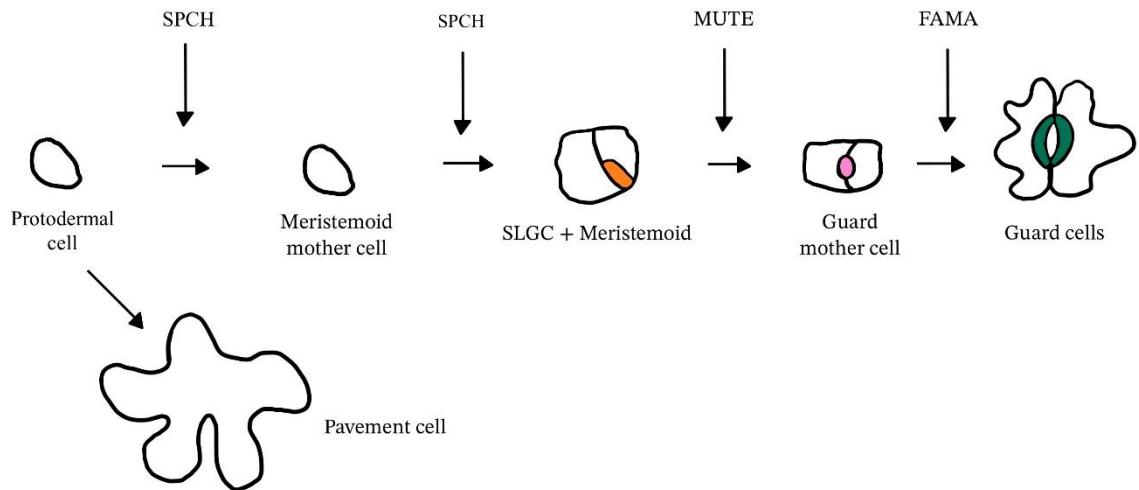


Figure 3. Stomatal lineage. Protodermal cells undergo a set of divisions to form guard cells, as well as pavement cells. The master transcription factors SPEECHLESS (SPCH), MUTE, and FAMA play an important role in stomatal development. The original figure (Jalakas, 2019) has been modified.

1.3.2 Regulation of stomata in response to abiotic environmental signals

Plants actively change their stomata aperture by adjusting the turgor pressure of the guard cells (Driesen et al., 2020). This turgor pressure is generated by ions and water entering guard cells via aquaporins (Daszkowska-Golec & Szarejko, 2013) (Figure 4). Stomatal opening or closing happens in response to the environment to minimize water loss, moderate CO₂ uptake, and maximize photosynthesis (Pillitteri & Torii, 2012).

Stomata open when there is high light intensity, low level of CO₂, high humidity, and higher temperatures. In contrast, stomata close in darkness, high CO₂ concentration, low humidity, and cold temperatures (Willmer & Fricker, 1996; Hetherington & Woodward, 2003; Driesen et al., 2020).

Moreover, a plant hormone, abscisic acid (ABA), controls stomata aperture in response to drought stress. ABA is produced when water availability decreases. It initiates a signaling pathway that promotes stomatal closure and thus water saving regime in plants (Liu et al., 2022).

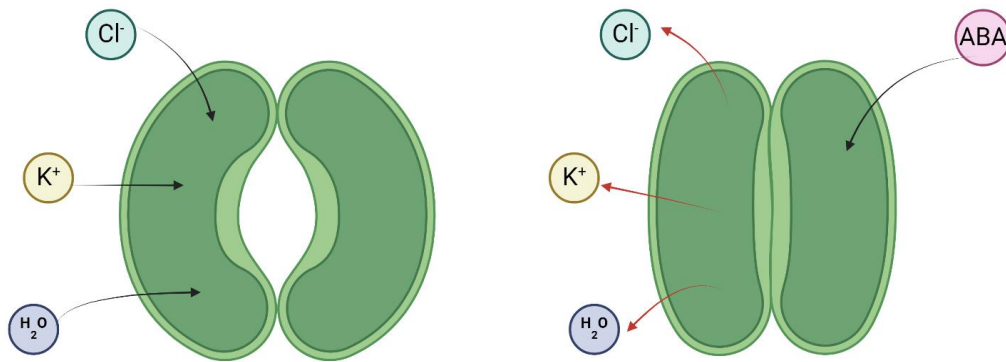


Figure 4. On the left, ions (K^+ and Cl^-) and water (H_2O) enter guard cells to create turgor pressure and open stomata. On the right, ions and water leave, guard cells become flaccid, and as a result, stomata close. Created with BioRender.com.

1.3.3 VPD-induced stomatal regulation

Plants close their stomata in response to high VPD (Jalakas et al., 2021). This process involves two primary mechanisms: active and passive. Hydropassive stomatal closure occurs when water content and potential in guard cells drop passively, together with the reduction of water availability in the surrounding environment caused by increased VPD, leading to decreased guard-cell turgor pressure, and, therefore, stomata close. This closure type does not require foliar ABA (McAdam & Brodribb, 2015; Merilo et al., 2017). The evolutionarily earliest response of stomata to VPD was a passive-hydraulic response. Nowadays, this response is found in the extant relatives of basal vascular plants such as lycophytes, ferns, and gymnosperms (McAdam & Brodribb, 2015).

The second mechanism is hydroactive, which involves a series of events within guard cells, such as signal perception, ion channel regulation, guard cell ion efflux, and a reduction in guard-cell solutes. It is then followed by water efflux and turgor pressure reduction, leading to stomata closure (Merilo et al., 2017). Research by Jalakas, Nuhkat et al. (2021) demonstrated that *Arabidopsis* plants lacking S- and R-type anion channels did not respond to various stimuli such as darkness, elevated CO_2 , reduced air humidity, and ABA, indicating the necessity of both channels for stomata closure. Similarly, Merilo et al. (2017) found that mutants lacking the main protein kinase OST1 showed small and slow stomata closure in response to high VPD. This suggests that the stomatal closure in *Arabidopsis* is primarily

hydroactive. While angiosperms exhibit several components contributing to VPD-induced stomatal closure, including hydropassive response, ABA-dependent, and ABA-independent active closures (Jalakas et al., 2021), the presence of hydropassive closure in all angiosperm species is still unknown.

1.3.4 ABA-mediated stomatal regulation

Abscisic acid (ABA) is a plant hormone which regulates plant biological activity, specifically responses to biotic and abiotic stresses, such as drought and pathogens (Christmann et al., 2006). Dry air leads to an increase in ABA concentration and, therefore, the closure of stomata in order to conserve water availability within a plant (McAdam & Brodribb, 2015).

ABA-activated guard cell S-type and R-type anion channels play an important role in stomatal closure. A slow S-type anion channel is associated with a slower activation, while a rapid R-type anion channel activates in milliseconds (Schroeder & Hagiwara, 1989).

In ABA signaling, guard cell anion channels can activate calcium-dependently or calcium-independently. In the calcium-dependent pathway, ABA promotes a rise in cytosolic Ca^{2+} in guard cells (McAinsh et al., 1990). Elevated calcium concentration activates S-type anion channels and inhibits K^+ channels (Schroeder & Hagiwara, 1989), resulting in the release of ions and a reduction in turgor pressure, therefore causing stomata to close.

The process of calcium-independent stomatal closure starts by binding of ABA to its receptors: PYRABACTIN RESISTANCE/PYR-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR), which are members of soluble ligand-binding proteins (Cutler et al., 2010) (Figure 5). This binding leads to the inactivation of 2C-type protein phosphatases (PP2Cs), which are a subclass of Mg^{2+} and Mn^{2+} dependent serine-threonine phosphatases type 2C (PP2Cs) (Yoshida et al., 2006). ABI1 and ABI2 are members of PP2Cs and act as negative regulators of ABA responses (Merlot et al., 2001). In the presence of ABA, they interact with ABA receptors (PYR/PYL/RCAR receptors), and this binding enables ABA signaling to proceed. In Arabidopsis, ABA receptors are part of a family consisting of 14 protein members that share structural similarities with class 10 pathogen-related proteins (Ma et al., 2009).

In the absence of ABA, the PP2Cs dephosphorylate and inactivate SNF1-RELATED PROTEIN KINASE 2 (SnRK2s), including OST1 (Umezawa et al., 2009). As a result, when there is no ABA, OPEN STOMATA1 (OST1) is inactive, but in the presence of ABA, PP2C-

related inactivation of OST1 is terminated, and it triggers activation of OST1 and downstream transcription factors and ion channels, such as SLOW ANION CHANNEL1 (SLAC1) (Vahisalu et al., 2008; Vahisalu et al., 2010). SLAC1 represents the slow, deactivating, weak voltage-dependent anion channel of guard cells controlled by phosphorylation/dephosphorylation (Vahisalu et al., 2008; Geiger et al., 2009). The activation of SLAC1 leads to the efflux of anions, which is followed by cation and water efflux and stomata closure (H. Kollist et al., 2014).

Moreover, ABA primarily inhibits seed germination (Schopfer & Plachy, 1985) and shoot growth (Quarrie & Jones, 1977) as well as resistance to pathogens by preventing them from entering the plant via stomata and increasing tolerance (Ton et al., 2009). ABA also contributes to embryo and seed development and reproduction (Cutler et al., 2010).

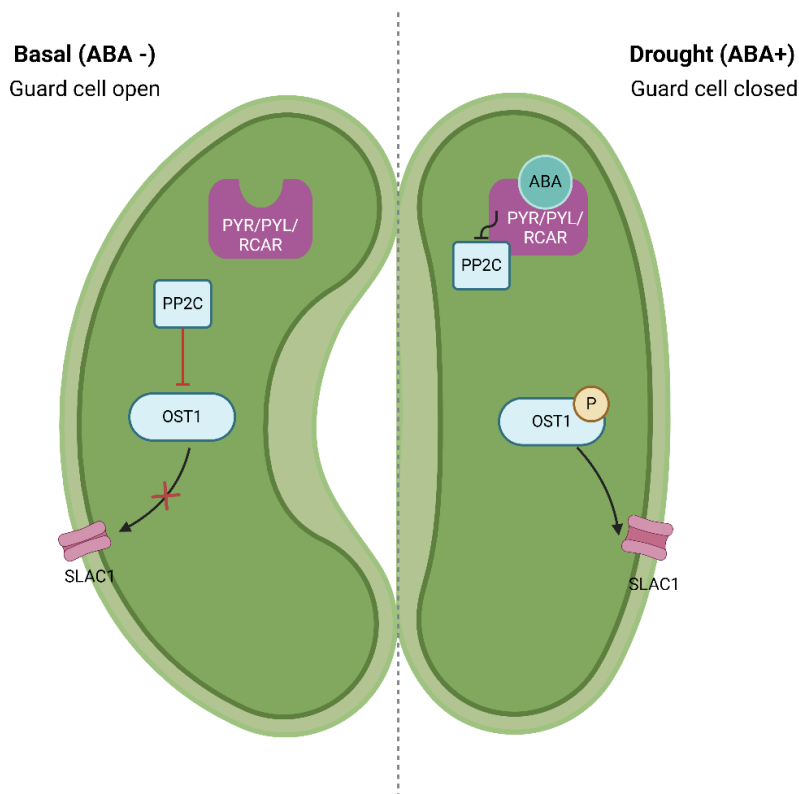


Figure 5. Simplified abscisic acid (ABA) signaling pathway in guard cells. In the absence of ABA (left), PYR/PYL/RCARs are not attached to PP2Cs; therefore, PP2Cs prevent the activation of OST1. In the presence of ABA (right), PYR/PYL/RCAR ABA receptors bind to and inhibit PP2Cs, leading to activation of OST1 by phosphorylation, followed by activation of S-type anion channel via OST1 and then, SLAC1 opens for anion efflux. Created with BioRender.com.

1.4 OST1 protein

OPEN STOMATA1 (OST1), also known as SnRK2.6, is the main protein kinase regulating stomatal aperture in response to various factors such as ABA, reduced air humidity, and darkness (Merilo et al., 2017). B3-family Raf-like M3Ks activate OST1 in response to ABA and osmotic stress (Lin et al., 2020). OST1 strongly controls stomatal sensitivity to VPD (Merilo et al., 2017).

In the study conducted by Tulva et al. (2023), the stomatal conductance of *ost1-3* mutants did not respond to VPD changes under well-watered conditions but showed a significant VPD response when grown under reduced soil water content. OST1 is a positive regulator in ABA signaling pathway (Mustilli et al., 2002). It possesses two regulatory domains in the C-terminus: domain II (ABA-box) for ABA-dependent activation and domain I (SnRk2-box) for ABA-independent osmotic stress and low humidity activation (Yoshida et al., 2006).

In the study by Hsu et al. (2018), it was suggested that OST1 does not participate in CO₂ signaling pathway. However, they discussed that the basal ABA signaling and OST1/SnRK2 activity is needed to initiate stomatal closure in response to elevated CO₂ level.

Notably, the steady-state whole-plant stomatal conductance is primarily controlled by ABA and PYR/RCAR receptors rather than by OST1: the loss-of-function plants of OST1 show moderately increased stomatal conductance, whereas – the stomatal conductance of ABA-deficient lines is much higher (Merilo et al., 2017). Additionally, OST1 is not responsible for regulating stomatal density (Jalakas et al., 2018).

1.5 Missing components in VPD-mediated stomata closure pathway

The stomatal closure pathway in response to high VPD involves various components (Jalakas et al., 2021; McAdam & Brodribb, 2015; Yaaran et al., 2019; Merilo et al., 2017). Understanding the VPD signaling pathway is important because it might help in breeding plants that are able to survive in drier air. However, our comprehension of VPD-induced stomatal closure pathway is still incomplete. One approach to studying this pathway is to conduct experiments with mutants defective in known genes to identify those with reduced responses to high VPD.

Ost1-3 plants lack protein kinase OST1 and can act as negative controls since they are seriously impaired in VPD-induced stomatal closure (Merilo et al., 2017). The triple mutant defective in three main guard cell anion channels, *quac1-1slac1-3slah3-1*, also showed

impaired high VPD responses, pointing to the crucial role of active stomatal closure in VPD-sensitivity (Jalakas, Nuhkat, et al., 2021). In contrast, the sextuple mutants lacking six PYR/RCAR ABA receptors showed a closure response to VPD. However, it was delayed, suggesting that ABA is involved in this process but may not be crucial in stomatal VPD response (Merilo et al., 2017).

Additionally, all ABA-deficient mutants showed higher stomatal conductance than wild-type Col-0 but then rapidly closed stomata under high VPD conditions, similar to Col-0 (Merilo et al., 2017). This could be explained by passive hydraulic VPD-induced stomatal closure of ABA-defective mutants, which have very high stomatal conductance and, thus, high water loss under high VPD. It is also possible that these ABA-deficient lines still have some ABA and can initiate a VPD-induced ABA-dependent closure pathway.

Furthermore, Raf-like M3ks, which control ABA and osmotic stress-induced activation of SnRK2s, are involved in VPD-induced stomatal regulation, as also receptor-like (pseudo)kinase GHR1 (Lin et al., 2020; Hsu et al., 2021). However, there are still components in the VPD-mediated stomata closure pathway that remain to be discovered, particularly the initiation of the response above OST1 and its activation by RAF-kinases.

2 THE AIMS OF THE THESIS

- To study VPD-induced stomatal regulation, *Arabidopsis* mutants defective in selected guard cell enriched and OST1-interacting genes were used in order to identify new components involved in VPD-induced stomatal closure.
- To find whether these selected genes are involved in determining steady-state stomatal conductance, stomatal ABA-response or stomatal density.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Genotyping of plant lines

In experiments, T-DNA insertion Arabidopsis mutants were used. T-DNA insertion mutants are a valuable tool in plant genetics research, and they are generated through a random insertion of transfer DNA (T-DNA) into the plant genome using *Agrobacterium tumefaciens*. The T-DNA-inserted mutants are used to identify whether the mutated genes caused specific phenotypes (Liu & Qin, 2013).

Seeds of plant lines defective in genes of interest were ordered from the European Arabidopsis Stock Centre (www.arabidopsis.info). Genes of interest were selected based on two criteria: 1) It is included in Wang et al. (2020) dataset of OST1 putative substrates based on a strategy of isotope-labeled *in vitro* phosphorylation reactions and 2) the target gene's expression level in guard cells compared to mesophyll cells is high (Table 1).

An essential step when working with seeds obtained from the seed bank is genotyping those lines to confirm their homozygosity and the presence of T-DNA in the target gene. For genotyping, each plant line was planted in ten pots and grown for 25-30 days in a growth room.

The first step was DNA extraction from the plant tissue (Weigel & Glazebrook, 2009). 2-3 small leaves were collected from a plant and placed in a 1.5 ml Eppendorf tube. Next, 600 µl of plant DNA extraction buffer and several glass beads were added to the tube. DNA extraction buffer consists of 100 mM TRIS pH= 8.0, 50 mM EDTA, and 500 mM NaCl. The leaves were then ground in a tissue grinder (FastPrep- 24, Zymo Research). After that, tubes were centrifuged at a maximum speed of 15000 rpm for 5 minutes to precipitate the buffer. Following centrifugation, the supernatant was carefully removed, and 500 µl of isopropanol was added to it. After centrifuging at maximum speed for 5 minutes, the supernatant was discarded, and the pellet was air-dried until the smell of the isopropanol disappeared. Lastly, the pellet was dissolved in 100 µl of mQ water, and DNA solutions were stored at -20 °C until further procedures.

Designing primers is crucial for genotyping using PCR (Polymerase Chain Reaction) experiments: the website <http://signal.salk.edu/tdnaprimers.2.html> was used to design reverse and forward primers for T-DNA insertion mutants of target genes.

For genotyping, PCR was performed using two separate mixtures containing water, 1x buffer B, 2.5 mM MgCl₂, 0.2 mM dNTP, 0.5 μM of each primer, 2 μl of isolated plant DNA, and 0.05 U/μl DNA polymerase. The first mix contained forward and reverse primers that amplified wild-type alleles and heterozygous plants if they were present, while the second mix consisted of reverse and SALK LBb1.3 primers that targeted specifically T-DNA insertion sites of mutant plants. Genotyping was conducted using a wild-type plant Col-0 together with each mutant line as a reference. The PCR protocol consisted of denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 57°C for 20 seconds, extension at 72°C for 90 seconds. Finally, extension at 72°C for 7 minutes followed.

Subsequently, gel electrophoresis was performed to separate DNA fragments by size. The agarose gel was 1%, consisting of 0.5x TAE buffer, and electrophoresis was carried out at 100 V/cm. Gel images are provided in supplementary materials. Genotyping was repeated for plants involved in gas exchange experiments to verify their genotype accuracy.

3.1.2 Studied plant lines

At the outset, there were a total of twelve lines. After genotyping, seven correct mutants, in which the presence of T-DNA insertion in the target gene was confirmed, were selected for further gas exchange experiments. The mutants involved in the study are described in Table 1.

Table 1. List of mutants used in the experiment. Mutants are shown with their gene identification and the ratio of gene expression in guard cells to mesophyll cells. The expressions in guard and mesophyll cells were obtained from the database (<https://bar.utoronto.ca/>). Both basal and ABA-induced relative expressions in guard cells are presented. The values in the database rely on two studies: Pandey et al. (2010) and Yang et al. (2008).

Gene ID	Gene	Pandey et al. (2010)		Yang et al. (2008)	
		ABA-	ABA+	ABA-	ABA+
AT1G03905 (N560356)	<i>ATP-binding cassette 119</i>	8,4	29,0	7,0	11,0
AT4G03320 (N523579)	<i>Translocon at the inner envelope membrane of chloroplasts</i>	10,5	3,5	25,7	3,4
AT3G09010 (N519665)	<i>Protein kinase superfamily protein</i>	5,3	10,5	4,2	2,5
AT5G24380 (N501348)	<i>YELLOW STRIPE like 2</i>	15,5	15,7	5,6	5,7
AT5G65470 (N529693)	<i>O-fucosyltransferase family protein</i>	4,2	5,7	9,5	5,0
AT3G22104 (N514590)	<i>Phototropic-responsive NPH3 family protein</i>	3,6	2,0	6,5	5,2
AT1G78530 (N559895)	<i>Protein kinase superfamily protein</i>	14,3	9,4	80,1	62,6

3.1.3 Plant growth

Seven verified mutant lines were kept in the growth room for seed propagation. Once collected, seeds were soaked in water in 1.5 ml Eppendorf tubes and stored at +4°C for 2-7 days to facilitate germination. Next, seeds of each mutant were planted through the hole in the glass covers of special gas exchange pots for further gas exchange measurements, as described by Kollist et al. (2007) (Figure 6). The cover glass on the pot served to separate the above-ground rosette from soil and below-ground parts of the plant to ensure accurate gas exchange measurements. The soil mixture contained peat, vermiculite, and water in a volume ratio of 2:1:1.5, with a weight of approximately 270 grams.

Plants were grown in a growth cabinet (AR-66LX and AR-22 L Percival Scientific) in specific conditions: 12/12-h photoperiod, 23°C/19°C temperature, 250/0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, and 70%/80% relative air humidity during day and night, respectively. After seven days, when plants began to sprout from seeds, thinning was performed to ensure that only one plant grew in each pot. Mutants were grown for 24-30 days for gas exchange experiments and watered regularly, once weekly, and the Percival was kept pest-free.



Figure 6. Plants were grown through a hole in the glass covering the pot in Percival growth cabinets. Photographed by Daana Morozova.

3.1.4 Apparatus

A gas exchange measurement device, as described by Kollist et al. (2007), was used for the stomatal conductance measurements (Figure 7). It measures the concentrations of CO₂ and water vapour in air entering and going out of measurement cuvettes, as well as cuvette air temperature. The gas exchange apparatus consists of eight flow-through whole-rosette

cuvettes, allowing the simultaneous measurement eight plants. Stomatal conductance value of one particular plant was measured every 4 or 8 minutes in these experiments, depending on the number of used cuvettes. Since four plants were inserted into cuvettes for high-VPD experiments, measurements of certain plants were taken in every 4 minutes. In the ABA experiment, all eight cuvettes were used.

Before gas exchange measurements, any openings between the plant and the hole in the cover glass were sealed with a special wax. The lamps above chambers were adjusted to obtain light intensity as in growth cabinets ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$). The air temperature was 23.5°C - 24°C in measurement chambers during experiments. The air flow rate in the chambers was $0.000813 \text{ mol s}^{-1}$.

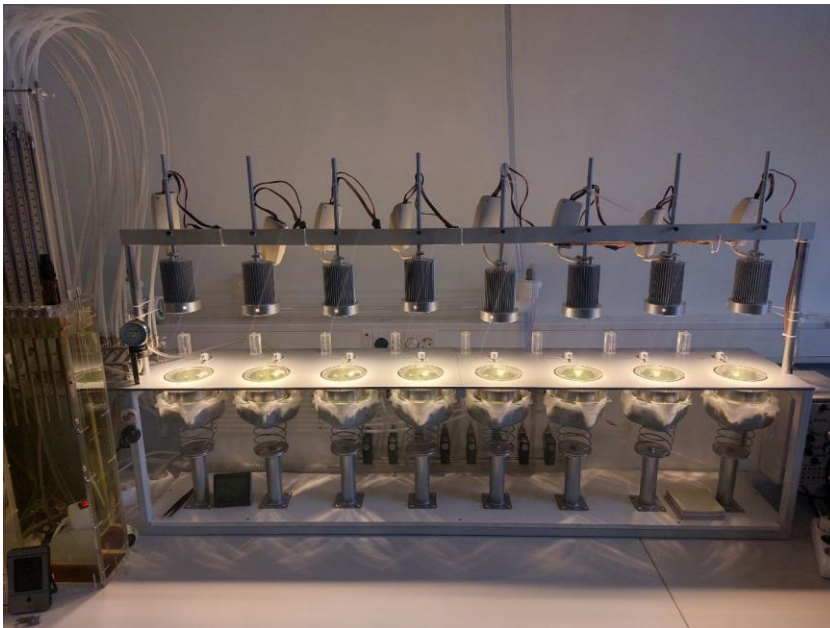


Figure 7. The gas exchange measurement device. In the picture, ABA spraying experiment is done: all eight cuvettes are used for measurements. Photographed by Daana Morozova.

3.1.5 Stomatal Conductance Measurements

In high-VPD experiments, four plants were inserted into four chambers: three mutants and one control plant, which was either wild-type Col-0 or VPD- and ABA-insensitive *ost1-3*. Conditions during stabilization period, which lasted 45-60 minutes were: ambient $\text{CO}_2 \sim 400 \mu\text{L L}^{-1}$, light $250 \mu\text{mol m}^{-2} \text{s}^{-1}$, and relative air humidity $\sim 70\%$. After stabilization, relative air humidity in the ingoing air was sharply reduced (i.e. VPD was increased) to 30-50%, and measurements continued for another 45 minutes. The average VPD during the stabilization period was 0.963 kPa ($\text{SE}=0.0168$), and during treatment, VPD was 1.73 kPa ($\text{SE}=0.0273$). In the ABA experiment, the average value of VPD was 1.03 kPa ($\text{SE}=0.02$).

In ABA-spraying experiments, eight plants were inserted into chambers. After stabilization, plants were removed from chambers one by one, sprayed with a 5 μ M ABA solution, and placed back into measurement cuvette for another 45 minutes of gas exchange measurements. ABA solution consisted of distilled water, 0.012% Silwet L-77, and 0.05% ethanol. Pictures of plants were taken after the experiments, and leaf areas were calculated using ImageJ 1.37v (National Institutes of Health).

The values of transpiration were calculated using special excel program as follows: Transpiration = air flow rate * ($H_2O_{chamber} - H_2O_{reference}$) / leaf area, where

$H_2O_{chamber}$ and $H_2O_{reference}$ are the water vapour concentrations of air coming out of measurement cuvette and entering it, respectively.

Stomatal conductance was calculated using the values of transpiration and leaf temperature, which was found from air temperature.

Experiments to measure stomatal conductance changes in response to high VPD or ABA spraying were performed in three rounds. In total, the number of plants examined per line were as follows: N501348 (6 plants), N514590 (7 plants), N519665 (6 plants), N523579 (5 plants), N529693 (6 plants), N559895 (7 plants), and N560356 (7 plants).

3.1.6 Measurement of Stomatal Density

After conducting gas exchange experiments, stomatal density of leaves was measured. Plants were ~5-6 weeks old. First, the sixth leaf from each plant, as recommended by my supervisor, was collected. The leaf was cut in half, and both the adaxial and abaxial surfaces of the leaves were covered with dental resin (Coltene, Zhermack). Once the resin had hardened and the leaves were removed, resin impressions were covered with nail varnish. The dried nail varnish imprints were affixed to a microscopic glass slide using transparent tape. Next, images of the adaxial and abaxial sides were taken with a microscope (Kern light microscope OBF 133C832 with Kern camera ODC 832). An image with an area of ~0.26 mm² was captured from the middle of the leaf, close to the middle vein. Stomata was counted in ImageJ 1.37v (National Institutes of Health).

Stomatal density (SD) was determined as: $SD = (\text{number of stomata}) / (\text{area of the image})$.

3.1.7 Data analysis

First, the courses of stomatal conductance (G_s) were detected for each plant. Then, the initial and total closures were calculated to characterize the magnitude of stomatal responses for high-VPD and ABA-spraying experiments as:

$$\text{Closure 1 (initial closure)} = G_{\text{spre}} - G_{\text{smid}}$$

$$\text{Closure 2 (total closure)} = G_{\text{spre}} - G_{\text{spost}}$$

In given formulas, G_{spre} represents the average of three last stomatal conductance values of the stabilization period, before any treatment was applied. G_{smid} is the average of two stomatal conductance values at 20 and 24 minutes after high VPD was applied, while in ABA experiment, it is only one value at 16 minutes. G_{spost} represents the average of last two stomatal conductance values after 30-40 minutes of applied treatments (Figure 8).

Statistical analyses were performed with Statistica, version 7.0 (StatSoft). Repeated measures ANOVA of General Linear Model [GLM] procedure was used to test whether the effects of VPD- and ABA- treatments on stomatal conductance values were significant for that line. One-way ANOVA of GLM procedure was used to compare the effect of a line on steady-state stomatal conductance (G_{spre}), stomatal density, VPD-, and ABA-induced stomatal closures. If the main effect of line was significant, post-hoc tests were used to identify what specific groups differed from each other using Tukey's HSD test. Simple regression test of GLM procedure was used to identify the significance of regression line.

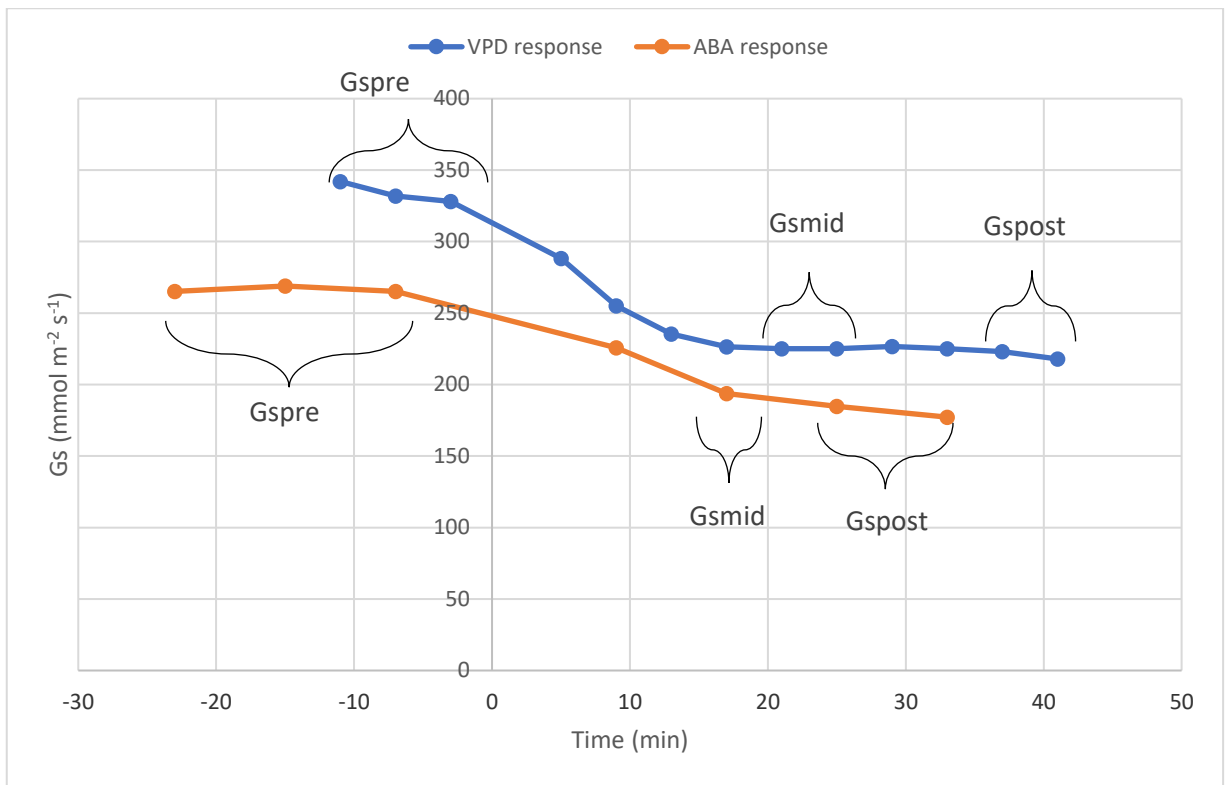


Figure 8. Courses of stomatal conductance (G_s) of wild type Col-0 before and after high VPD or ABA treatment. Figure shows how different G_s values were averaged together to get the values of G_{spre} , G_{smid} and G_{spost} , which were then used to calculate Closures 1 and 2.

3.3 RESULTS

3.3.1 Stomatal closure due to high VPD

This experiment studied the stomatal conductance of different plant lines under both low and high Vapour Pressure Deficit (VPD) conditions (Figure 9). Under stable low VPD conditions (G_{spre}), stomatal conductance ranged from 250 to 380 $\text{mmol m}^{-2} \text{s}^{-1}$, with lower values detected in N559895 and N519665 and higher values in N501348 and *ost1-3*. Even though the main effect of line was significant, post-hoc test revealed no significant differences in G_{spre} between the lines. Under high VPD (G_{spost}), stomatal conductance decreased significantly in all lines except *ost1-3*.

Additionally, the decreases in stomatal conductance under high VPD were calculated to characterize initial and total stomatal closures, respectively (Figure 10). *Ost1-3* demonstrated no stomatal closure to high VPD and this response was significantly different from all other lines, which displayed stomata closure in response to high VPD. Although there were differences between the lines as regards closures (e.g., line N501348 had the largest closure, and line N523579 had the lowest), they were not statistically significant. Figure 10 also shows that for the most part, stomatal closure took place during the first 20-24 minutes at high VPD, since the values of Closure1 and Closure2 were very similar for most genotypes (repeated measures ANOVA did not detect significant differences between Closures 1 and 2 for any plant line).

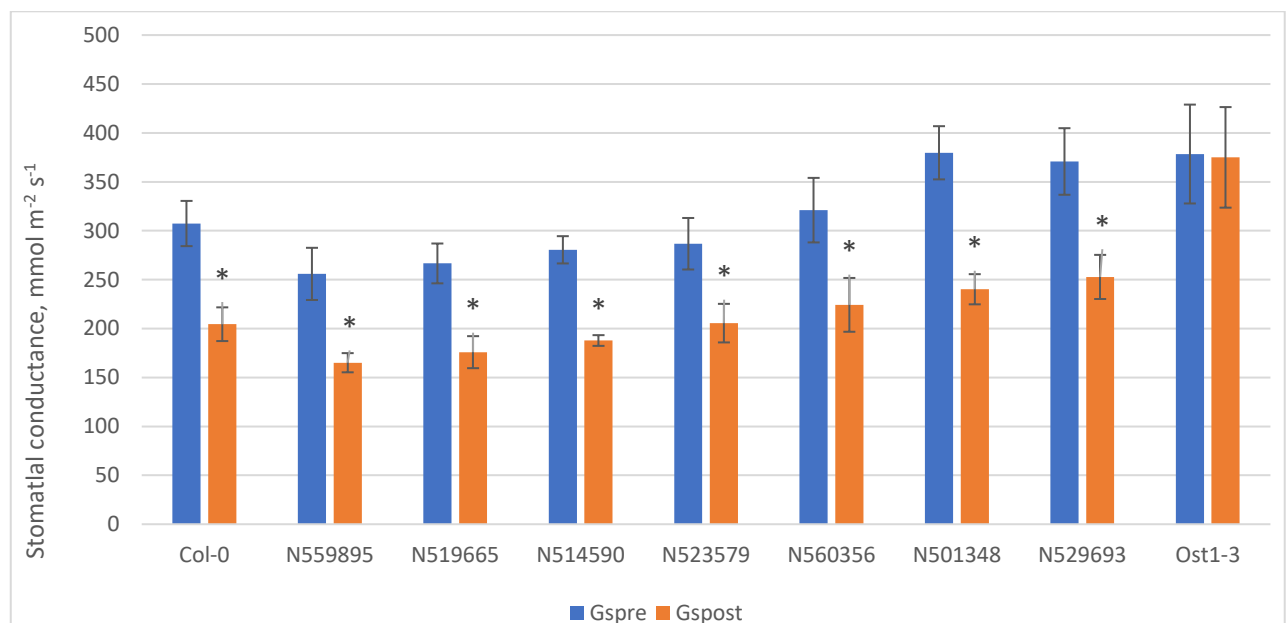


Figure 9. Stomatal conductance of wild-type Col-0 and mutants under low and high VPD conditions (G_{spre} and G_{spost} , respectively). No statistically significant differences were found among the nine

plant lines in initial stomatal conductance, G_{spre} (one-way ANOVA and Tukey HSD test). The stars above G_{spost} values indicate a significant difference between G_s values measured under low and high VPD conditions (G_{spre} and G_{spost}) for that plant line (repeated-measures ANOVA).

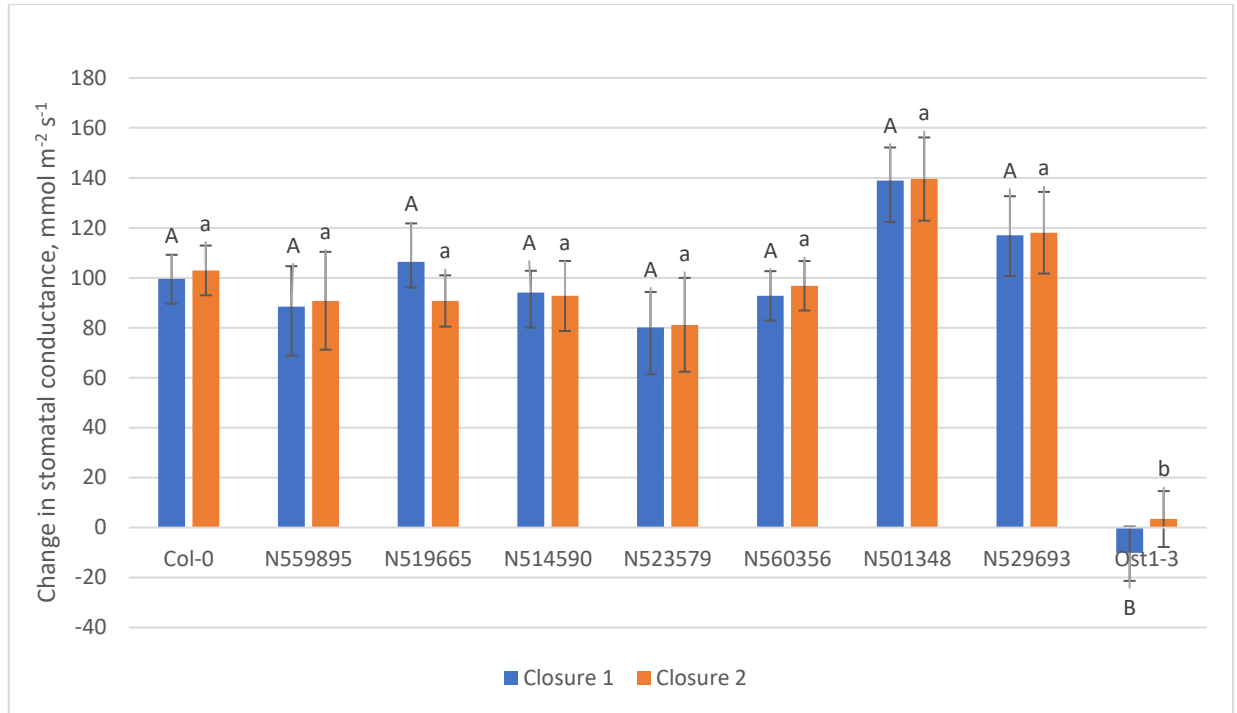


Figure 10. High VPD-induced initial and total stomatal closures of wild-type and mutants. Capital letters indicate statistically significant differences between studied lines in initial closing (Closure 1), while small letters in total closing (Closure 2) (one-way ANOVA and Tukey HSD test).

3.3.2 Stomatal closure induced by ABA spraying

This experiment examined the effect of the drought hormone abscisic acid (ABA) on wild-type and different *Arabidopsis* mutants' stomatal conductance, which was measured both before and after spraying with ABA.

In the ABA experiment, the main effect of line on G_{spre} was significant and post-hoc test showed that G_{spre} values of line N519665 and *ost1-3* were significantly different from each other. (Figure 11). After ABA spraying, stomatal conductance decreased significantly in all plant lines except *ost1-3*.

ABA-induced initial and total stomata closures were also calculated (Figure 12). ABA-insensitive *ost1-3* displayed no ABA-induced stomatal closure. All other plant lines closed their stomata in response to ABA. However, plant lines N519665 and N560356 showed larger variation in initial stomatal response to ABA treatment: their initial stomatal closures were not significantly different from *ost1-3*. The total closure of N519665 was not

significantly different from *ost1-3* either (Figure 12). Even though for most plant lines Closure2 was larger than Closure1, repeated measures ANOVA did not detect any significant differences between the values of Closure1 and Closure2 for any of the lines.

A correlation was detected between the total VPD-induced closure and ABA-induced closure (Figure 13), showing that the stronger the stomatal response to high VPD, the stronger the ABA response.

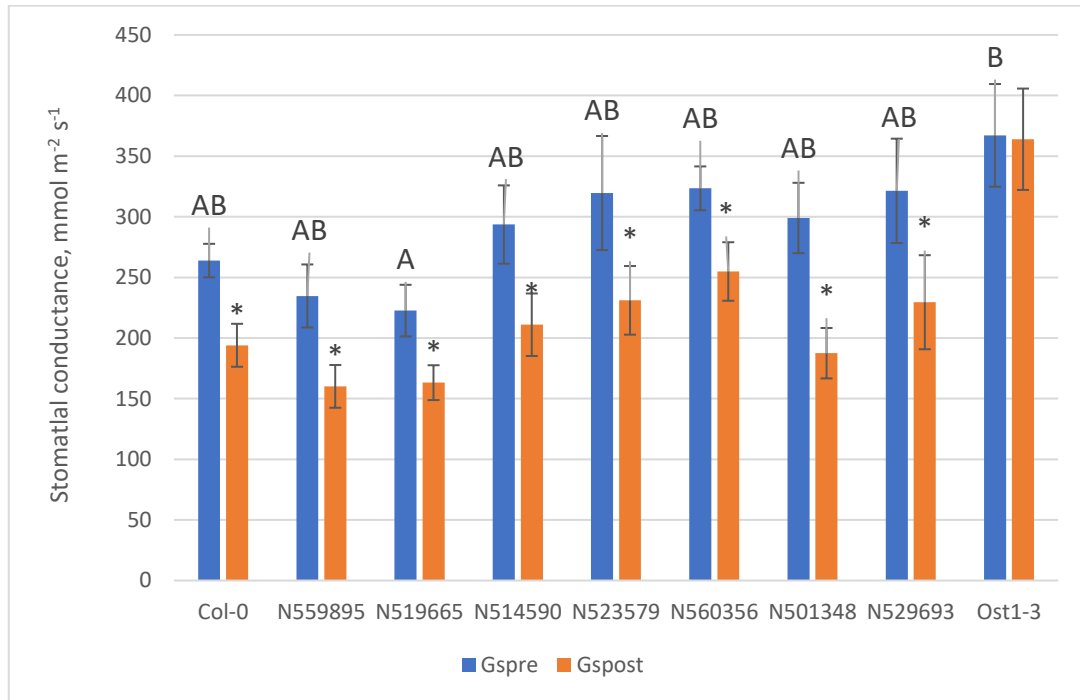


Figure 11. Values of stomatal conductance of wild-type and mutants before and after ABA treatment (Gspre and Gspost). Different capital letters above Gspre values indicate statistically significant differences in Gspre between the plant lines (one-way ANOVA and Tukey HSD test). Stars above Gspost values indicate significant differences between stomatal conductance values before and after ABA treatment for that plant line (Gspre and Gspost, repeated measurements ANOVA).

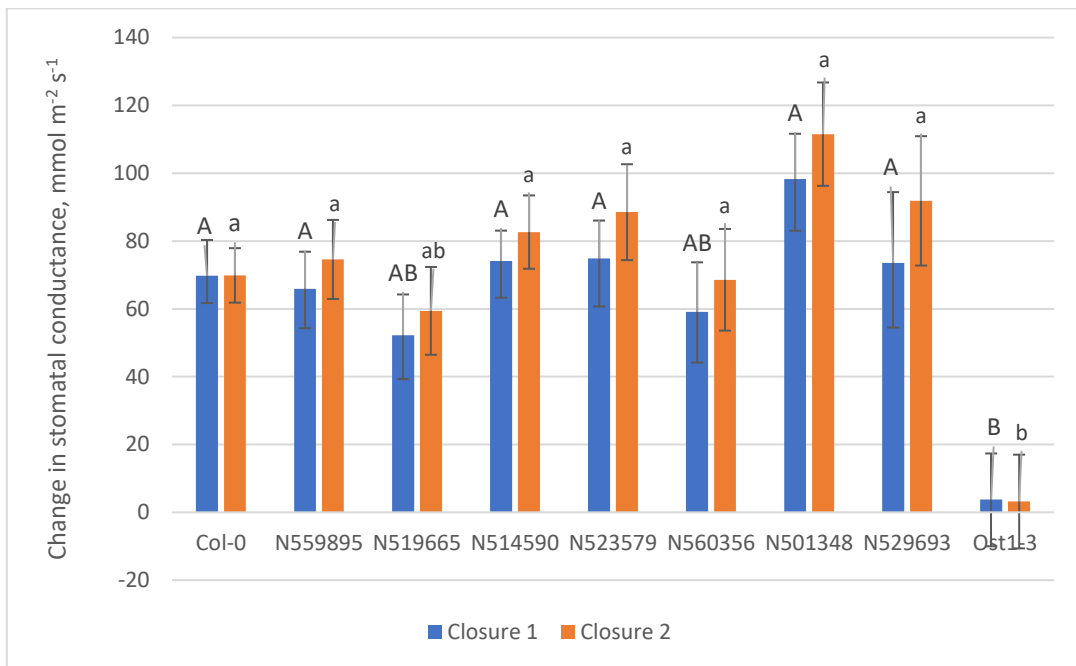


Figure 12. ABA-induced initial and total stomatal closures of wild-type and mutants. Capital letters indicate statistically significant differences in the initial stomatal closure (Closure 1), while small letters in the total stomatal closure (Closure 2; one-way ANOVA and Tukey HSD test).

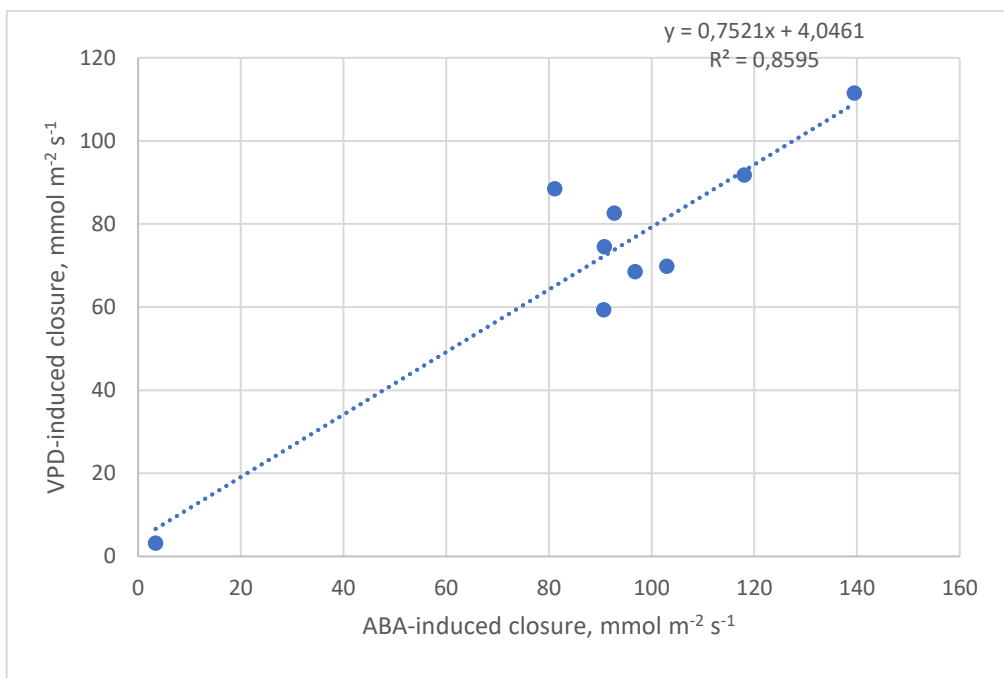


Figure 13. The correlation between total VPD- and ABA-induced closures (Closure 2). The correlation is significant with *ost1-3* included ($P < 0.005263$) and without it ($P < 0.003755$).

3.3.3 Stomatal density

In this experiment, the abaxial and adaxial stomatal densities of the studied lines were detected. (Figure 14). There was a significant main effect of leaf side on stomatal density:

higher density of stomata was found on the abaxial compared to the adaxial leaf surface for all studied lines. Although there were differences in the values of abaxial and adaxial stomatal densities between the lines, e.g. N523579 and N501348, these were not statistically significant.

Furthermore, the ratio of adaxial to abaxial stomatal density was calculated (Figure 15). Again, no statistically significant differences in the adaxial to abaxial stomatal ratio between lines were found.

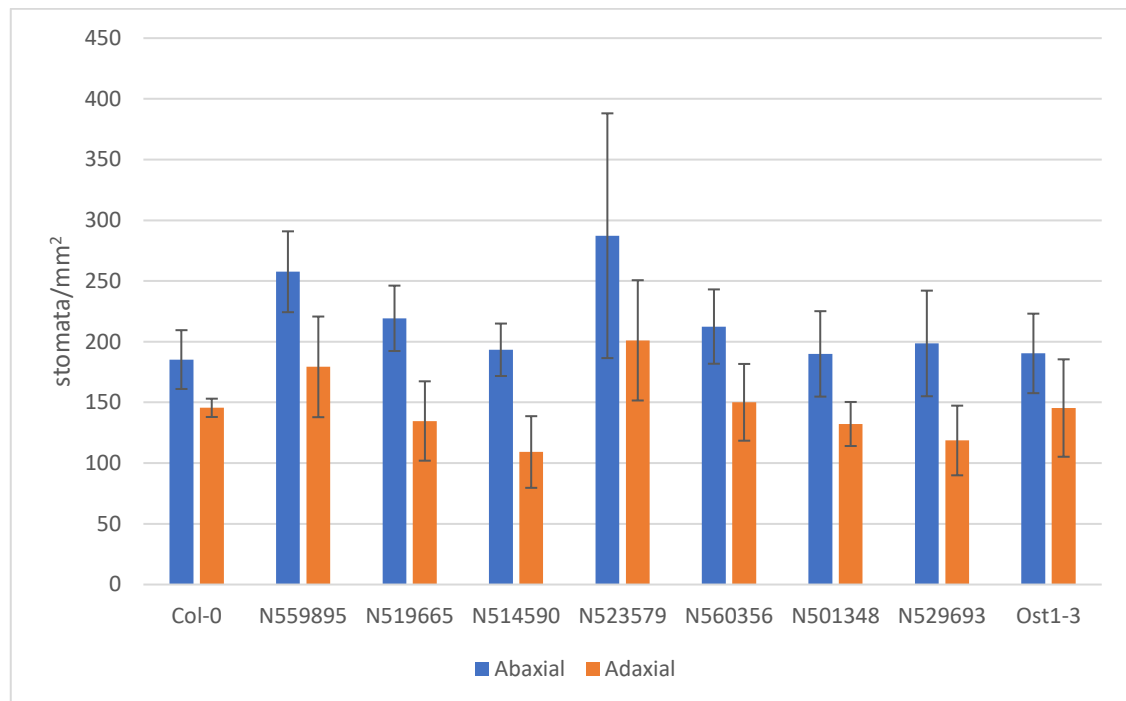


Figure 14. Stomatal densities of abaxial and adaxial leaf sides in the studied mutants. No statistically significant differences were found between the lines as regards their abaxial and adaxial densities (GLM and Tukey HSD test).

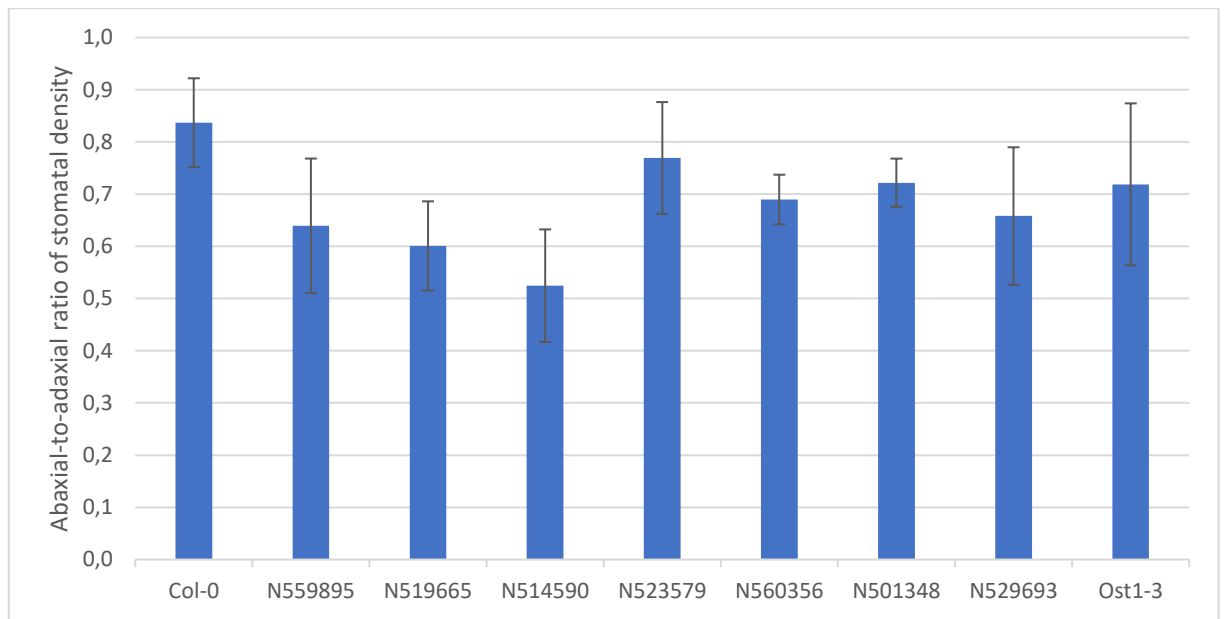


Figure 15. The abaxial to adaxial stomatal density ratios of mutants and wild-type. No statistically significant differences were found between the lines (one-way ANOVA and Tukey HSD test).

3.4 DISCUSSION

This study aimed to learn more about VPD-induced stomatal signaling pathways using loss-of-function mutants of seven genes. These genes are more expressed in guard cells relative to mesophyll cells, and they are coding proteins that represent putative substrates of OST1 (Wang et al., 2020), an important regulator of VPD-induced stomatal closure.

Our experiments confirmed that, as a rule, plants close their stomata in response to high VPD to minimize water loss and prevent plant dehydration (Buckley, 2016; López et al., 2021). Namely, when plants are exposed to high VPD, their transpiration increases due to the rising difference between air and leaf water vapour concentrations. It results in a considerable water loss, counteracted by rapid stomatal closure under high VPD (López et al., 2021). Similarly, exogenous ABA treatment resulted in the stomatal closure of all mutants in our experiments. Only stomatal conductance of mutants with disrupted OPEN STOMATA 1 (OST1), an essential protein kinase in stomatal regulation pathways, did not decrease under high VPD and ABA treatments since *ost1-3* is insensitive to VPD and ABA. Loss-of-function mutants of OST1-3 demonstrated the highest stomatal conductance among nine plant lines in both VPD and ABA experiments. Its average stomatal conductance was 1.3 times higher than in Col-0, and it changed only a little when high VPD conditions were applied or plants were sprayed with ABA. It confirms that OST1 is a key component in VPD- and ABA-mediated stomatal closure.

A significant correlation was detected between the total VPD-induced and ABA-induced stomatal closures. It suggests that the VPD response partially happens via the ABA pathway. Moreover, the VPD response turned out to be stronger than the ABA response. According to the linear regression formula, the VPD response of 100 units corresponds to the ABA response of 79 mmol m⁻² s⁻¹. This indicates that other components may contribute to the VPD response in addition to ABA (Jalakas et al., 2021; Merilo et al., 2017). In the study conducted by Merilo et al. (2017), ABA signaling and synthesis mutants responded to VPD changes, suggesting either the presence of the passive hydraulic response or ABA-independent component of OST1-activation in VPD-response. Moreover, experiments with guard cell-specific ABA-insensitive Arabidopsis plants (GCabi) showed that ABA does not play an important role in VPD-induced closure, suggesting ABA-independent stomata closure (Yaaran et al., 2019).

To investigate the genetic basis of stomatal conductance regulation, seven plant lines with defective genes listed in Table 1 were used to determine if their absence affected stomatal conductance.

- **At1G03905 (N560356)**: Annotated as ATP-binding cassette protein ABCI19, which interacts with ABCI20 and ABCI21 to regulate the cytokinin responses in early seedling development. This regulation occurs at the endoplasmic reticulum under the control of HY5 in response to light (Kim et al., 2020).
- **AT4G03320 (N523579)**: Defective in TIC20, a protein, which functions at the inner envelope membrane of chloroplasts and participates in photosynthesis (Campbell et al., 2014). In guard cells, ATP is produced in photosynthesis, and it provides plasma membrane H⁺-ATPase with energy to move hydrogen ions, which in turn is necessary to open stomata (Tominaga et al., 2001).
- **AT3G09010 (N519665)** and **AT1G78530 (N559895)**: Disrupted in protein kinases, which play important roles in the perception of biotic and abiotic agents, light quality and quantity, and plant hormones (Lehti-Shiu & Shiu, 2012). Protein kinases are also involved in regulating activities of proteins involved in stomatal regulation (Wang et al., 2007); however, these two kinases had no significant role in stomatal closure pathways.
- **AT5G24380 (N501348)**: Impaired in the YELLOW STRIPE like 2 (YSL2), responsible for the transport of iron (Fe) and copper (Cu), when they form a complex with nicotianamine (NA) (DiDonato et al., 2004). These heavy metals are essential for different enzymes involved in mitochondrial respiration, photosynthesis, and oxidative stress protection (Burkhead et al., 2009). In the study conducted by DiDonato et al. (2004), the mutants lacking YSL2 did not show any noticeable phenotypes, which might indicate that other proteins in the YSL family are compensating for the absence of YSL2.
- **AT5G65470 (N529693)**: Mutated in O-fucosyltransferase, which modifies DELLA protein, a repressor of phytohormone gibberellin (GA) signaling. O-fucosylation activates DELLA by interaction with regulators in brassinosteroid- and light-signaling pathways (Zentella et al., 2017). DELLA proteins regulate basal stomata aperture in Arabidopsis (Sukiran et al., 2020).
- **AT3G22104 (N514590)**: Defective in the phototropic-responsive NPH3 family protein, which is involved in stomatal blue light response signaling pathway and

interacts with the blue light photoreceptor NPH1 (NPH3 Phototropic-responsive NPH3 Family Protein [Arabidopsis Thaliana (Thale Cress)] - Gene - NCBI, n.d.-b). Blue light promotes stomatal opening by activating a plasma membrane H⁺ pump in guard cells (Assmann et al., 1985).

The study revealed that the mutants defective in those genes showed wildtype-like stomatal responses to high VPD and ABA spraying, thus these genes do not contribute considerably to stomatal closure. This result can be explained in several ways:

- 1) Single gene knockouts will not enable to reveal the genes/proteins involved in stomatal regulation due to potential redundancy in the protein function, particularly in the case of large protein families.
- 2) Minor to moderate changes in stomatal regulation may have been missed due to the small sample size (5-7 plants per line). For example, the higher steady-state stomatal conductance of lines N501348 and N529693 could have turned statistically significant in the case of a higher number of replications.
- 3) The genes to be tested were chosen using the Wang et al. (2020) database of putative substrates of OST1 and relative guard cell versus mesophyll cell expression using the database <https://bar.utoronto.ca/>. Both these databases should be regarded as showing preliminary results.

Moreover, my analysis revealed that studied genes were not involved in stomatal development either. While stomatal density measurements detected no significant differences between genotypes in either abaxial or adaxial stomatal density, more stomata were detected on abaxial than adaxial leaf side, a common result in *Arabidopsis* (Willmer & Fricker, 1996b). Stomatal conductance, a key determinant of gas exchange, is influenced by the stomatal density and aperture (Jalakas et al., 2018). For instance, in the study by Büssis et al. (2006), the lower stomata density was compensated by increased stomata aperture and *vice versa*, maintaining similar levels of stomata conductance to the wild-type. Additionally, the mutant *ost1-3* exhibited high stomatal conductance in our experiment because of the larger aperture width and unchanged stomatal density.

In conclusion, the research aimed to identify new components involved in VPD-induced stomata closure. Based on measurements of stomatal conductance in response to high VPD and ABA treatment in gas exchange experiments, it can be concluded that studied genes do not participate in the stomatal closure caused by high VPD or ABA. Additionally, the

stomatal response was larger in high VPD experiments than in ABA-spraying experiments, proposing that ABA is partially involved in VPD signaling and that some other components besides ABA are involved in VPD-pathway. Even though gas exchange experiments did not reveal any major role for studied proteins in stomatal VPD- and ABA-induced closure, these proteins may still participate in stomatal regulation, e.g., in stomatal opening or light/dark responses. The stomatal regulation mechanism under rising VPD levels is still not fully understood, as the pathway above OST1 activation is unclear. Understanding how current climate change and increasing temperatures worldwide affect plants is essential. This knowledge might be used in plant breeding in order to modify plants better suited to soil and atmospheric drought, as well as in crop agronomy to optimize irrigation systems in the fields and contribute to feasible farming.

SUMMARY

The Earth's climate has undergone an increase in global temperatures, resulting in a rise in atmospheric vapour pressure deficit (VPD). High VPD means the air is relatively dry, negatively impacting plants. When plants grow in high VPD conditions, they close their stomata, leading to decreased stomatal conductance and photosynthesis and changes in stomatal anatomy, root growth, and plant biochemical composition, ultimately reducing biomass production and yield. OST1 is the primary protein kinase involved in stomatal closure under high VPD. It is still unclear whether all components participating in the VPD signaling pathway above the activation of OST1 are known.

This study aims to identify new components of the stomatal closure pathway mediated by high VPD.

The central questions for this research were:

1. Are seven highly guard cell-expressed genes reported as putative OST1 substrates selected for the study involved in VPD-induced stomata closure?
2. Are these disrupted genes involved in determining steady-state stomatal conductance?

Arabidopsis genes to be knocked out were selected based on the OST1 interactor table by Wang et al. (2020) and their expression levels in guard cells. The stomatal conductance and its change were measured in gas exchange experiments in response to high VPD and ABA treatment. Results showed no statistically significant differences between the studied lines and wild type Col-0 as regards steady-state stomatal conductance values or stomatal VPD- and ABA-responses, meaning that these genes are not involved in the VPD- or ABA-signaling pathway. Moreover, the stomatal response in high VPD experiments was higher than in ABA-spraying experiments, suggesting that ABA is partially involved in VPD signaling. To conclude, this study showed no major role of studied proteins in stomatal closure. However, their role in stomatal opening is not excluded. The study aims to fill the gaps in our knowledge of stomata closure in response to dry air, which is valuable for plant adaptation for future environment, crop breeding, optimizing water delivery systems, and ultimately contributing to sustainable agriculture. Further research on different disrupted genes is needed due to the current study's limitations.

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REFERENCES

- Ackerson, R. C.** (1982). Synthesis and movement of abscisic acid in Water-Stressed cotton leaves. *Plant Physiology*, *69*(3), 609–613. <https://doi.org/10.1104/pp.69.3.609>
- Assmann, S. M., Simoncini, L., & Schroeder, J. I.** (1985). Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba*. *Nature*, *318*(6043), 285–287. <https://doi.org/10.1038/318285a0>
- Betts, A. K., Desjardins, R. L., Worth, D. E., & Beckage, B.** (2014). Climate coupling between temperature, humidity, precipitation, and cloud cover over the Canadian Prairies. *Journal of Geophysical Research. Atmospheres*, *119*(23). <https://doi.org/10.1002/2014jd022511>
- Brodribb, T. J., & Holbrook, N. M.** (2004). Diurnal depression of leaf hydraulic conductance in a tropical tree species. *Plant, Cell & Environment/Plant, Cell and Environment*, *27*(7), 820–827. <https://doi.org/10.1111/j.1365-3040.2004.01188.x>
- Buckley, T. N.** (2016). Stomatal responses to humidity: has the ‘black box’ finally been opened? *Plant, Cell & Environment*, *39*(3), 482–484. <https://doi.org/10.1111/pce.12651>
- Burkhead, J. L., Reynolds, K. a. G., Abdel-Ghany, S. E., Cohu, C. M., & Pilon, M.** (2009). Copper homeostasis. *New Phytologist*, *182*(4), 799–816. <https://doi.org/10.1111/j.1469-8137.2009.02846.x>
- Büßis, D., Von Groll, U., Fisahn, J., & Altmann, T.** (2006). Stomatal aperture can compensate altered stomatal density in *Arabidopsis thaliana* at growth light conditions. *Functional Plant Biology*, *33*(11), 1037. <https://doi.org/10.1071/fp06078>
- Campbell, G. S., & Norman, J. M.** (1998). An introduction to environmental biophysics. In *Springer eBooks*. <https://doi.org/10.1007/978-1-4612-1626-1>
- Campbell, J. H., Hoang, T., Jelokhani-Niaraki, M., & Smith, M. D.** (2014). Folding and self-association of atTic20 in lipid membranes: implications for understanding

- protein transport across the inner envelope membrane of chloroplasts. *BMC Biochemistry*, 15(1). <https://doi.org/10.1186/s12858-014-0029-y>
- Chen, L., Wu, Z., & Hou, S.** (2020). SPEECHLESS speaks loudly in stomatal development. *Frontiers in Plant Science*, 11. <https://doi.org/10.3389/fpls.2020.0011>
- Choat, B., Brodribb, T. J., Brodersen, C. R., Duursma, R. A., López, R., & Medlyn, B. E.** (2018). Triggers of tree mortality under drought. *Nature*, 558(7711), 531–539. <https://doi.org/10.1038/s41586-018-0240-x>
- Christmann, A., Moes, D., Himmelbach, A., Yang, Y., Tang, Y. Y., & Grill, E.** (2006). Integration of Abscisic Acid Signalling into Plant Responses. *Plant Biology*, 8(3), 314–325. <https://doi.org/10.1055/s-2006-924120>
- Cutler, S. R., Rodriguez, P. L., Finkelstein, R., & Abrams, S. R.** (2010). Abscisic acid: emergence of a core signaling network. *Annual Review of Plant Biology*, 61(1), 651–679. <https://doi.org/10.1146/annurev-arplant-042809-112122>
- Daszkowska-Golec, A., & Szarejko, I.** (2013). Open or close the gate – Stomata action under the control of phytohormones in drought stress conditions. *Frontiers in Plant Science*, 4. <https://doi.org/10.3389/fpls.2013.00138>
- DiDonato, R. J., Roberts, L. A., Sanderson, T. L., Easley, R. B., & Walker, E. L.** (2004). Arabidopsis Yellow Stripe-Like2 (YSL2): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine–metal complexes. *Plant Journal*, 39(3), 403–414. <https://doi.org/10.1111/j.1365-313x.2004.02128.x>
- Driesen, E., Van Den Ende, W., De Proft, M., & Saeys, W.** (2020). Influence of environmental factors light, CO₂, temperature, and relative humidity on stomatal opening and development: a review. *Agronomy*, 10(12), 1975. <https://doi.org/10.3390/agronomy10121975>
- Ficklin, D. L., & Novick, K. A.** (2017). Historic and projected changes in vapor pressure deficit suggest a continental-scale drying of the United States atmosphere. *Journal of Geophysical Research: Atmospheres*, 122(4), 2061–2079. <https://doi.org/10.1002/2016jd025855>
- Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., Ache, P., Matschi, S., Liese, A., Al-Rasheid, K. a. S., Romeis, T., & Hedrich, R.** (2009). Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling

kinase-phosphatase pair. *Proceedings of the National Academy of Sciences of the United States of America*, 106(50), 21425–21430.
<https://doi.org/10.1073/pnas.0912021106>

Hartmann, D. L., Tank, A. M. G. K., Rusticucci, M., Alexander, L. V., Brönnimann, S., Charabi, Y., Dentener, F., Dlugokencky, E. J., Easterling, D. R., Kaplan, A., Soden, B. J., Thorne, P., Wild, M., & Zhai, P. (2014). Observations: atmosphere and surface. In *Cambridge University Press eBooks* (pp. 159–254).
<https://doi.org/10.1017/cbo9781107415324.008>

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

Hsu, P. K., Takahashi, Y., Merilo, E., Costa, A., Zhang, L., Kernig, K., Lee, K. H., & Schroeder, J. I. (2021). Raf-like kinases and receptor-like (pseudo)kinase GHR1 are required for stomatal vapor pressure difference response. *Proceedings of the National Academy of Sciences of the United States of America*, 118(47).
<https://doi.org/10.1073/pnas.2107280118>

Hsu, P.-K., Takahashi, Y., Munemasa, S., Merilo, E., Laanemets, K., Waadt, R., Pater, D., Kollist, H., & Schroeder, J. I. (2018). Abscisic acid-independent stomatal CO₂ signal transduction pathway and convergence of CO₂ and Aba signaling downstream of ost1 kinase. *Proceedings of the National Academy of Sciences*, 115(42). <https://doi.org/10.1073/pnas.1809204115>

Jalakas, P. (2019). *Unravelling signalling pathways contributing to stomatal conductance and responsiveness*. <https://dspace.ut.ee/handle/10062/64780>

Jalakas, P., Merilo, E., Kollist, H., & Brosché, M. (2018). ABA-mediated regulation of stomatal density is OST1-independent. *Plant Direct*, 2(9).
<https://doi.org/10.1002/pld3.82>

Jalakas, P., Nuhkat, M., Vahisalu, T., Merilo, E., Brosché, M., & Kollist, H. (2021). Combined action of guard cell plasma membrane rapid- and slow-type anion

channels in stomatal regulation. *Plant Physiology*, 187(4), 2126–2133.
<https://doi.org/10.1093/plphys/kiab202>

- Jalakas, P., Takahashi, Y., Waadt, R., Schroeder, J., & Merilo, E.** (2021). Molecular mechanisms of stomatal closure in response to rising vapour pressure deficit. *New Phytologist*, 232(2), 468–475. <https://doi.org/10.1111/nph.17592>
- Kim, A., Chen, J., Khare, D., Jin, J., Yamaoka, Y., Maeshima, M., Zhao, Y., Martinoia, E., Hwang, J., & Lee, Y.** (2020). Non-intrinsic ATP-binding cassette proteins ABCI19, ABCI20 and ABCI21 modulate cytokinin response at the endoplasmic reticulum in *Arabidopsis thaliana*. *Plant Cell Reports*, 39(4), 473–487.
<https://doi.org/10.1007/s00299-019-02503-0>
- Kim, T., Böhmer, M., Hu, H., Nishimura, N., & Schroeder, J. I.** (2010). Guard Cell Signal Transduction Network: Advances in understanding Abscisic Acid, CO₂, and CA₂+Signaling. *Annual Review of Plant Biology*, 61(1), 561–591.
<https://doi.org/10.1146/annurev-arplant-042809-112226>
- Kollist, H., Nuhkat, M., & Roelfsema, M. R. G.** (2014). Closing gaps: linking elements that control stomatal movement. *New Phytologist (Print)*, 203(1), 44–62.
<https://doi.org/10.1111/nph.12832>
- Kollist, T., Moldau, H., Rasulov, B., Oja, V., Rämme, H., Hüve, K., Jaspers, P., Kangasjärvi, J., & Kollist, H.** (2007). A novel device detects a rapid ozone-induced transient stomatal closure in intact *Arabidopsis* and its absence in *abi2* mutant. *Physiologia Plantarum*, 129(4), 796–803. <https://doi.org/10.1111/j.1399-3054.2006.00851.x>
- Lehti-Shiu, M. D., & Shiu, S. H.** (2012). Diversity, classification and function of the plant protein kinase superfamily. *Philosophical Transactions - Royal Society. Biological Sciences*, 367(1602), 2619–2639. <https://doi.org/10.1098/rstb.2012.0003>
- Lin, Z., Li, Y., Zhang, Z., Liu, X., Hsu, C., Du, Y., Sang, T., Chen, Z., Wang, Y., Satheesh, V., Pratibha, P., Zhao, Y., Song, C., Tao, W. A., Zhu, J., & Wang, P.** (2020). A RAF-SnRK2 kinase cascade mediates early osmotic stress signaling in

higher plants. *Nature Communications*, 11(1). <https://doi.org/10.1038/s41467-020-14477-9>

- Liu, H., Song, S., Zhang, H., Li, Y., Niu, L., Zhang, J., & Wang, W.** (2022). Signaling transduction of ABA, ROS, and Ca^{2+} in plant stomatal closure in response to drought. *International Journal of Molecular Sciences*, 23(23), 14824. <https://doi.org/10.3390/ijms232314824>
- Liu, Q., & Qin, G.** (2013). Generation and characterization of Arabidopsis T-DNA insertion mutants. In *Methods in molecular biology* (pp. 241–258). https://doi.org/10.1007/978-1-62703-580-4_13
- Lobell, D. B., Roberts, M. J., Schlenker, W., Braun, N., Little, B. B., Rejesus, R. M., & Hammer, G. L.** (2014). Greater sensitivity to drought accompanies maize yield increase in the U.S. Midwest. *Science*, 344(6183), 516–519. <https://doi.org/10.1126/science.1251423>
- López, J. R., Way, D. A., & Sadok, W.** (2021). Systemic effects of rising atmospheric vapor pressure deficit on plant physiology and productivity. *Global Change Biology*, 27(9), 1704–1720. <https://doi.org/10.1111/gcb.15548>
- Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., & Grill, E.** (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science*, 324(5930), 1064–1068. <https://doi.org/10.1126/science.1172408>
- McAdam, S. a. M., & Brodribb, T. J.** (2015). The evolution of mechanisms driving the stomatal response to vapor pressure deficit. *Plant Physiology*, 167(3), 833–843. <https://doi.org/10.1104/pp.114.252940>
- McAinsh, M. R., Brownlee, C., & Hetherington, A. M.** (1990). Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature*, 343(6254), 186–188. <https://doi.org/10.1038/343186a0>
- Merilo, E., Yarmolinsky, D., Jalakas, P., Parik, H., Tulva, I., Rasulov, B., Kilk, K., & Kollist, H.** (2017). Stomatal VPD response: There is more to the story than ABA. *Plant Physiology*, 176(1), 851–864. <https://doi.org/10.1104/pp.17.00912>
- Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A., & Giraudat, J.** (2001). The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the

abscisic acid signalling pathway. *Plant Journal* (Print), 25(3), 295–303.
<https://doi.org/10.1046/j.1365-313x.2001.00965.x>

Mustilli, A., Merlot, S., Vavasseur, A., Fenzi, F., & Giraudat, J. (2002). Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *The Plant Cell*, 14(12), 3089–3099. <https://doi.org/10.1105/tpc.007906>

Novick, K. A., Ficklin, D. L., Grossiord, C., Konings, A. G., Martínez-Vilalta, J., Sadok, W., Trugman, A. T., Williams, A. P., Wright, A. J., Abatzoglou, J. T., Dannenberg, M. P., Gentine, P., Guan, K., Johnston, M. R., Lowman, L., Moore, D. J., & McDowell, N. G. (2024). The impacts of rising vapour pressure deficit in natural and managed ecosystems. *Plant, Cell & Environment*.
<https://doi.org/10.1111/pce.14846>

NPH3 Phototropic-responsive NPH3 family protein [Arabidopsis thaliana (thale cress)] – Gene - *NCBI*. (n.d).
<https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=836554>

Pandey, S., Wang, R., Wilson, L., Li, S., Zhao, Z., Gookin, T. E., Assmann, S. M., & Albert, R. (2010). Boolean modeling of transcriptome data reveals novel modes of heterotrimeric G-protein action. *Molecular Systems Biology*, 6(1).
<https://doi.org/10.1038/msb.2010.28>

Pillitteri, L. J., & Torii, K. U. (2012). Mechanisms of stomatal development. *Annual Review of Plant Biology*, 63(1), 591–614. <https://doi.org/10.1146/annurev-arplant-042811-105451>

Quarrie, S. A., & Jones, H. G. (1977). Effects of abscisic acid and water stress on development and morphology of wheat. *Journal of Experimental Botany*, 28(1), 192–203. <https://doi.org/10.1093/jxb/28.1.192>

Schönbeck, L., Grossiord, C., Geßler, A., Gisler, J., Meusbürger, K., D’Odorico, P., Rigling, A., Salmon, Y., Stocker, B. D., Zweifel, R., & Schaub, M. (2022). Photosynthetic acclimation and sensitivity to short- and long-term environmental changes in a drought-prone forest. *Journal of Experimental Botany*, 73(8), 2576–2588. <https://doi.org/10.1093/jxb/erac033>

- Schopfer, P., & Plachy, C.** (1985). Control of seed germination by abscisic acid. *Plant Physiology*, 77(3), 676–686. <https://doi.org/10.1104/pp.77.3.676>
- Schroeder, J. I., & Hagiwara, S.** (1989). Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature*, 338(6214), 427–430. <https://doi.org/10.1038/338427a0>
- Sukiran, N. A., Steel, P. G., & Knight, M. R.** (2020). Basal stomatal aperture is regulated by GA-DELLAs in *Arabidopsis*. *Journal of Plant Physiology*, 250, 153182. <https://doi.org/10.1016/j.jplph.2020.153182>
- Tominaga, M., Kinoshita, T., & Shimazaki, K.** (2001). Guard-Cell chloroplasts provide ATP required for H⁺ pumping in the plasma membrane and stomatal opening. *Plant & Cell Physiology/Plant and Cell Physiology*, 42(8), 795–802. <https://doi.org/10.1093/pcp/pce101>
- Ton, J., Flors, V., & Mauch-Mani, B.** (2009). The multifaceted role of ABA in disease resistance. *Trends in Plant Science*, 14(6), 310–317. <https://doi.org/10.1016/j.tplants.2009.03.006>
- Tulva, I., Vålbe, M., & Merilo, E.** (2023). Plants lacking OST1 show conditional stomatal closure and wildtype-like growth sensitivity at high VPD. *Physiologia Plantarum*, 175(5). <https://doi.org/10.1111/ppl.14030>
- Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., & Shinozaki, K.** (2009). Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 106(41), 17588–17593. <https://doi.org/10.1073/pnas.0907095106>
- Vahisalu, T., Kollist, H., Wang, Y., Nishimura, N., Chan, W., Valerio, G., Lamminmäki, A., Brosché, M., Moldau, H., Desikan, R., Schroeder, J. I., & Kangasjärvi, J.** (2008). SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature*, 452(7186), 487–491. <https://doi.org/10.1038/nature06608>

- Vahisalu, T., Puzõrjova, I., Brosché, M., Valk, E., Lepiku, M., Moldau, H., Pechter, P., Wang, Y., Lindgren, O., Salojärvi, J., Loog, M., Kangasjärvi, J., & Kollist, H.** (2010). Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. *Plant Journal*, *62*(3), 442–453. <https://doi.org/10.1111/j.1365-3113x.2010.04159.x>
- Vicente-Serrano, S. M., Nieto, R., Azorín-Molina, C., Drumond, A., Kenawy, A. E., Domínguez-Castro, F., Tomàs-Burguera, M., & Peña-Gallardo, M.** (2018). Recent changes of relative humidity: regional connections with land and ocean processes. *Earth System Dynamics Discussions*, *9*(2), 915–937. <https://doi.org/10.5194/esd-9-915-2018>
- Wang, H., Ngwenyama, N., Liu, Y., Walker, J. C., & Zhang, S.** (2007). Stomatal development and patterning are regulated by environmentally responsive Mitogen-Activated Protein kinases in Arabidopsis. *the Plant Cell*, *19*(1), 63–73. <https://doi.org/10.1105/tpc.106.048298>
- Wang, P., Hsu, C., Du, Y., Zhu, P., Zhao, C., Fu, X., Zhang, C., Paez, S. J., Macho, A. P., Tao, W. A., & Zhu, J.** (2020). Mapping proteome-wide targets of protein kinases in plant stress responses. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(6), 3270-3280. <https://doi.org/10.1073/pnas.1919901117>
- Weigel, D., & Glazebrook, J.** (2009). Quick miniprep for plant DNA isolation. *CSH Protocols*, *2009*(3), pdb.prot5179. <https://doi.org/10.1101/pdb.prot5179>
- Williams, A. P., Seager, R., Berkelhammer, M., Macalady, A. K., Crimmins, M. A., Swetnam, T. W., Trugman, A. T., Buening, N. H., Hryniw, N., McDowell, N. G., Noone, D., Mora, C. I., & Rahn, T.** (2014). Causes and Implications of Extreme Atmospheric Moisture Demand during the Record-Breaking 2011 Wildfire Season in the Southwestern United States. *Journal of Applied Meteorology and Climatology*, *53*(12), 2671–2684. <https://doi.org/10.1175/jamc-d-14-0053.1>
- Willmer, C. M., & Fricker, M. D.** (1996). Stomata. In *Springer eBooks*. <https://doi.org/10.1007/978-94-011-0579-8>

- Willmer, C. M., & Fricker, M. D.** (1996b). The distribution of stomata. In *Springer eBooks* (pp. 12–35). https://doi.org/10.1007/978-94-011-0579-8_2
- Xiao, M., Yu, Z., Kong, D., Gu, X., Mammarella, I., Montagnani, L., Arain, M. A., Merbold, L., Magliulo, V., Lohila, A., Buchmann, N., Wolf, S., Gharun, M., Hörtnagl, L., Beringer, J., & Gioli, B.** (2020). Stomatal response to decreased relative humidity constrains the acceleration of terrestrial evapotranspiration. *Environmental Research Letters*, *15*(9), 094066. <https://doi.org/10.1088/1748-9326/ab9967>
- Yaaran, A., Negin, B., & Moshelion, M.** (2019). Role of guard-cell ABA in determining steady-state stomatal aperture and prompt vapor-pressure-deficit response. *Plant Science*, *281*, 31–40. <https://doi.org/10.1016/j.plantsci.2018.12.027>
- Yang, Y., Costa, A., Leonhardt, N., Siegel, R. S., & Schroeder, J. I.** (2008). Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. *Plant Methods*, *4*(1). <https://doi.org/10.1186/1746-4811-4-6>
- Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., & Shinozaki, K.** (2006). The Regulatory Domain of SRK2E/OST1/SnRK2.6 Interacts with ABI1 and Integrates Abscisic Acid (ABA) and Osmotic Stress Signals Controlling Stomatal Closure in Arabidopsis. *Journal of Biological Chemistry*, *281*(8), 5310–5318. <https://doi.org/10.1074/jbc.m509820200>
- Yuan, W., Zheng, Y., Piao, S., Ciais, P., Lombardozzi, D., Wang, Y., Ryu, Y., Chen, G., Dong, W., Zhong-Ming, H., Jain, A. K., Jiang, C., Kato, E., Li, S., Lienert, S., Liu, S., Nabel, J. E. M. S., Qin, Z., Quine, T. A., Yang, S.** (2019). Increased atmospheric vapor pressure deficit reduces global vegetation growth. *Science Advances*, *5*(8). <https://doi.org/10.1126/sciadv.aax1396>
- Zentella, R., Sui, N., Barnhill, B., Hsieh, W. P., Hu, J., Shabanowitz, J., Boyce, M., Olszewski, N. E., Zhou, P., Hunt, D. F., & Sun, T.** (2017). The Arabidopsis O-fucosyltransferase SPINDLY activates nuclear growth repressor DELLA. *Nature Chemical Biology*, *13*(5), 479–485. <https://doi.org/10.1038/nchembio.2320>

- Zhang, S., Tao, F., & Zhang, Z.** (2017). Spatial and temporal changes in vapor pressure deficit and their impacts on crop yields in China during 1980–2008. *Journal of Meteorological Research*, *31*(4), 800–808. <https://doi.org/10.1007/s13351-017-6137-z>
- Zhuang, Y., Fu, R., Santer, B. D., Dickinson, R. E., & Hall, A.** (2021). Quantifying contributions of natural variability and anthropogenic forcings on increased fire weather risk over the western United States. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(45). <https://doi.org/10.1073/pnas.2111875118>
- Zoulias, N., Harrison, E., Casson, S. A., & Gray, J. E.** (2018). Molecular control of stomatal development. *Biochemical Journal*, *475*(2), 441–454. <https://doi.org/10.1042/bcj20170413>

Appendix

Table S1. Sequences of used primers. Primer design was based on the T-DNA Primer Design database (<http://signal.salk.edu/tdnaprimers.2.html>).

Primer	Sequence
LBb1.3	ATTTTGCCGATTCGGAAC
N560356- forward	AGAAAAACCGACCGTCTATGG
N560356- reverse	TGTTATCATCATTGCTGACGC
N523579- forward	TGGGTGGTGAAGAACAAAGAG
N523579- reverse	CAAAGAACTCATCGAGGTTGC
N519665- forward	TTCGACCTGTCTCCTCGATAC
N519665- reverse	GTTACATGCCACAACCTCCACC
N501348- forward	ATGAGACCACAAGCAACCATC
N501348- reverse	TTATCCTAGTGGAACGGCAAC
N529693- forward	AAAAGGACGAGAAGCGAGAAG
N529693- reverse	AGAGAGAGAGCGTGTGAATGC
N514590- forward	TTGGGATATGGAAGCTTGTTG
N514590- reverse	GGCTAAAGAATCAACTTCGGG
N559895- forward	AATACCTCTGCGTTGACGTTG
N559895- reverse	CTGGAAAAGCGACAATGAAAG

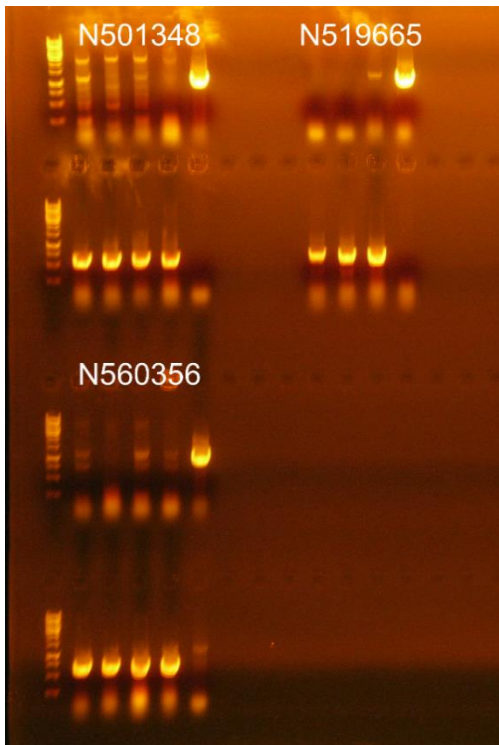


Figure S1. Gel image of plant lines N501348, N519665, and N560356, illustrating that three are mutants. The top row shows results using forward and reverse primers, while bottom row- with SALK LBb1.3 and reverse primers. In each row, the last plant was wild type as a reference. The image was made by Daana Morozova.

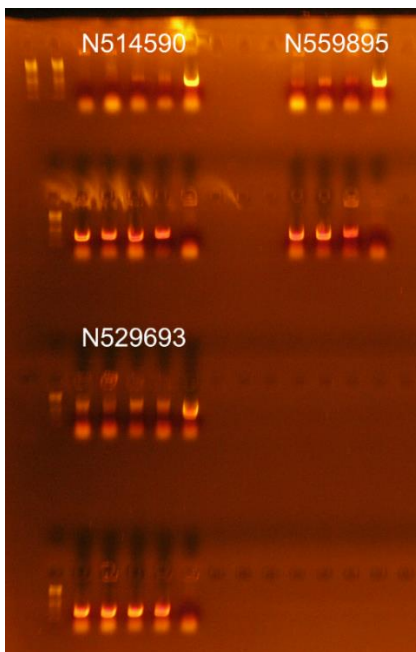


Figure S2. Gel image of plant lines N514590, N559895, and N529693, illustrating that three are mutants. The top row shows results using forward and reverse primers, while bottom row- with SALK LBb1.3 and reverse primers. In each row, the last plant was wild type as a reference. The image was made by Daana Morozova.

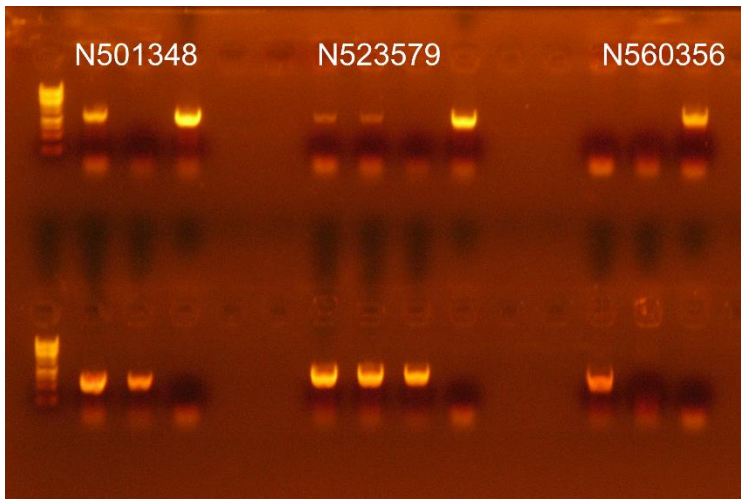


Figure S3. Gel image of plant lines N523579, illustrating it is a mutant, while lines N501348 and N560356 are not correct mutants, since there are heterozygous plant and probably a plant without DNA. The top row shows results using forward and reverse primers, while bottom row- with SALK LBb1.3 and reverse primers. In each row, the last plant was wild type as a reference. The image was made by Daana Morozova.

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