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Institute of Bioengineering, Faculty of Science and Technology, University of Tartu, Estonia

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Supervisors: Hannes Kollist, PhD
Professor of Molecular Plant Biology
Institute of Bioengineering, University of Tartu, Estonia

Hanna Hõrak, PhD
Associate Professor of Molecular Plant Physiology
Institute of Technology, University of Tartu, Estonia

Reviewer: Tiina Tosens, PhD
Associate Professor in Plant Biology
Institute of Agricultural and Environmental Sciences
Estonian University of Life Sciences, Estonia

Opponent: Matthew Gilliham, PhD
Director, ARC Centre Plants for Space
University of Adelaide, Australia

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Author's contribution

i. Participated in conducting the experiments.

ii. Designed and conducted most of the experiments, analysed and visualised the data, and wrote most of the article.

iii. Analysed part of the data and commented on final draft of the article.

ABBREVIATIONS

ABA	ABSCISIC ACID
AKT1	ARABIDOPSIS THALIANA K^+ TRANSPORTER 1
CA1	CARBONIC ANHYDRASE 1
CA4	CARBONIC ANHYDRASE 4
CBC1	CONVERGENCE OF BLUE LIGHT AND CO_2 1
CBC2	CONVERGENCE OF BLUE LIGHT AND CO_2 2
CO_2	CARBON DIOXIDE
EPF	EPIDERMAL PATTERNING FACTOR
EPF1	EPIDERMAL PATTERNING FACTOR 1
EPF2	EPIDERMAL PATTERNING FACTOR 2
GHR1	GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1
GMC	GUARD MOTHER CELL
GORK	GATED OUTWARDLY-RECTIFYING K^+ CHANNEL
HT1	HIGH LEAF TEMPERATURE 1
KAT1	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1
KAT2	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2
MMC	MERISTEMOID MOTHER CELL
MPK12	MITOGEN ACTIVATED PROTEIN KINASE 12
MPK4	MITOGEN ACTIVATED PROTEIN KINASE 4
OST1	OPEN STOMATA 1
PHOT1	PHOTOTROPIN 1
PHOT2	PHOTOTROPIN2
PIP1	PLASMA MEMBRANE INTRINSIC PROTEIN 1
PIP2	PLASMA MEMBRANE INTRINSIC PROTEIN 2
PP2C	TYPE 2C PROTEIN PHOSPHATASES
PYR/PYL	PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE-LIKE
QUAC1	QUICK ACTING ANION CHANNEL
SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1
SPCH	SPEECHLESS
STP1	SUGAR TRANSPORT PROTEIN 1
STP4	SUGAR TRANSPORT PROTEIN 4
SUC1	SUCROSE-PROTON SYMPORTER 1
TMM	TOO MANY MOUTHS
VPD	VAPOR PRESSURE DEFICIT
WUE	WATER USE EFFICIENCY

1. INTRODUCTION

Photosynthesis is the basis of most life on Earth, using solar energy to fix atmospheric CO_2 (carbon dioxide) into organic compounds that fuel ecosystems. Over millions of years, plants have adapted to diverse environments ranging from humid rainforests to arid deserts. However, the pace of current climate change is increasingly rapid, challenging plants capabilities to adapt and threatening agricultural productivity. One major driver of climate change is the rise in atmospheric CO_2 concentration. Elevated CO_2 can stimulate photosynthesis by providing more substrate, but it also contributes to global warming, altering precipitation patterns and increasing evapotranspirative demand (vapor pressure deficit, VPD). Higher VPD means reduced air humidity and thus, greater humidity gradient between plant leaf and atmosphere, which causes increased water loss. Plants respond to these conditions by adjusting the apertures of their stomatal pores to balance CO_2 uptake and water loss.

Stomata are small adjustable openings on plant surfaces, mostly on leaves. They are formed by pairs of specialised cells, termed as guard cells. By modulating their turgor, guard cells open or close the stomatal pore in response to multiple environmental signals, in an effort to find optimal balance of CO_2 uptake and water loss. Stomata allow plants to rapidly adapt and optimize their gas exchange depending on environmental conditions, this greatly increases plant survival and productivity. Open stomata allow CO_2 entry for photosynthesis but also increase water loss through stomata, whereas closed stomata reduce water loss but restrict carbon intake. In an era of rising CO_2 and more frequent droughts, understanding and manipulating stomatal regulation has become increasingly important. Improved stomatal control can potentially enhance plant water-use efficiency (WUE), which is crucial for crop yield stability under water-limited conditions. Indeed, while elevated CO_2 alone can reduce stomatal opening and improve instantaneous WUE, this benefit may be offset by higher temperatures and drought stress in future climates. Plants may face scenarios where they need to close stomata to conserve water at the cost of photosynthesis, which directly impacts productivity and nutrient acquisition. Thus, research in guard cell physiology and stomatal signal transduction is fundamental for breeding climate-resilient crops.

2. REVIEW OF LITERATURE

2.1. Stomatal development and density

Plants can adapt to their environment by adjusting stomatal density and distribution. Stomatal development follows the one-cell spacing rule: each stoma is separated by at least one pavement cell (Sachs and Novoplansky, 1993; Geisler et al., 2000; Nadeau and Sack, 2002). In Arabidopsis and most dicots, stomata are formed by series of asymmetric divisions of precursor cells. A meristemoid mother cell (MMC) divides to form a small meristemoid and a larger sister cell (Geisler et al., 2000). The meristemoid can undergo a few rounds of asymmetric divisions, producing satellite meristemoids, before finally differentiating into a guard mother cell (GMC). The GMC then divides once symmetrically to produce the two guard cells of a stoma (Geisler et al., 2000). Stomatal development is controlled by a signalling pathway involving EPF/EPFL (EPIDERMAL PATTERNING FACTOR) family peptide signals (Hunt and Gray, 2009), ERECTA family receptor kinases and TMM (TOO MANY MOUTHS) as their receptors, and downstream transcription factors SPCH (SPEECHLESS), MUTE and FAMA (Bergmann and Sack, 2007; Hara et al., 2007; Torii, 2021). These components are responsible for proper stomatal patterning and density.

Environmental conditions can influence stomatal density, for example, plants grown in elevated CO_2 conditions display decreased stomatal density (Haworth et al., 2013). It has been shown that mature leaves detect changes in CO_2 concentration and developing leaves are affected by these signals (Lake et al., 2001). Relative air humidity has similar effects on stomatal development with higher or lower air humidity leading to increased stomatal density (Fanourakis et al., 2013; Carins Murphy et al., 2014). The developmental effect of CO_2 and air humidity seems to be very species specific and more research is needed to draw clear conclusions about this topic (Chua and Lau, 2024). In addition, light levels also positively affect stomatal development, leading to higher stomatal densities in higher light conditions (Casson and Hetherington, 2010). Such plasticity in plant stomatal development demonstrates their ability to optimize stomatal conductance for their environment.

While increasing stomatal density might improve CO_2 uptake capacity, it has a drawback. More stomata mean greater resource cost for developing and maintaining larger number of guard cells forming stomatal pores, it has also been shown to increase water loss (Lawson and Blatt, 2014), but recent studies have shown the opposite – plants with higher stomatal density in combination with smaller stomata appear to be more drought resilient (Caine et al., 2023). Excess and cluttered stomata can reduce photosynthetic productivity due to disrupted CO_2 diffusion (Dow et al., 2014).

2.2. EPF1/2

EPF1 (EPIDERMAL PATTERNING FACTOR 1) and EPF2 are key regulatory peptides in Arabidopsis stomatal development. They encode small secreted cysteine-rich peptides that act as intercellular signals, functioning as negative regulators of stomatal formation (Hara et al., 2007; Hara et al., 2009; Hunt and Gray, 2009). Both EPF1 and EPF2 inhibit the progression of stomatal lineage cells, thereby controlling stomatal density and enforcing proper spacing between stomata (Hara et al., 2007; Hara et al., 2009). EPF2 is expressed in MMCs and young meristemoids, it restricts the initiation and proliferation of stomatal precursors (Hara et al., 2009). EPF1 is expressed later, in developing meristemoids and guard mother cells, where it reinforces the "one-cell spacing rule" that prevents adjacent stomata from forming side by side (Hara et al., 2009). This temporal separation suggests that EPF2 primarily limits the number of stomata that form, while EPF1 ensures any stomata that do form are properly spaced apart.

Mutant phenotypes of EPF1 and EPF2 demonstrate their different roles in stomatal development and patterning. Plants lacking EPF2 function develop greatly increased amount of stomata due to excessive asymmetric divisions of epidermal progenitor cells (Hara et al., 2009; Hunt and Gray, 2009). In contrast, loss of EPF1 disrupts stomatal spacing as *epf1* mutants often form paired or clustered stomata since the signal enforcing a one-cell spacing rule is absent (Hara et al., 2007). An *epf1epf2* double mutant combines phenotypes from both single mutant lines, it shows an additive increase in stomatal density while also producing clustered stomata similar to the *epf1* phenotype (Hara et al., 2009; Hunt and Gray, 2009). These findings indicate that EPF1 and EPF2 functions are partially overlapping but have their distinct role in the developmental pathway.

2.3. Stomatal responses

Stomata, as adjustable valves, respond to a multitude of environmental stimuli. Generally, stomatal opening is promoted by conditions favourable for photosynthesis (light, ample humidity, low CO_2) whereas stomatal closure is triggered by stress or conditions where water conservation is prioritized (darkness, drought, high CO_2, pathogenic signals). This dynamic behaviour optimizes the plant carbon gain while minimizing water loss and possible damage by pathogens. A stoma is formed by two guard cells and an aperture formed between them. Stomatal aperture size is controlled by modifying turgor pressure inside the guard cells (Figure 1).

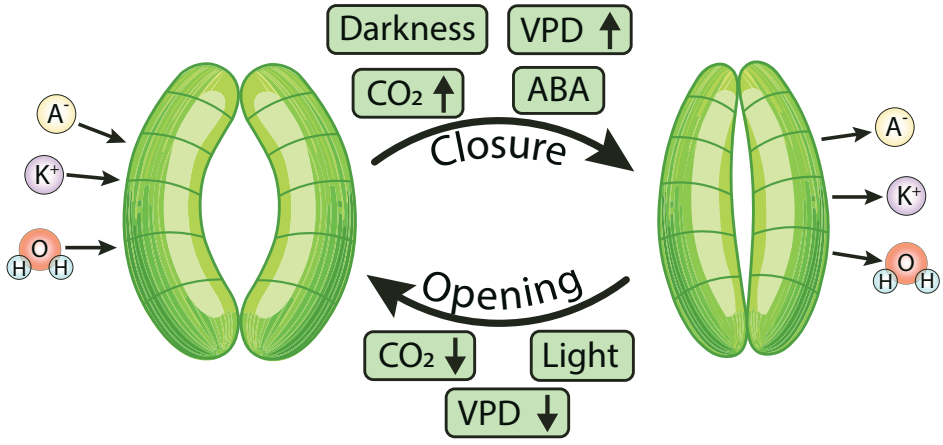

Figure 1. Stomatal complex and guard cell movements.
Stomatal complex formed by two guard cells and an aperture between them. Influx of anions, K^+ and water causes turgor pressure to increase and stomata to open. Outflow of anions, K^+ and water causes turgor pressure to drop and stomata to close.

2.4. Mechanisms of stomatal movements

Stomatal opening is driven by an increase in guard cell turgor pressure. Opening is initiated by the hyperpolarization of the guard cell plasma membrane, which is achieved through the activation of H^+-ATPase proton pumps that extrude H^+ from the cell. The hyperpolarization opens inward-rectifying K^+ channels, such as KAT1 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1), KAT2 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2), and AKT1 (ARABIDOPSIS THALIANA K^+ TRANSPORTER 1) allowing K^+ influx into guard cells (Schachtman et al., 1992; Pilot et al., 2001; Szyroki et al., 2001). The accumulation of K^+ also causes several anions to enter the cell and creates increase in the osmotic potential. Due to osmotic potential, water flows into the guard cells and the stomatal pore opens. In addition to inorganic ions, guard cells also accumulate organic osmolytes, such as malate^{2-} and sucrose (Hedrich and Marten, 1993). Guard cells import sucrose via specific sugar transporters, such as SUC1 (SUCROSE-PROTON SYMPORTER 1), STP1 (SUGAR TRANSPORT PROTEIN 1), STP4 (SUGAR TRANSPORT PROTEIN 4) and also produce it photosynthetically or mobilize it from starch (Talbott and Zeiger, 1998; Daloso et al., 2016). This sucrose not only contributes to osmotic pressure but also fuels guard cell metabolism (Talbott and Zeiger, 1998; Daloso et al., 2016).

Light is a primary trigger for stomatal opening. Blue light is especially effective for stomatal opening, it is sensed by phototropin receptors PHOT1 (PHOTOTROPIN 1) and PHOT2 (PHOTOTROPIN 2) (Briggs and Christie, 2002; Christie, 2007; Boccalandro et al., 2012). Upon sensing blue light, phototropins initiate a signalling cascade that activates the H^+-ATPase proton pumps. This blue-light specific pathway leads to rapid stomatal opening even under constant ambient CO_2 (Hiyama et al., 2017). Studies have identified Raf-like protein kinases CBC1

(CONVERGENCE OF BLUE LIGHT AND CO_2 1) and CBC2 (CONVERGENCE OF BLUE LIGHT AND CO_2 2) as downstream components in the blue-light opening pathway (Hiyama et al., 2017; Hayashi et al., 2020). In Hiyama et al., 2017, CBC1 and CBC2 were found to stimulate stomatal opening by inhibiting S-type anion channel activity. However, Hayashi et al., 2020 showed that CBC-s negatively regulate H^+-ATPase phosphorylation and thus, stomatal opening. Later it was demonstrated that CBC1 and CBC2 are phosphorylated by HT1 (HIGH LEAF TEMPERATURE 1) in low CO_2 environment, which promotes stomatal opening (Takahashi et al., 2022). Additionally, blue light dependent phosphorylation sites were found to have no clear role in HT1 mediated CBC activation (Takahashi et al., 2022), this could mean that blue light and CO_2 response both function through CBCs, but use different mechanisms.

During stomatal closure, guard cells lose turgor through ion efflux followed by water loss, leading to stomatal closure. Stomata close in response to various environmental stimuli, including darkness, elevated CO_2 concentration, high VPD (vapor pressure deficit), soil water deficit, air pollutants like ozone and biotic stress such as pathogen attack. Stomatal closure is initiated by depolarization of the guard cell membrane and efflux of anions through anion channels. The SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) is a major slow-type anion channel that releases Cl^- and NO_3^- from guard cells during closure (Negi et al., 2008; Vahisalu et al., 2008), mutations in SLAC1 result in disrupted stomatal closure in response to all major stimuli. Another anion channel, QUAC1 (QUICK ACTING ANION CHANNEL), primarily mediates $malate^{2-}$ efflux (Meyer et al., 2010; Imes et al., 2013). Activation of these anion channels causes membrane depolarization, which in turn activates outward K^+ channels such as GORK (GATED OUTWARDLY-RECTIFYING K^+ CHANNEL) (Ache et al., 2000; Hosy et al., 2003), allowing K^+ to exit. The combined loss of anions and cations reduces the osmotic pressure inside guard cells, causing water efflux and the guard cells to lose turgor, which leads to stomatal closure.

A central hormone in stomatal closure is abscisic acid (ABA), which is needed for rapid stomatal closure during drought stress, high VPD (Hsu et al., 2021a). ABA binds to PYR/PYL (PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE-LIKE) receptors in guard cells, triggering a signalling cascade that inhibits PP2Cs (TYPE 2C PROTEIN PHOSPHATASES) and thereby activates SnRK2 family protein kinase OST1 (OPEN STOMATA 1) (Figure 2) (Park et al., 2009; Gonzalez-Guzman et al., 2012; Imes et al., 2013). Recent studies have also shown that Raf-kinases directly phosphorylate OST1 and by this modulate its activity (Fàbregas et al., 2020; Soma et al., 2020; Lin et al., 2021). Active OST1 phosphorylates SLAC1, promoting anion efflux (Mustilli et al., 2002; Acharya et al., 2013).

Elevated CO_2 induces stomatal closure through a pathway that can function independently of ABA. While ABA and CO_2 both converge on activating anion channels, CO_2 does not appear to directly activate OST1 kinase (Hsu et al., 2018). Instead, CO_2 employs a distinct upstream signalling branch involving protein kinases HT1 and MPK4/MPK12 (MITOGEN ACTIVATED PROTEIN KINASE 4

AND 12) (Hashimoto et al., 2006; Marten et al., 2008; Jakobson et al., 2016). Arabidopsis mutants lacking OST1 can still partially close stomata in elevated CO_2 (Merilo et al., 2013; Jalakas et al., 2018), whereas mutants in the CO_2-specific kinases show a much stronger impairment (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al. 2016; Jakobson et al. 2016). This indicates two partly independent pathways for stomatal closure: an ABA-OST1 pathway and a CO_2-specific pathway, which can synergize under certain conditions, for example basal ABA levels enhance CO_2 induced closure (Hsu et al., 2018). The GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1 (GHR1) was more recently demonstrated to act as a receptor protein required for stomatal closure in response to elevated CO_2 and air pollutant ozone (Hua et al., 2012a; Sierla et al., 2018).

2.5. CO_2 signalling in guard cells

CO_2 fluctuations in the environment cause plant stomatal response, inducing narrowing the stomatal pores at elevated CO_2 concentrations while triggering opening at lower concentrations. This behaviour optimizes CO_2 concentration inside the leaf and its availability for photosynthesis – if external CO_2 is abundant, stomata can partially close to conserve water, whereas if CO_2 availability is limited, stomata open to maintain photosynthesis. The guard cell CO_2 response is vital for plant WUE and carbon gain, and it has likely played an important role in plant evolution.

CO_2 enters guard cells by diffusing across the plasma membrane and also via aquaporin channels (Groszmann et al., 2017). Once inside, CO_2 is converted to bicarbonate (HCO_3^-), this happens passively in the cell, but is also catalyzed by CA1 (CARBONIC ANHYDRASE 1) and CA4 (CARBONIC ANHYDRASE 4) (Figure 2) for a much more rapid conversion (Hu et al., 2010), these two β-carbonic anhydrases are highly expressed in guard cells and are shown to contribute to CO_2 induced stomatal movements. Bicarbonate is believed to be the molecule sensed by CO_2 signalling pathway (Xue et al., 2011).

Primary CO_2/bicarbonate sensing complex in guard cells consists of the protein kinase HT1 and MAP kinases MPK12 or MPK4 (Figure 2) (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016; Jakobson et al., 2016; Takahashi et al., 2022). HT1 is a Raf-like protein kinase that has long been known as a negative regulator of CO_2 induced stomatal closure (Hashimoto et al., 2006;). MPK4 or MPK12 binds to HT1 at elevated CO_2 levels, and inhibit its kinase activity (Takahashi et al., 2022; Yeh, et al. 2023). Active HT1 phosphorylates downstream targets such as the CBC1/CBC2 kinases (Figure 2), which in their active state promote stomatal opening by inhibiting anion channels (Takahashi et al., 2022). Active HT1 normally promotes stomatal opening**,** inhibiting HT1 releases downstream signalling pathway components from inhibition, causing stomatal closure (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). Under low CO_2, the absence of the bicarbonate signal means MPK4/12 do not bind HT1, and thus HT1 remains active.

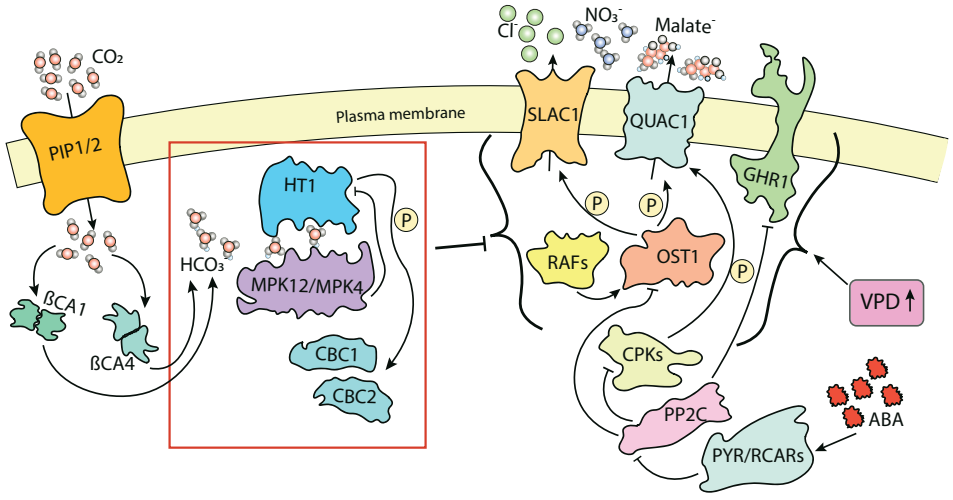

Figure 2. Model of stomatal CO_2 and VPD signalling pathway.
CO_2 enters cell through PIP1/2 aquaporins (Groszmann et al., 2017), carbonic anhydrases CA1 and CA4 catalyze CO_2 conversion to HCO^{3-} (Hu et al., 2010). HCO^{3-} activates MPK12/MPK4 kinases (Takahashi et al., 2022; Yeh et al., 2023), which inhibit HT1 (Hashimoto et al., 2006) and consequently stops its activation of CBC1 and CBC2 kinases (Hiyama et al., 2017; Hayashi et al., 2020), which in their active state promote stomatal opening by inhibiting anion channels SLAC1 (Vahisalu et al., 2008) and QUAC1 (Meyer et al., 2010; Imes et al., 2013). OST1 functions in CO_2 signalling pathway by controlling anion channels (Xue et al., 2011; Hsu et al., 2018), RAF kinases have been shown to activate OST1 in elevated CO_2 conditions (Hsu et al., 2021b) and it is also necessary in VPD response (Merilo et al., 2018). GHR1 pseudokinase is also part of stomatal CO_2 signalling pathway, in addition to contributing in VPD, ABA, darkness and ozone responses (Sierla et al., 2018). ABA-OST1-SLAC1 pathway is crucial for VPD responses (Merilo et al., 2018)and GHR1 is also needed for fast VPD responses (Hsu et al., 2021b). PYR/PYL receptors inhibit PP2Cs in the presence of ABA which leads to OST1 activation (Park et al., 2009; Gonzalez-Guzman et al., 2012; Imes et al., 2013).

OST1 and other kinases function downstream of the HT1-MPK12/4 module (Figure 2). Once HT1 is inhibited under high CO_2 conditions, its suppression of the closure mechanism is lifted, allowing OST1 to activate SLAC1 and QUAC1 for stomatal closure (Imes et al., 2013; Tian et al., 2015). OST1 is well-known for ABA signalling, but studies have shown OST1 also contributes to CO_2-induced closure (Xue et al., 2011). This is supported by findings that basal ABA/OST1 activity is required for efficient CO_2 response, even if CO_2 does not directly activate OST1 (Hsu et al., 2018). GHR1 pseudokinase is another stomatal CO_2 signalling pathway component, it is phosphorylated by HT1 and is involved in stomatal responses to CO_2, darkness, ozone and VPD (Sierla et al., 2018).

This complex signalling system allows plants to finely tune stomatal aperture in response to CO_2 fluctuations without losing the ability to close stomata under drought. It also suggests that manipulating specific signalling pathway components could adjust how stomata responds to rising CO_2 levels. For example, the *ht1-2* mutant, which has loss of HT1 function, has low stomatal conductance and

no response to changes in CO_2 concentration (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016), whereas mutants with constitutively active HT1, such as *ht1-8D*, have elevated stomatal conductance and are also insensitive to CO_2 concentration changes (Hõrak et al., 2016). These phenotypes show the potential of modifying plant water use: removal of HT1 leads to a water-conserving plant with potential loss in photosynthetic productivity, which would be suitable for water limited conditions where survival is crucial. While constitutively active HT1 results in a plant that has increased water use but also maximized CO_2 uptake, which would be suitable when water is abundant and increased production is preferred. This knowledge can be used to improve crop performance in specific conditions.

2.6. Stomatal VPD regulation

Water content in the air, referred also as atmospheric relative humidity, is another environmental factor influencing plant stomatal behaviour. VPD defines the difference between the water vapor pressure inside the leaf and that of the surrounding air, high VPD corresponds to dry air that increases transpiration while low VPD corresponds to humid air which decreases transpiration. Plants typically respond to elevated VPD by reducing stomatal conductance, which reduces water loss. Under low VPD conditions, the evaporative demand is reduced and stomata can remain more open without losing too much water (Oren et al., 1999; Buckley, 2005; Buckley, 2019).

The stomatal response to VPD functions by hydro-passive and hydro-active mechanisms (Buckley, 2005). In high VPD condition, leaves can lose water faster than it can be supplied by the xylem and this can lead to a drop in leaf turgor that can physically cause stomatal closure, such process is defined as hydro-passive mechanism (Franks et al., 1998; Buckley, 2005). However, plants also actively sense VPD levels and use hormonal signals to close stomata before wilting occurs (Jalakas et al., 2021b). Main hormone that controls stomatal signalling under elevated VPD is ABA, which is produced during drought and low relative air humidity (Merilo et al., 2013; Merilo et al., 2018). Guard cells under high VPD conditions show activation of the ABA-OST1-SLAC1 pathway (Figure 2) and experiments demonstrate crucial role for OST1 in VPD-driven stomatal closure (Merilo et al., 2018). OST1 deficient mutants are impaired in high-VPD-induced stomatal closure, and ABA-deficient plants display WT-like stomatal closure in response to high VPD, but their stomatal conductance remains much higher than that of WT-plants (Merilo et al., 2018). OST1 phosphorylates SLAC1 and QUAC1 anion channels (Figure 2) in response to elevated VPD, which leads to ion efflux and stomatal closure (Imes et al., 2013; Deng et al., 2021). In a recent study it was also shown that OST1 function is not completely ABA dependent, plants that expressed OST1 with no ABA activation site retained greater stomatal response to elevated VPD than OST1 deficient plants (Tulva et al., 2023). This

shows that although ABA is an important component in VPD signalling, it is not the sole mechanism.

Short-term responses to a sudden increase in VPD can occur too rapidly to be solely explained by ABA biosynthesis and redistribution (Merilo et al., 2018). This indicates that hydraulic mechanisms are responsible during sudden VPD changes, which leads to partial stomatal closure before ABA specific responses take place. Experiments support that stomatal responses to rapid VPD changes involve ABA-independent signalling, whereas ABA maintains stomatal closure during sustained high VPD levels (Merilo et al., 2018; Jalakas et al., 2021b). Meaning that immediate hydro-passive response causes initial closure, and ABA response is used during long term stomatal adjustment to VPD stress. GHR1 has been identified as an important component in high VPD induced stomatal closure response, GHR1 deficient mutants show a weaker and slower response to suddenly elevated VPD (Hsu et al., 2021b). Additionally, GHR1 deficient plants display ABA insensitivity in stomatal responses (Sierla et al., 2018), which shows that GHR1 is needed for stomatal responses to ABA, whether or not this connects ABA to stomatal VPD regulation is a question for future research. Another component in guard cell response to VPD are PIP1 (PLASMA MEMBRANE INTRINSIC PROTEIN 1) and PIP2 (PLASMA MEMBRANE INTRINSIC PROTEIN 2) aquaporins, which transport water to and from guard cells, causing stomatal movements under varying VPD conditions (Israel et al., 2021). During low VPD, water is moved though aquaporins into guard cells to open stomata; during elevated VPD, aquaporins are used for rapid water efflux. Studies have shown that mutants in certain PIP aquaporins have altered stomatal responses to VPD changes (Israel et al., 2021; Ding et al., 2022), this suggests their involvement in VPD induced stomatal responses. B3 family Raf-kinases M3Kδ1, M3Kδ5, M3Kδ6 and M3Kδ7 also function in elevated VPD induced stomatal closure with M3Kδ5 having the central role, these B3 kinases activate OST1 in guard cells and plants deficient in M3Kδ5 showed slower stomatal response to elevated VPD (Hsu et al., 2021b).

2.7. HT1 – central component in CO_2 signalling

HT1 kinase was first discovered by a genetic screen applying thermal imaging of plants at different CO_2 concentrations. Mutant plants deficient in HT1 showed significantly higher leaf temperatures (Hashimoto et al., 2006). HT1 is the central CO_2 signalling component in guard cells. It has been shown that HCO_3^- enhances HT1 to form dimers with MPK12 or MPK4 (Yeh et al., 2023), which leads to inhibition of HT1 kinase activity and triggers stomatal closure by SLAC1 activation (Figure 2). In addition to CO_2 pathway, HT1 is also involved in stomatal responses to red light (Matrosova et al., 2015) and temperature (Pankasem et al., 2024). Pankasem et al., 2024 used HT1 mutants with severely disrupted kinase function (*ht1-2*) and with constitutively active kinase (*ht1-8D*), stomatal responses to temperature change were severely disrupted in both mutants. Plants with

deficient HT1, such as the *ht1-2* mutant, exhibit low stomatal conductance and insensitivity to atmospheric CO_2 changes. Alternatively, a constitutively active HT1 mutation like *ht1-8D* leads to very open stomata which are insensitive to CO_2 changes (Hõrak et al., 2016). This makes it possible to use variations in HT1 mutations to modify plant stomatal behaviour and conductance.

2.8. Carbonic anhydrases CA1 and CA4

Carbonic anhydrases CA1 and CA4 have an important part in stomatal CO_2 signalling pathway as they catalyse the conversion of CO_2 to bicarbonate, which serves as the initial signal in CO_2-induced stomatal closure (Figure 2) (Hu et al., 2010; Xue et al., 2011). The importance of carbonic anhydrase activity is demonstrated by *ca1ca4* double mutants that have slower and weaker elevated CO_2 induced stomatal closure response. This shows that HCO_3^- is required to initiate the guard cell CO_2 signalling cascade (Xue et al., 2011). The HCO_3^- generated by CA1/CA4 is sensed by MPK12/4 complex which then inhibits HT1 activity (Figure 2) (Takahashi et al., 2022). Inhibition of HT1 by elevated CO_2 leads to OST1 activation, which results in S-type anion channels releasing anions from the guard cell, thereby promoting stomatal closure (Zhang et al., 2018). Thus, by catalysing the conversion of CO_2 into HCO_3^-, CA1 and CA4 are at the top of the pathway that leads to elevated CO_2 induced stomatal closure response.

2.9. QUAC1

QUAC1, encoded by the Arabidopsis *ALMT12* gene, is a guard cell plasma membrane anion channel mediating the rapid (R-type) anion efflux for stomatal movement (Meyer et al., 2010). R-type channels like QUAC1 activate within milliseconds and conduct primarily organic anions (Hedrich et al., 1990). Activation of QUAC1 triggers membrane depolarization and initiates stomatal closure by anion efflux, which in turn activates K^+ efflux channels and guard cell water loss (Meyer et al., 2010; Imes et al., 2013; Jalakas et al., 2021a). QUAC1 operates alongside the slower S-type anion channels: the S-type channels preferentially release Cl^- and NO_3^-, whereas QUAC1 enables efflux of organic acids like malate that accumulate during stomatal opening and must be exported for closure (Figure 2) (Meyer et al., 2010; Sasaki et al., 2010). Together, these anion channels provide the required ion movement for guard cells to rapidly lose turgor and close the stomatal pore. Recent studies have shown that QUAC1 action is indispensable for full stomatal responsiveness. While QUAC1 knockout mutants exhibit only partial defects, the loss of both, QUAC1 and SLAC1, virtually abolishes stomatal closure responses to ABA, elevated CO_2, darkness, and VPD changes and *slac1-3quac1-1* double mutants had high steady-state stomatal conductance (Jalakas et al., 2021a; Jaślan et al., 2023).

2.10. GHR1

GHR1 is a pseudokinase in *Arabidopsis thaliana* that plays a central role in guard cell signalling and stomatal closure (Sierla et al., 2018). It was first identified in a genetic screen for hydrogen peroxide insensitive stomatal responses, and *ghr1* mutants have disrupted ABA and H_2O_2 induced stomatal closure response (Hua et al., 2012a), later several mutations in GHR1 were identified in a genetic screen for plant ozone sensitivity (Sierla et al., 2018). GHR1 encodes a plasma membrane-localized receptor-like pseudokinase that interacts with the guard cell S-type anion channel SLAC1 (Hua et al., 2012b). While GHR1 can activate SLAC1 in heterologous systems, it carries substitutions in kinase-domain motifs and acts mostly as a scaffold protein rather than an active kinase (Sierla et al., 2018). GHR1 participates in elevated CO_2 induced stomatal closure, *ghr1* loss-of-function mutants exhibit weakened stomatal response to elevated CO_2 levels, indicating that GHR1 is a key component of the CO_2-induced stomatal closing pathway (Hõrak et al., 2016). In the CO_2 signalling pathway, GHR1 functions alongside the OST1 kinase to activate SLAC1, and their action is normally inhibited by the protein kinase HT1 until it is inactivated by upstream MAP kinases MPK4 or MPK12 (Hõrak et al., 2016). GHR1 is also used in stomatal responses to VPD changes, GHR1 lack of function mutants exhibit weakened response to high VPD and also lack the initial wrong way opening response (Hsu et al., 2021b).

2.11. MPK12 kinase

Guard cell MAP kinase MPK12 and its paralog MPK4 have recently been shown to be a pivotal component of the stomatal CO_2 signalling machinery (Jakobson et al., 2016). At high CO_2 levels, MPK12 interacts with HT1 kinase (Figure 2), directly binding and inhibiting HT1 activity (Takahashi et al., 2022). MPK12 deficient mutants have higher transpiration and lower water use efficiency, in addition to slower and weaker stomatal CO_2 responses, which suggests that HT1 inhibition is not purely dependent on MPK12. MPK4 kinase is similar to MPK12 and can complement MPK12 loss of function mutants when expressed under MPK12 promoter control (Yeh et al., 2023). MPK12 forms heterodimers with HT1 in the presence of bicarbonate (Yeh et al., 2023). This MPK12/HT1 module forms the core of the guard-cell CO_2 sensor. In contrast, under low CO_2 the uninhibited HT1 phosphorylates downstream CBC1/2 kinases, which redundantly promote stomatal opening by suppressing S-type anion channel activity (Hiyama et al., 2017; Takahashi et al., 2022). Thus, MPK12 acts as a CO_2-triggered switch, when CO_2 rises, MPK12 inhibits HT1 and consequently CBC1/2 (Figure 2), causing anion channel activation and stomatal closure. Recent structural and genetic studies show that kinase function of MPK12 is not required for MPK12/HT1 complex to function, binding with HT1 is sufficient for stomatal regulation (Takahashi et al., 2022; Yeh et al., 2023). These findings place MPK12 at the centre of stomatal CO_2 regulation.

3. AIMS OF THE STUDY

Optimizing stomatal behaviour is a promising approach to develop climate-resilient plants. By tuning how and when stomata open or close, we could create crops that use water more efficiently under drought or take full advantage of elevated CO_2 in the atmosphere. The goal of this PhD study was to deepen our understanding of stomatal CO_2 signalling pathways and their interaction with other factors like humidity, and to leverage this knowledge to improve plant water-use efficiency and drought tolerance. The specific aims were:

- **Aim 1:** Determine stomatal CO_2 responses at sub-ambient and above-ambient CO_2 levels, and study how these responses depend on other environmental factors such as vapor pressure deficit (air humidity). We studied whether the same signalling components control stomatal movements in different CO_2 ranges, and whether low air humidity (high VPD) has an effect on these responses. Additionally, we evaluated the possible role of MPK4 in stomatal CO_2 signalling pathway.
- **Aim 2:** Study possibilities of using key targets in the stomatal CO_2 signalling pathway to improve drought resistance and water use efficiency in plants. We focused on the HT1 kinase, a central component of the guard cell CO_2 response, hypothesizing that altering HT1 function could yield plants with reduced water loss without severely impacting growth.
- **Aim 3:** Evaluate the combined effects of stomatal density and air humidity on plant growth and physiology. Whether having higher numbers of more closed stomata confers any advantage or disadvantage under low-humidity conditions, thereby linking developmental stomatal traits with environmental stress outcomes.

To address these aims, we used the various stomatal signalling mutants of the model plant *Arabidopsis thaliana* for detailed physiological experiments. Next, we extended our findings to *Solanum lycopersicum* (tomato) as a crop model to test the applicability of HT1 for modulating tomato water management. Aim 2 was primarily tackled by experiments with tomato CRISPR/Cas9 mutants for HT1 and by assessing their gas exchange, growth, fruit quality and water use characteristics. Aim 1 was addressed by performing gas-exchange experiments on Arabidopsis stomatal mutants (*ht1-2*, *mpk12-4*, *slac1-3*, *ost1-3*, *ht1-8D*, *ghr1-3*, *ca1ca4*,) under controlled CO_2 and VPD conditions. Aim 3 was investigated via growth experiments using Arabidopsis lines with altered stomatal densities and apertures under different humidity regimes. Through these objectives, the study integrates fundamental discoveries in guard cell signal transduction with practical strategies for crop improvement.

4. MATERIALS AND METHODS

Detailed materials and methods of publications are described in materials and methods sections of respective manuscripts (I–III). Materials and methods for experiments not shown in manuscripts are described below.

4.1. Plant material

For Arabidopsis, the Columbia-0 (Col-0) line was used as wild type. For studying QUAC1 effect in stomatal CO_2 regulation we used *slac1-3quac1-1* (derived from SALK_099139 and WiscDsLox329_D04) double-mutant line.

For Tomato, *Solanum lycopersicum* line M82 (Moneymaker) was used as WT in experiments. Mutant lines were generated by Prof. Assaf Mosquna research group (The Hebrew University of Jerusalem, Israel), using Crispr-Cas9 mediated Golden Gate cloning method in M82 background. Seeds were grown and used to propagate new plants for gas exchange analysis. Plants for gas exchange measurements were grown in Plant Signal Research Group, Tartu. Seeds were kept in water at +4 °C for 24 hours, after which they were pre-germinated for 3–4 days in the dark between wet filter papers at room temperature. Healthy seedlings were transferred into growth pots, two seedlings per pot (11 × 10 × 10 cm) were sown into opposite corners.

4.2. Growth conditions

Arabidopsis plants were grown in a controlled environment growth chamber on a peat-vermiculite soil mix (2:1 ratio by volume). Growth conditions were 10 h light/14 h dark, ~22 °C day and ~18 °C night, 60/80% relative humidity and a light intensity (PAR) of ~250 μmol m^{-2} s^{-1}.

Tomato experiments used growth substrate mixture of peat, vermiculite and water in a 4/2/3 ratio by volume. The tomato plants were grown for approximately 20 days in plant growth chambers (Percival Intellus environmental controller AR-66LX and CLF PlantClimatics SE-41AR2cLED) at light intensity of 300 μE, temperature 22 °C day/ 19 °C night, RH 40–70%, 12/12 h day cycle.

4.3. Gas exchange measurements

For Arabidopsis, we employed a custom-built multi-cuvette gas exchange system (Kollist et al. 2007; Jyrkki, Plantinvent Ltd) to monitor whole-plant gas exchange. Arabidopsis plants (24–28 days old) were placed in cuvettes with controlled temperature, CO_2 concentration, light intensity and humidity. To test stomatal responses, we subjected plants to changes in CO_2 concentrations, 400-100-400-

800 ppm. Each experiment typically started at ~12:00 to minimize circadian and diurnal effects.

Tomato plants were measured in Plantinvent multichannel gas-exchange system (Kollist et al., 2007). A mature plant leaf was sealed into the gas-exchange chamber and chamber was made air-tight. Plants were left to stabilize for ~2 hours under same conditions as in growth chamber before stimuli were applied. For ABA response, gas-exchange chamber was temporarily opened to apply exogenous 10 µM ABA treatment via spray bottle (10µl Sigma-Aldrich 2-cis,4-trans-Abscisic acid dissolved in 96% EtOH, 2,4 µl SILWET, brought to 10ml with distilled water). For elevated VPD response, an ice-cooled metal spiral was used to bring in-flowing air humidity down ~20–30%. Elevated VPD treatment was ended by bringing the ambient air humidity in measuring cuvette back to growth condition levels.

4.4. Drought experiments

For the drought experiments, plants were grown in aforementioned conditions and substrate while pot positions in the growth cabinet were constantly rotated throughout the experiment to avoid effects from light and air humidity distribution. Pots were weighed and watered to fixed mass regularly; during drought period half of the used genotypes' pots received regular watering and half were entirely cut off from water. "Wilting" was observed when plant leaves had lost some turgor and started to droop, "plant collapse" was observed when plant leaves had lost vast majority of the leaf turgor and some leaf edges began to dry out.

4.5. Experiments in Helmholtz Zentrum Munich

In Helmholtz Zentrum Munich, the tomato seeds were germinated and grown in a greenhouse for 2 weeks, after which the plants were grown in ExpoScreen phytotrons with complete environmental simulation (light intensity 600 µE, temperature 24 °C day/ 18 °C night, RH day 70%/ night 90%, 12/12 h day cycle). To assess the long-term effect of different CO_2 concentrations, plants were grown in ambient (400ppm CO_2) and above-ambient CO_2 (1000ppm CO_2) conditions for 21 days. The plants were watered daily via automatic system, using distilled water.

4.6. Tomato fresh and dry weight measurements

At 35 days of age, the whole shoot together with leaves but without the roots was harvested for fresh weight, after which shoots and leaves were dried in 70 °C oven for at least 72 h before dry weight was measured.

4.7. Fruit harvest and analysis

After experiments in phytotron, 1/3 of the tomato plants were transferred to greenhouse for fruit production. As the CO_2 levels in greenhouse were not controlled, only fruits from plants that had previously grown in ambient CO_2 conditions were used in final data analysis. Fruits were harvested (1–3 fruits per plant) and measured for fresh weight, volume and pH upon fruit ripening. Afterwards the fruits were cut up into even slices and dried at 70–65 °C for 72 h before dry weight was measured. For assessing lycopene content, ~10g of pulverized fruit material was used to measure optical density at E = 503 nm, where lycopene content is shown µg per g in dry weight (µg/g Dry Weight).

4.8. Stomatal density

A small leaf sample was taken from the tomato plants after transfer to greenhouse. A thin layer of transparent commercial nail polish was applied to the abaxial side of the leaf. The dried impressions were peeled from the leaves and transferred to glass slides for photographing under microscope with 40× magnification. Fixed area on the photo was selected where visible stomata were counted using ImageJ (Schneider et al., 2012). Stomatal density was expressed as stomata mm^{-2}.

Experiments were arranged in completely randomized designs with multiple replicates.

5. RESULTS AND DISCUSSION

5.1. MPK12 and MPK4 are key components in CO_2-induced stomatal movements (I)

Previous research suggested that MPK12 has an important role in CO_2 signaling, as in MPK12 deficient plants, CO_2-induced stomatal closure and opening responses are partially impaired (Jakobson et al., 2016). MPK12 was demonstrated to interact with HT1 and by this inhibit its kinase activity (Jakobson et al. 2016; Hõrak et al. 2016). MPK4 is a close paralalogue to MPK12 and it was demonstrated that MPK4 is also capable to inhibit HT1 kinase activity (Hõrak et al., 2016). To further examine involvement of MPK12 and MPK4 in CO_2 specific stomatal signalling, we used Arabidopsis *mpk12-4* mutant and also a *mpk12-4 mpk4GC* double mutant. Because *mpk4* null mutants have extreme dwarfism and this makes experiments with them difficult, we silenced *MPK4* specifically in guard cells to rescue *mpk4* lines dwarf growth. Such approach provided healthy plants that allowed to assess MPK role in stomatal CO_2 responses. Gas-exchange measurements revealed that the double mutant *mpk12 mpk4GC* completely lacked CO_2 induced stomatal movements, its stomatal conductance remained high and had minimal change in response to CO_2 increase or decrease (I, Figure 1A–C). This shows that stomata of plants deficient in both MPK12 and MPK4 in guard cells are insensitive to CO_2- triggered stomatal movements. Plants in which only MPK4 was silenced in guard cells were also generated to study MPK4 function for stomatal regulation separately. These plants grew relatively normally and their stomatal responses to CO_2 were similar to WT, although steady-state conductance remained higher (I, Figure 3A–D). These results demonstrate that MPK4 and MPK12 are both required for stomatal CO_2 responses. Gas exchange experiments with ABA treatment also showed that stomatal response to ABA is unaffected in *mpk12 mpk4GC* plants (I, Figure 1D–F), showing that MPK12 and MPK4 are not involved in ABA signalling.

While MPK4 can complement MPK12 and partially recover guard cell CO_2 responses, MPK4 function is not rescued by MPK12. We complemented plants deficit in MPK4 with MPK12 (proMPK4:MPK12), but the dwarf phenotype was not rescued (I, Figure 3D). This means that MPK12 functions specifically in guard cells in the context of CO_2-dependent stomatal regulation. To test if MPK4 or MPK12 kinase activity is directly modulated by CO_2, we conducted *in vitro* kinase assays. CO_2/bicarbonate treatment did not alter the kinase activity of MPK4 or MPK12 in isolated protein assays. This experiment suggested that guard cell CO_2 sensing does not rely on the regulation of MPK4 and MPK12 kinase activity. Later studies showed that formation of either MPK12:HT1 or MPK4:HT1 dimer that leads to inhibition of HT1 kinase activity and suppresses HT1-dependent phosphorylation of downstream Raf-kinase CBC1 is dependent on CO_2/HCO_3^- concentration (Takahashi et al., 2022; Yeh et al., 2023). Thus, CO_2/HCO_3^- dependent interaction of MPK12/4:HT1 forms the primary mechanism by which guard cells sense changes in CO_2 concentration.

5.2. Stomatal response kinetics to different CO_2 concentration ranges (II)

Stomatal closure in response to elevated CO_2 is a common experiment featured in many studies, whereas a CO_2 shift from ambient (400 ppm) to above-ambient (800 ppm) has become standard experimental protocol. However, one might suggest that in field conditions, stomatal CO_2 responses typically take place in sub-ambient (100 ppm) to ambient (400 ppm) concentration range. During the day photosynthesis causes a reduction of CO_2 concentration inside the leaves and plants open their stomata to ensure sufficient CO_2 supply for carbon assimilation. While ambient to above-ambient CO_2 response happens very rarely in nature, it is still relevant during the night when photorespiration increases CO_2 concentration in leaves (Hanstein et al., 2001), which closes plant stomata and leads to lower water loss. Here, we studied stomatal responses in Arabidopsis to CO_2 changes across a wide CO_2 range (from 100 ppm up to 800 ppm) and found that response kinetics differed markedly depending on the concentration range.

Gas exchange experiments with Col-0 plants were done to address if there are any differences in response kinetics depending on the used CO_2 concentration ranges. Four different series of CO_2 changes were used: 400-100-400-800 ppm, 400-800-400-100 ppm, 400-800-100 ppm and 400-100-800 ppm. These experiments showed that stomatal responses in the above-ambient CO_2 concentrations have different response curves and are significantly faster than in sub-ambient ranges (Figure 3A,B). The 400–800 ppm CO_2 shift had very fast stomatal closure response while 100–400 was significantly slower (Figure 3B) while 100–800 ppm response speed was somewhere between the former two (Figure 3H). These results indicate that the stomatal CO_2 response is not linear across concentrations, above-ambient CO_2 levels induce a more rapid closure than would be predicted from the sub-ambient response. This raises questions about the underlying mechanisms: different CO_2 ranges may trigger distinct signalling processes or sensitivities in guard cells. Our findings show the importance of experimental setup, stomatal responses observed from ambient to above ambient CO_2 levels may involve mechanisms that are less dominant in the sub ambient to ambient range and vice versa. Plants evolved under generally sub ambient CO_2 conditions (Sage and Coleman, 2001) and typically experience CO_2 decrease during photosynthesis rather than elevated CO_2 spikes, this would suggest that CO_2 sensing mechanisms should be tuned for high sensitivity in sub ambient to ambient levels (Franks et al., 2013). For plants, it makes sense to focus more on changes in sub ambient CO_2 levels as active photosynthesis continually reduces intercellular CO_2 concentration and CO_2 responsiveness allows plants to increase CO_2 uptake as needed. Stomatal closure at above ambient CO_2 levels benefits plants as a means to reduce transpiration in high CO_2 environments. These conditions would rarely occur naturally in daylight, but during darkness, intracellular CO_2 concentration can quickly exceed 600 ppm due to photorespiration (Hanstein et al., 2001; Engineer et al., 2016). This allows plants to greatly lower their stomatal apertures during night and conserve water.

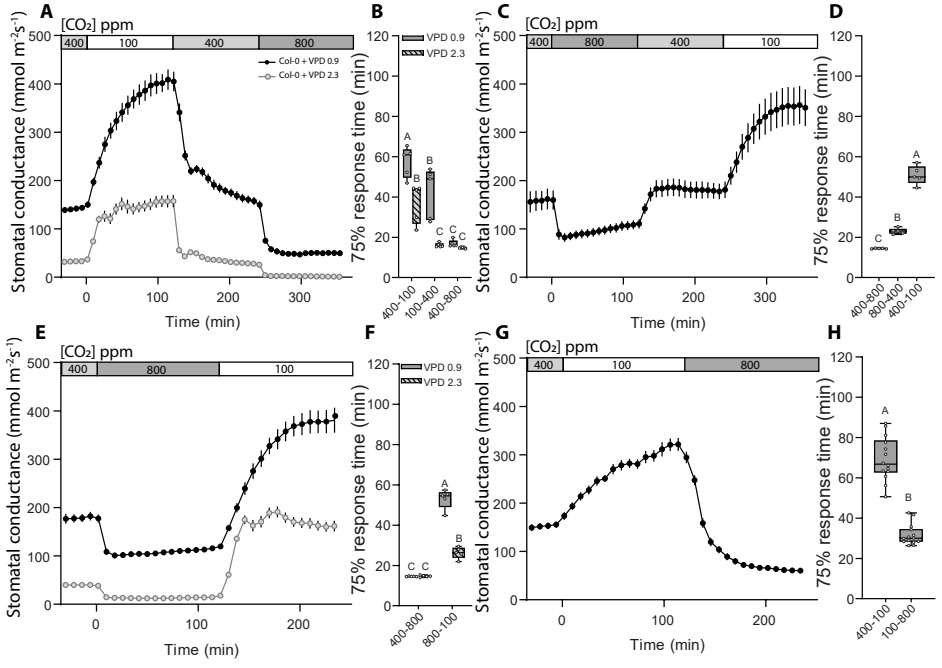

Figure 3. Kinetics of CO_2-induced stomatal responses in wild-type Arabidopsis with regular and elevated vapor pressure deficit (VPD).

Article I. Col-0 wild-type Arabidopsis stomatal response to sequential changes in CO_2 concentration under regular (A,C,E,G) and elevated (A,E) VPD conditions. Mean stomatal conductance ± SEM is shown. (B,D,F,H) Boxplot of 75% response time (minutes) of stomatal response to CO_2 concentration changes. Boxes represent 25–75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. (B,F) Statistically significantly different groups are indicated with different letters (Two-way ANOVA with Tukey post-hoc test, $p < 0.05$). (D,H) Statistically significantly different groups are indicated with different letters (One-way ANOVA with Tukey post-hoc test, $p < 0.05$). Sample size was 5 in (A,B,C,D,E,F) and 14 in (G,H). Start of first treatment was between 11:30 to 12:30.

5.2.1. Genetic dissection of CO_2 signalling pathways

To clarify which guard cell signalling components are responsible for different stomatal movements under different CO_2 concentration ranges we compared plant lines carrying mutations in thus far identified guard cell CO_2 signalling components. Following mutant lines were selected: *ht1-2*, *ca1ca4*, *mpk12-4* and *ht1-8D.* Another group of mutants was chosen for their role in anion channel activation: *slac1-3*, *ghr1-3, quac1-3* and *ost1-3*. Each genotype was subjected to sequential CO_2 changes from sub-ambient to elevated levels. These experiments showed that *ht1-2* and *ht1-8D* were insensitive to CO_2 changes regardless of the concentration (Figure 4A,C), which was expected outcome, as several previous studies have reported such phenotype (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). The guard cell carbonic anhydrases CA1 and CA4 facilitate CO_2 conversion to bicarbonate (HCO_3^-), and the *ca1 ca4* double mutant showed a slow and low amplitude response particularly in the sub-ambient 100–400 ppm transition (Figure 4A,C,E). The MAP kinase mutant *mpk12-4* exhibited a similarly shallow closure response to 100–400 ppm (Figure 4C) and a somewhat slower response time, in line with the known role of MPK12 as a regulator of CO_2 induced stomatal closure (Jakobson et al., 2016). Stomatal responses to 400–800 ppm CO_2 concentration change were less disrupted, total change in stomatal conductance was greater than WT for both mutants (Figure 4C), this could be explained by high initial stomatal conductance, which led to stronger response.

Conversely, mutants affecting the anion transport displayed relatively larger defects when stomata were exposed to above-ambient CO_2 levels. In *slac1-3*, *ost1-3*, and *ghr1-3* mutants, the closure response to 400–800 ppm was slow compared to WT (Figure 4B,D,F). The *slac1-3* mutant had slow, almost linear, stomatal response in 400–800 ppm transition (Figure 4F), but total response amplitude was WT like (Figure 4D). SLAC1 is the principal S-type anion channel required for stomatal closure (Negi et al., 2008; Vahisalu et al., 2008) and this explains the slow response, the similarity in closure amplitude might mean that some other mechanisms compensate for SLAC1 deficiency, this could potentially be done by QUAC1 R-type anion channel. Overall weakest response to 400–800 ppm CO_2 change was observed in *ghr1-3* (Figure 4D). GHR1 was suggested to function as pseudokinase possibly acting as a scaffolding protein facilitating access of protein kinases involved in SLAC1 anion channel activation by phosphorylation (Sierla et al., 2018).

Interestingly, in response to sub-ambient 100–400 ppm transition, *slac1-3* and *ghr1-3* mutants were less affected; their stomata responded fairly well to CO_2 changes, albeit with some delay or reduced magnitude. This indicates that other regulators and possibly rapid-type (R-type) anion channels can partially compensate their function at sub-ambient CO_2 ranges. OST1 deficient plants had slow responses to CO_2 changes in both ambient and above ambient ranges and severely inhibited response amplitude in sub ambient to ambient CO_2 response (Figure 4BDF). These results indicate that GHR1 and SLAC1 could have a greater role specifically in above-ambient CO_2 conditions.

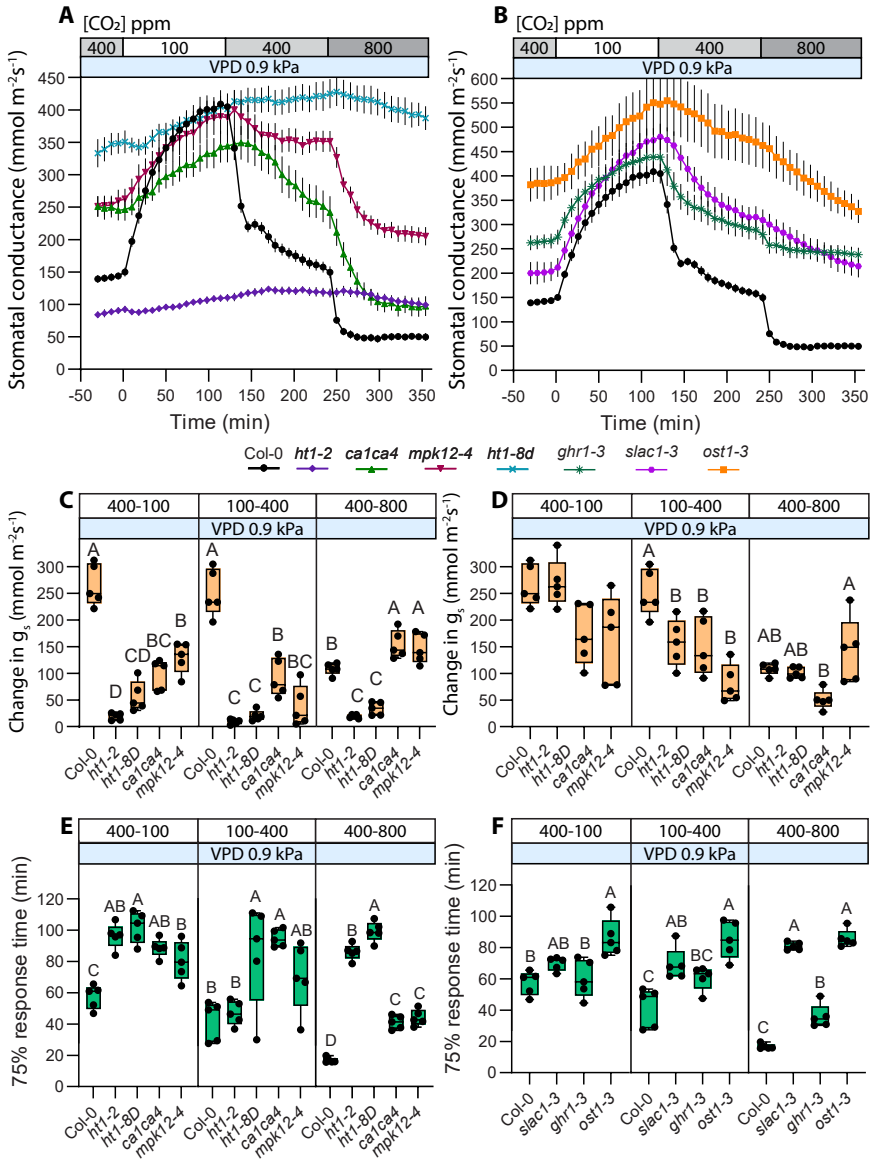

Figure 4. CO_2 response patterns.
Modified from article I. (A) and (B) Stomatal response to CO_2 concentration changes from 400 to 100 parts per million (ppm), 100 to 400 ppm and 400 to 800 ppm, mean stomatal conductance ± SEM is shown. (C,D) Boxplot of stomatal conductance (g_s) change (mmol $m^{-2}s^{-1}$) in response to CO_2 concentration changes from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. (E,F) Boxplot of 75% response time (minutes) of stomatal response to CO_2 concentration changes from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. (C-F) Boxes represent 25–75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey post hoc test, $p < 0.05$). (A–F) Sample size was 5 for all plant lines. Start of first treatment was between 11:30 to 12:30. Col-0 data is same as used in Figure 3, experiments with Col-0 and mutant lines were done together.

To investigate the role of QUAC1 (also known as ALMT12) which is the major R-type anion channel in guard cells (Meyer et al., 2010) we carried out additional experiments. Prior studies have shown that QUAC1 primarily transports malate across guard cell plasma membrane and defective QUAC1 affects stomatal responses to different stimuli less than defective SLAC1, but additive effect with SLAC1 has also been demonstrated with *slac1-3 quac1-1* double mutants, showing no stomatal response in above ambient CO_2-induced stomatal closure (Jalakas et al., 2021a). We used *slac1-3 quac1-1* double mutant and subjected it to the same 400-100-400-800 ppm CO_2 shifts and monitored these plants whole rosette gas exchange to see if there are differences in sub-ambient and above-ambient stomatal CO_2 responses. Results showed that the double mutant does not close stomata in response to CO_2 either at above ambient nor at below ambient increase in CO_2 levels (Figure 5).

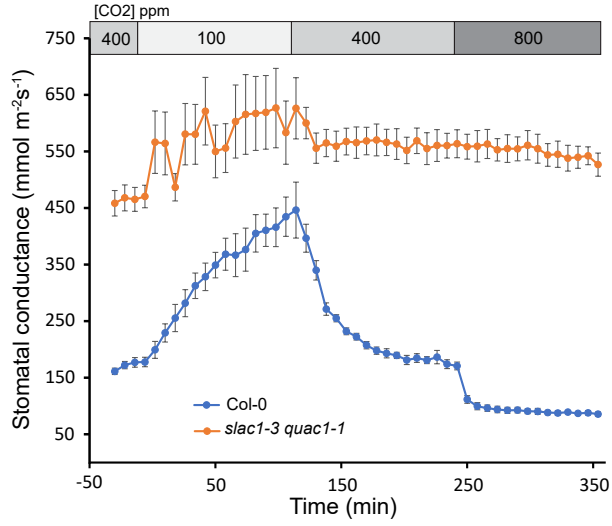

Figure 5. CO_2 response in *slac1-3quac1-1* double-mutant.
Stomatal response to CO_2 concentration changes from 400 to 100 parts per million (ppm), 100 to 400 ppm and 400 to 800 ppm. Sample size was 4 for all plant lines. Start of first treatment was between 11:30 to 12:30.

Thus, while QUAC1 alone has a minor role during CO_2 shifts, it can compensate SLAC1 function at sub-ambient CO_2 levels. Studies have shown QUAC1 to be primarily a malate transport channel (Hedrich and Marten, 1993; Meyer et al., 2010) and malate was previously thought to be highly important in stomatal CO_2 signalling (Meyer et al., 2010), until bicarbonate was identified as the main signal for stomatal closure in elevated CO_2 (Takahashi et al., 2022). This explains how QUAC1 loss of function does not significantly affect stomatal responses. Nearly fully blocked stomatal responses to CO_2 shifts in the *slac1-3 quac1-1* double mutant show that anion efflux through QUAC1 and SLAC1 is required for stomatal closure across all CO_2 ranges. This CO_2 insensitive phenotype was similar to HT1 deficient mutants, especially to *ht1-8D* as stomatal conductance in double

mutants was also relatively high. According to current model the HT1 kinase with A109V mutation (*ht1-8D*) is always in its active state regardless of CO_2 concentration, which means that anion channel activity is constantly inhibited. Thus, the final outcome is similar, but through different mechanisms – anion channels do not function and stomatal regulation is hampered. This suggests that HT1 also inhibits QUAC1 as otherwise it should compensate for the SLAC1 inhibition. Additionally, OST1 has been shown to activate both, SLAC1 and QUAC1 (Imes et al., 2013), this could explain why OST1 deficient plants have disrupted CO_2 responses in sub and above ambient CO_2 ranges.

5.2.2. Interplay of CO_2 and vapor pressure deficit in stomatal responses

Rising atmospheric CO_2 is often accompanied by changes in vapor pressure deficit, as warmer temperatures reduce relative humidity and increase the drying power of air. High VPD is a potent driver of stomatal closure, and thus how VPD might modulate CO_2 responsiveness is an important question. By conducting CO_2 response experiments under both regular VPD (~0.9 kPa) and elevated VPD (~2.3 kPa) conditions, we observed that baseline stomatal conductance was lower in all genotypes in elevated VPD conditions as expected (Merilo et al., 2018). Certain CO_2-induced stomatal responses were accelerated at high VPD. For instance, the speed of stomatal closure upon a 100–400 ppm CO_2 increase was faster under elevated VPD compared to ambient VPD in wild-type plants (Figure 4E, Figure 6E). While 400–800 ppm CO_2 response speed was unchanged, the response magnitude was much smaller (Figure 3A,B), this can be caused by limitations in stomatal movements as the plants in elevated VPD achieved minimal stomatal conductance and thus room for additional closure was more limited. Mutant lines generally also showed trend for faster responses under elevated VPD, although *ost1-3* response speed did not increase meaningfully, further indicating that OST1 has a crucial role in VPD-induced stomatal regulation (Merilo et al. 2018). Plant lines with mutations in HT1 (*ht1-2* and *ht1-8D*) remained unresponsive to CO_2 changes also under high VPD conditions (Figure 6A,B,E,F). Interestingly, *mpk12-4* and *ca1ca4* plants, in which 400–800 ppm response was slower than WT in ambient VPD (Figure 4E), had comparable to WT stomatal response speed in 400–800 ppm CO_2 change under elevated VPD (Figure 6E). This could be caused by increased ABA concentration in plants due to VPD stress (McAdam and Brodribb, 2015) or the fact that stomata were more closed to begin with and response reached culmination faster. Elevated VPD also affected *slac1-3* response magnitude, which was similar to WT in 100–400 ppm response (Figure 6D), whereas in normal VPD conditions *slac1-3* had smaller than WT response amplitude (Figure 4D). Elevated VPD had a relatively large effect on *ghr1-3* behaviour, in 100–400 ppm CO_2 change the stomatal response was faster but also shallower than in ambient VPD (Figure 6D,F). Ambient VPD 400–800 ppm stomatal closure response was small but consistent in *ghr1-3*, while in elevated VPD conditions there was virtually no response (Figure 6D,F).

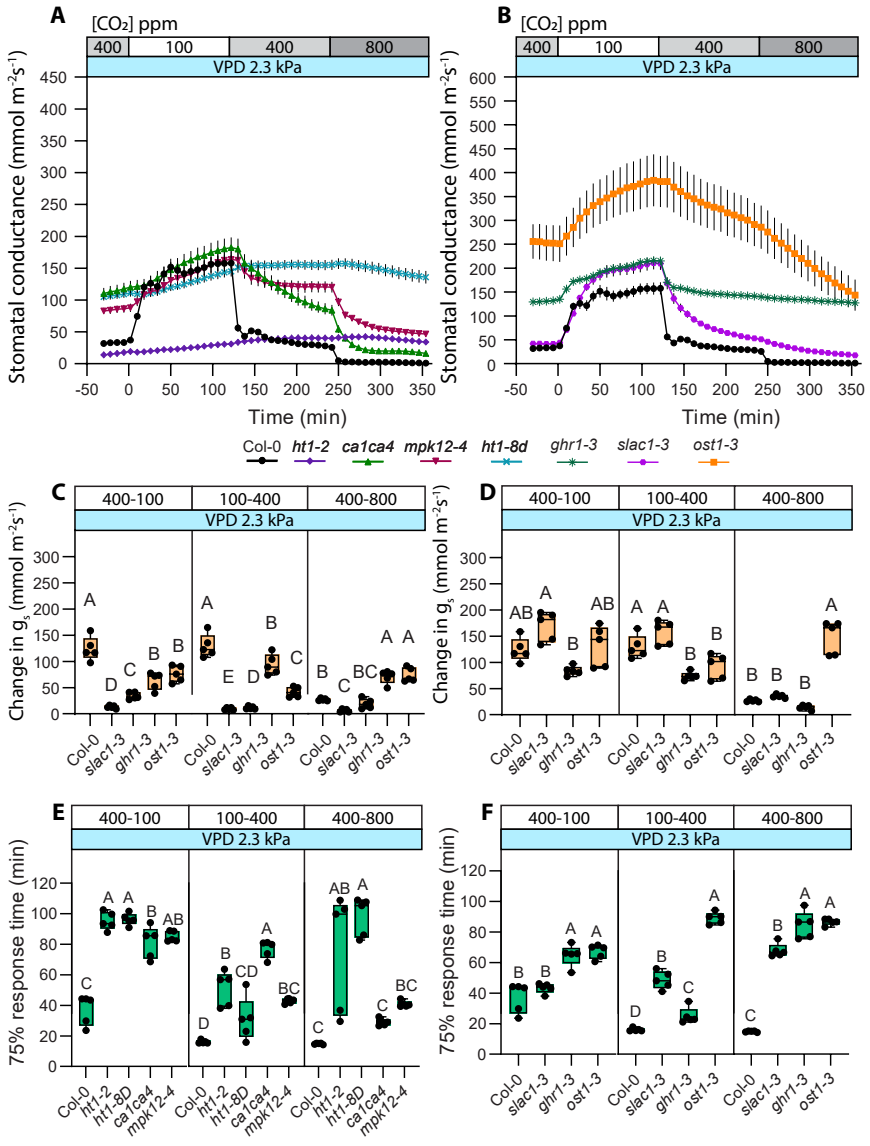

Figure 6. CO_2 response patterns in elevated VPD.
Modified from article I. (A) and (B) Stomatal response to CO_2 concentration changes from 400 to 100 parts per million (ppm), 100 to 400 ppm and 400 to 800 ppm at elevated VPD conditions (2.3 kPa), mean stomatal conductance ± SEM is shown. (C,D) Boxplot of stomatal conductance (g_s) change (mmol $m^{-2}s^{-1}$) in response to CO_2 concentration changes from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. (E,F) Boxplot of 75% response time (minutes) of stomatal response to CO_2 concentration changes from 400 to 100 ppm, 100 to 400 ppm and 400 to 800 ppm, respectively. (C–F) Boxes represent 25–75% quartiles and median as the horizontal lines, whiskers indicate the smallest and largest values, points show individual plant values. Statistically significantly different groups are marked with different letters (One-way ANOVA with Tukey post hoc test, $p < 0.05$). (A–F) Sample size was 5 for all plant lines. Start of first treatment was between 11:30 to 12:30. Col-0 data is same as used in Figure 3, experiments with Col-0 and mutant lines were done together.

It has been shown that GHR1 is required for ABA and rapid VPD responses, although elevated VPD response still functions in reduced capacity and speed (Hua et al., 2012b; Sierla et al., 2018; Hsu et al., 2021b), which suggests that elevated VPD had already triggered stomatal closure in *ghr1-3* and additional CO_2 had little effect. These results show that elevated VPD could trigger overlapping intracellular signals – such as elevation of ABA or Ca^{2+} – that can prime the CO_2 signalling pathway, effectively enhancing the guard cell sensitivity to CO_2 (Laanemets et al., 2013; Pantin et al., 2013).

5.3. Application of HT1 for improving plant water use efficiency and drought resilience

Previous studies have identified HT1 as potential target for modulating plant water use efficiency. Loss-of-function mutations in HT1 causes plants to have lower steady-state stomatal conductance (Hashimoto 2006, Hõrak et al., 2016) and recently it was demonstrated that such plant lines display improved water use efficiency in drought conditions (Xiao et al., 2024). Moreover, Arabidopsis HT1 orthologues have been found in many crop species, which suggests that HT1 has great potential for improving water use efficiency and drought performance in many crop species (Xiao et al., 2024).

Tomato is a crop with high water demand and requires irrigation throughout growing season (Patanè et al., 2011), while containing only 34.67 kcal/100 g (Ali et al., 2020). Tomatoes are the third most important vegetable crop after potato and cassava, with yearly worldwide production of nearly 200 million tons (FAO, 2025) and thus, technologies that would allow improving tomato water-use characteristics would be highly beneficial for worldwide food production. Since water availability is a major constraint of crop yield and is the single most important factor limiting food production, any improvement in tomato WUE, considered in terms of harvestable biomass per unit of water used would imply significant economic and environmental benefits (Mueller et al., 2012, Sinclair and Rufty, 2012, van Ittersum et al., 2013). However, to date, few studies have aimed to improve WUE in tomato (Cantero-Navarro et al., 2016).

Improving yield and WUE of tomatoes is highly relevant globally. One way to improve WUE is to grow tomatoes in greenhouses with high relative air humidity environments (>80% RH) which leads to lower transpiration (Suzuki et al., 2015). Conversely, reduced transpiration also causes a decrease in nutrient uptake from soil, although fruits seem to be unaffected (Suzuki et al., 2015). Elevated CO_2 levels can also increase plant WUE and decrease transpiration (Leakey et al., 2009; Pazzagli et al., 2016), yield benefits from elevated CO_2 as well, but also decreases fruit nutrient content (Mamatha et al., 2014; Myers et al., 2014). Thus, many greenhouses use CO_2 fumigation to increase production. Although, the increase in yield from elevated CO_2 has remained lower than theoretically possible (Long et al., 2006), part of this is likely caused by lowered stomatal conductance

in elevated CO_2 conditions, which suggests the possibility for improvement through modifying plant stomatal behaviour.

Light is another factor that impacts plant water use – in low light conditions (<200 μE) elevated CO_2 seems to have minimal effect on plant gas exchange, but elevated CO_2 combined with higher light intensities reduces plant gas exchange (Pan et al., 2020). All the previously mentioned methods for improving tomato WUE are only applicable in greenhouses, thus improving tomato WUE in open fields requires a different approach.

5.3.1. Arabidopsis HT1 homologues in tomato were edited with CRISPR/Cas9

We decided to modify HT1 homologues in *S.lycopersicum* to see if nonfunctional HT1 provides benefits in water use efficiency and drought tolerance. Tomato is a good candidate for model-to-crop approach, as its diploid genome with the size of 950 Mb, makes it easier to identify and manipulate genes of interest. We looked for HT1 homologues in tomato genome and found two potential candidates. Solyc11g012050.1 displays 83.6% identity with *A.th*aliana HT1 at protein level and Solyc04g014690.2 demonstrated 85.6% identity with *A.th*aliana HT1 at protein level. Next in collaboration with prof. Assaf Mosquna group (The Hebrew University of Jerusalem, Israel) we applied CRISPR/Cas9 system with guide RNA that would target both of these homologues (Figure 7A–C). This approach provided knockout double mutant tomato lines deficient in two HT1 homologues in tomato (SLHT1– Solyc04g014690.2 and SLHT2 – Solyc11g012050.1, which were named as SLHT1 and SLHT2, respectively). To assess the potential benefits of the applied mutation we did several experiments with this plant line: gas exchange measurements were used to measure stomatal responses to CO_2 changes, ABA treatment and elevated VPD. Additionally, we also subjected plants to drought conditions and evaluated their resilience, measured biomass of plants grown in ambient or elevated CO_2 conditions and grew plants to fruiting in greenhouse to analyze fruit properties.

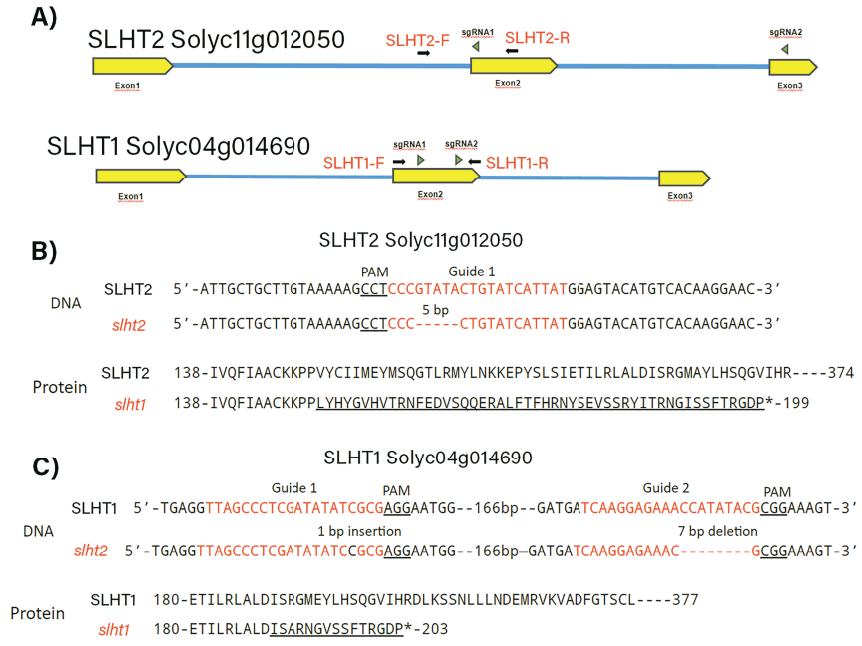

Figure 7. Tomato HT1 homologues.
A) The Crispr-Cas9 was targeted at SLHT1 (total protein length 377 AA) and SLHT2 (total protein length 374 AA) second exon and corresponding changes in **B)** SLHT2, where *slht2* has a 5bp deletion in second exon which results in frame-shift and a shorten protein ending at 199 amino acids. In **C)** SLHT1, *slht1* (*slht1*) has a 1bp insertion and a 7bp deletion in second exon which results in frame-shift and a shorten protein ending at 203 amino acids.

5.3.2. Loss of SLHT1 and SLHT2 function causes stomatal insensitivity to CO_2

In gas exchange experiments we subjected *slht1slht2* and WT tomatoes to sequential CO_2 concentration changes (400ppm to 800ppm to 400ppm to 100ppm). Our tomato *slht1slht2* line stomata did not respond to any change in CO_2 concentration (Figure 8A). This phenotype is very similar to results observed in Arabidopsis *ht1-2* mutants in publication II of this thesis (Figure 4A) and other previous work (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016).

Next, we tested stomatal responses to stress factors with ABA spray treatment and elevated VPD conditions. Stomatal responses to ABA and elevated VPD were WT-like, showing strong stomatal closure response in both ABA and elevated VPD experiments (Figure 8B,C). This shows that modifications in HT1 do not affect general stomatal stress responses, similar results have also been found previously in Arabidopsis (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016) and supports the claim that HT1 is a great target for improving drought performance (Xiao et al., 2024), as it does not affect stress responses and plants are still capable of closing their stomata in low water availability.

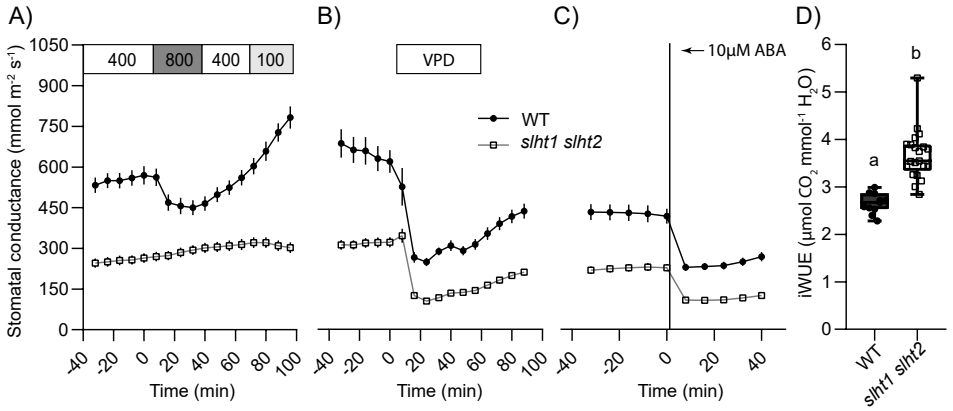

Figure 8. Tomato wild-type (WT) and *slht1 slht2* mutant stomatal conductances.
The *slht1slht2* plants does not respond to A) elevated (800ppm) and low (100ppm) CO_2, while reactions to B) vapor pressure deficit and C) exogenous treatment with 10 µM ABA solution remain intact. D) iWUE – ratio of net CO_2 assimilation to stomatal conductance (5 gas-exchange datapoints taken right before the application of 800ppm CO_2 were used in calculations). T-test p (same mean) = 1,49E-08, so $p<0.05$). WT n=11, double n=21; Error bars ± SEM.

In order to assess water use efficiency, we calculated iWUE (iWUE was calculated from gas exchange measurements as the ratio of net CO_2 assimilation to stomatal conductance ($iWUE=A/g_s$) from stomatal steady-state gas exchange measurements. This analysis demonstrated that double mutant tomatoes had higher iWUE, meaning that less water was used per assimilated CO_2 (Figure 8D). Improved iWUE was one of the goals for making this plant line and these results confirm what was theorized in Xiao et al., 2024.

5.3.3. *slht1slht2* tomato line has decreased water usage and possibly improved drought resilience

Based on gas exchange experiments we reasoned that *slht1slht2* tomato lines might have improved drought resilience. To address this we first grew all plants in well-watered conditions for 38 days and then stopped watering entirely and observed plants for wilting and loss of turgor based on visual assessment. During this experiment, each pot was watered to same mass and amount of water used was recorded (Figure 9A). Double mutants needed significantly less water than WT during our experiment (Figure 9B), which is likely caused by their generally lower stomatal conductance (Figure 8A). Lower water usage is directly beneficial for agricultural applications, a larger field could be irrigated with same amount of water, which would lower production costs and increase food security. After stopping watering at the onset of drought treatment, we visually observed our plants and noticed that *slht1slht2* plant line looked healthier than WT in drought (Figure 9E), while all plants in well-watered control group were healthy looking (Figure 9D).

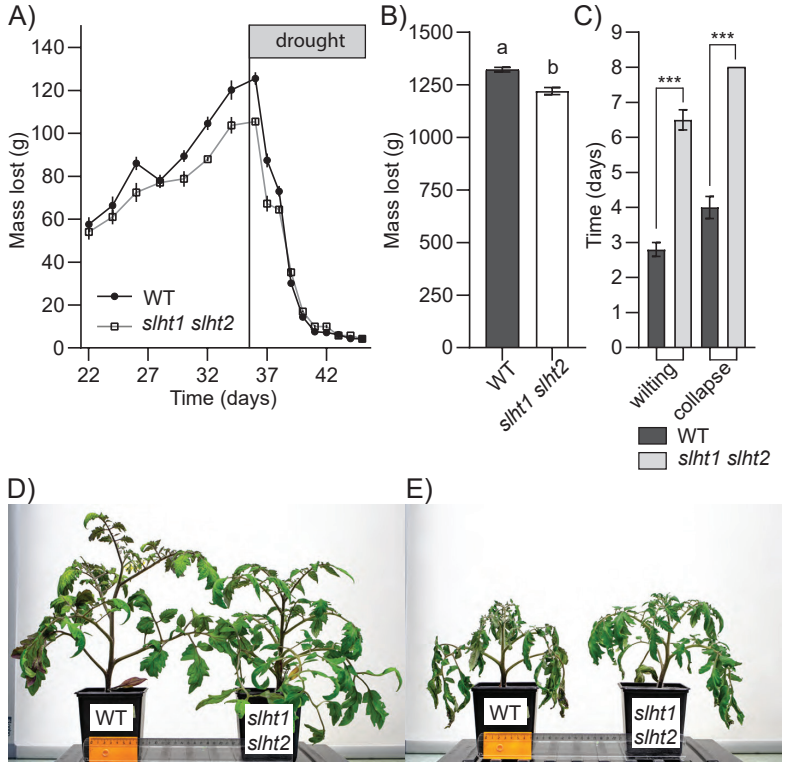

Figure 9. Tomato wild-type (WT) and *slht1 slht2* double-mutant drought experiment. Pots lost their mass at the expense of water, A) mass loss over 25 days and B) amount of total lost mass per plant in 45 days. At the age of 38 days, plants were exposed to complete drought (watering was stopped) and the rate of wilting was observed. C) Wild-type plants wilted 3,3 days after being exposed to drought and collapsed at 4,4 days into drought period, while double mutant plants started to wilt at 6,5 days and collapsed 8 days into drought ($p < 0{,}001$). Visually, there was no observable major turgor difference between D) well-watered WT and double mut plants while E) WT plant in drought looked significantly more drooped than double mutant plant. WT n=5, double n=4; Error bars ± SEM

We recorded the time when each plant was showing signs of wilting and total loss of turgor and observed that WT tomatoes started wilting after 3–4 days and collapsed completely 4–5 days after beginning of drought while *slht1slht2* line started to wilt 6–7 days and total collapse happened in 8 days (Figure 9C). This suggests that these tomatoes would have better chance to survive during sudden drought or limited water conditions. Although, soil water content was not controlled in this experiment and therefore *slht1slht2* plants could have used their soil water slower than WT. This means that *slht1slht2* phenotype in this experiment does not strictly demonstrate better drought tolerance and improved performance might be caused by lower water use. This phenotype is still clearly beneficial from an agricultural perspective, especially in regions where tomatoes are grown in open field conditions and where water availability is typically scarce. Conversely, such tomato lines would make little sense in commercial greenhouse

conditions, where watering is controlled and CO_2 concentration is elevated for increase in production. Variant of tomato with constitutively active HT1 mutation would theoretically be more practical in this case. Plants with constitutively active HT1 have very high stomatal conductance and no response to CO_2 changes (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). This would allow plants to benefit more from CO_2 fumigation as their stomata do not close and more CO_2 is available for use in photosynthesis. This should be explored more thoroughly in the future to see if more open stomata would translate into increased production capability.

5.3.4. HT1 and MPK12 could be conserved proteins

HT1 and MPK12 could be highly conserved and therefore have similar function in most plants. Previous research suggests that HT1 and MPKs could be highly conserved in vascular plants (Jiang et al., 2022) and HT1 has also been found to be highly expressed in *P.patens* sporophytes (Chater et al., 2013). The HT1 deficient tomato lines behaved very similarly to respective Arabidopsis HT1 loss-of-function lines, displaying disrupted stomatal CO_2 signalling and increased drought tolerance. Another study in which a complete deletion line of HT1 was made in Arabidopsis showed very similar results – increased drought tolerance, leaf temperature and iWUE (Xiao et al., 2024). Furthermore, they complemented their HT1 deletion line with HT1 sequences from *O.sativa* and *B.napus* and managed to recover the phenotype, this further indicates the conserved function of HT1 protein (Xiao et al., 2024).

5.3.5. Effects of long-term high CO_2 treatment

As *slht1slht2* plants had lower than WT stomatal conductance, we wanted to address if it also has an effect on plant biomass production. In Joerg-Peter Schnitzler laboratory at Helmholtz Zentrum Munich (Germany) the plants were grown in phytotorons at two CO_2 concentrations for 21 days, after which the plants were harvested. CO_2 concentration in control chambers was 400 ppm and 1000 ppm in elevated treatment chambers. Despite the CO_2 concentration difference, the plant fresh weight was similar for all treatments and genotypes (Figure 10A). This means that lower stomatal conductance of *slht1slht2* did not result in worse than WT growth. Dry weight measurements revealed that in control conditions, *slht1slht2* plants had lower biomass than elevated CO_2 treated WT and mutant plants (Figure 10B), yet WT plants in control CO_2 conditions were not significantly different from any of the former. This indicates that *slht1slht2* plant line has higher benefit from elevated CO_2 concentration, which suggests that it could be suitable for use in CO_2 fumigated greenhouse, although most likely not as efficient as plants with constitutively active HT1, as discussed previously.

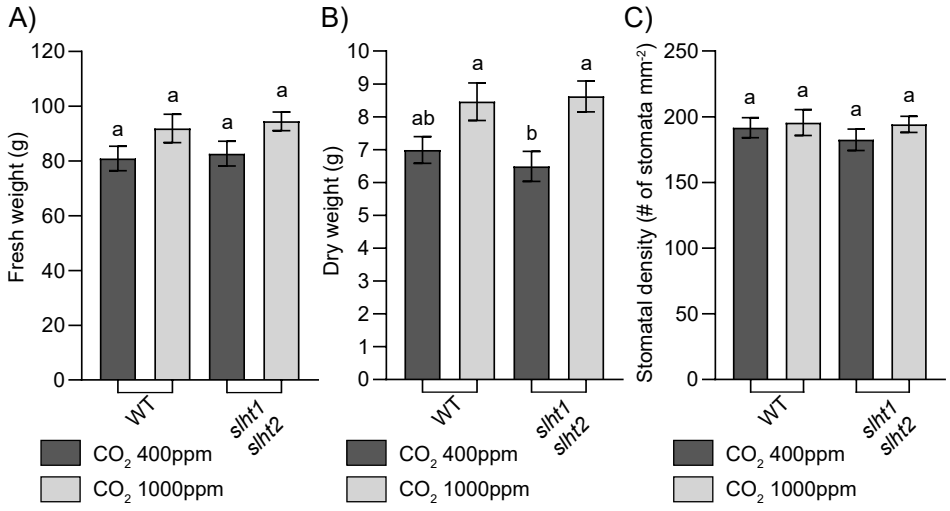

Figure 10. Tomato wild-type (WT) and *slht1 slht2* (double) mutant 42 days old plants' A) fresh and B) dry weight, measured from green parts of the plant/ roots excluded. No statistically significant differences were observed in fresh weight and C) stomatal density on the abaxial side of the leaf, however there were differences in plant dry weights. Statistically significant groups are denoted with different letters (one-way ANOVA with Tukey HSD post hoc test, $P < 0.05$). Error bars ± SEM.

Differences in stomatal density could play a role in these results, in publication III it is shown that stomatal density has effect on stomatal conductance and growth. Stomatal density changes in elevated CO_2 is a controversial topic, there is evidence that elevated CO_2 could decrease stomatal density in tomatoes (Pan et al., 2025) as well as results that show no significant effect of elevated CO_2 on stomatal density (Ainsworth and Rogers, 2007). This could mean that the effect is dependent on species or yet to be identified. In our experiments we took stomatal imprints from leaves fully developed during 400 ppm and 1000 ppm CO_2 treatment period to address whether growth under elevated CO_2 for 21 days could affect this trait. Results showed no major differences between plant lines or CO_2 treatments (Figure 10C), which shows that stomatal conductance was not a significant factor in growth. This also shows that elevated CO_2 concentration does not have significant effect on stomatal density in tomatoes.

5.3.6. Fruit properties of *slht1slht2*

To address yield characteristics, 1/3 of plants were transferred to a greenhouse after CO_2 treatment experiment and grown there until fruits were ripened and key characteristics used to define tomato fruits were documented, such as fruit volume, water content and pH, to detect potential changes in nutrient content, we also measured lycopene in fruits. CO_2 concentration was not controlled in greenhouse and thus CO_2 treatments were not applicable to this dataset and only plants grown at 400 ppm CO_2 were used. Data from fruit analyses shows that *slht1slht2*

double-mutants produce fruits with water content (Figure 11A) and volume (Figure 11B) in ambient CO_2 growth conditions. Fruit pH (Figure 11C) and lycopene content (Figure 11D) were comparable to WT, these results mean that *slht1slht2* plants produced fruits that were of equal or better quality than WT. Differences in water content and volume could be caused by lower transpiration of *slht1slht2* plants, which left more water available to be used in fruit development. Previous studies have shown that elevated VPD reduces relative growth rate of tomato fruits (Guichard et al., 2005), fruit transpiration is also increased in this case. As *slht1slht2* tomatoes have overall lower transpiration, the increased fruit water content and volume observed in our experiments could be caused by similar effect. Our edited tomatoes could have increased fruit production In CO_2 fumigated greenhouses, but this aspect needs further testing. With that in mind, this genetic editing produces similar benefits as growing tomatoes in very high relative air humidity environment (Suzuki et al., 2015), which also reduces water use but has no effect on fruit nutrient content.

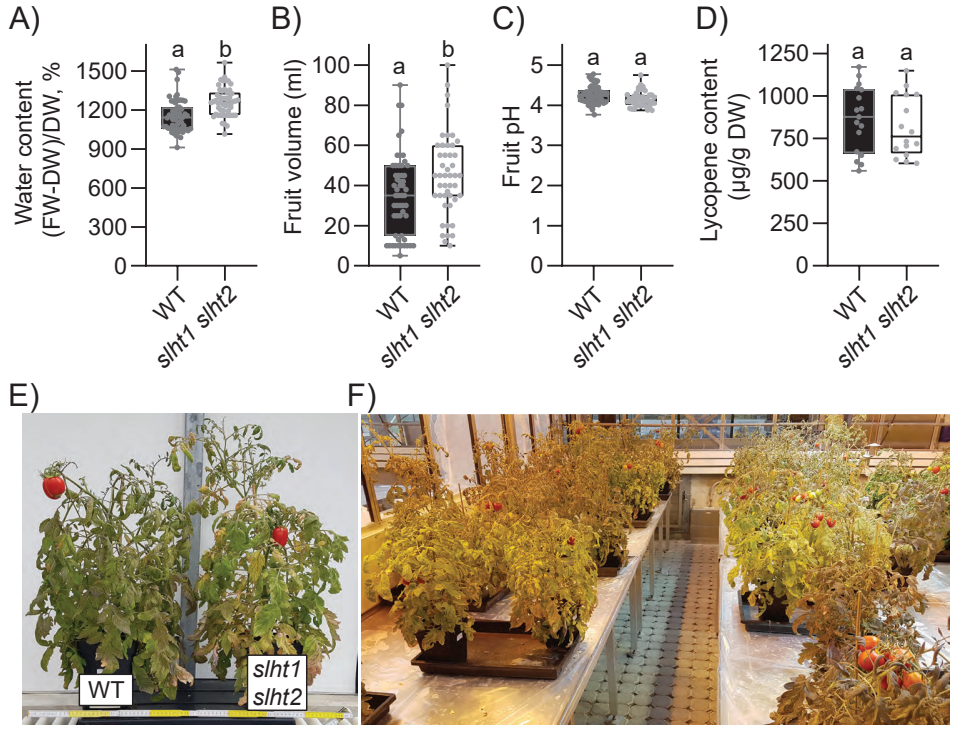

Figure 11. Tomato wild-type (WT) and *slht1slht2* (double) mutant fruit analysis A) fruit water content, B) fruit volume, C) pH and D) lycopene content. Box-and-whisker boxes mark the first to the third quartiles with a horizontal line showing the median, whiskers indicate the minimum and maximum, and circles are individual data points. Statistically significant groups are denoted with different letters (one-way ANOVA with Tukey HSD post hoc test, $P < 0.05$). Photos provide a visualisation how plants were producing fruits, where E) wild-type and *slht1slht2* plants of the same age are compared side-by-side and F) shows how the plants were kept in the greenhouse at ambient CO_2 levels (~400 ppm) to produce fruits.

5.3.7. MPK12 could be future target for improving plant WUE

MPK12 is known to inhibit HT1 (Hõrak et al., 2016; Jakobson et al., 2016) and would thus be another valuable tool for modifying plant water usage. MPK12 homologues have been found in tomato (Tõldsepp, 2019) and biochemistry in this study confirms that MPK12 functions in tomato are analogous to what is known for *A.thaliana*. Tõldsepp, 2019 generated tomato MPK12 and SLHT1 variants of mutations known to disturb HT1-MPK12 interactions, such as *mpk12 G53R* (Jakobson et al., 2016), *HT1 A109V* (Hõrak et al., 2016) and *HT1 R102K* (Hashimoto-Sugimoto et al., 2016). Tomato MPK12-induced inhibition of SLHT1 activity was clearly reduced by these mutations (Tõldsepp, 2019), suggesting that tomato lines with these mutations should have similar phenotype as respective Arabidopsis HT1 and MPK12 lines. Based on knowledge gained in Arabidopsis (Hõrak et al. 2016; Jakobson et al. 2016), plant lines carrying these mutations should have reduced stomatal sensitivity to CO_2 shifts and more open stomata phenotype. Such characteristics could have practical application in industrial greenhouses where CO_2 levels are typically doubled to maximize photosynthesis, whereas humidity and watering is carefully controlled.

Using MPK12-HT1 module for improving tomato WUE shows great potential, in *slht1slht2* double mutants we successfully altered stomatal CO_2 responses while stress responses remained intact. Future research into this topic should include searching for additional HT1 and MPK12 mutations in tomato, although finding out the primary SLMPK12 should be the first priority.

5.4. Effects of low RH and increased stomatal density on plant growth (III)

Current approach for genetical plant modification has mostly focused on single trait, yet it has been proposed that modifying singular trait might not be enough to have meaningful improvements in crops (Flexas, 2016). Here we designed Arabidopsis lines, which combine increased stomatal density (*epf1*, *epf2*) and high sensitivity (*ht1-2* or *cyp707a1/a3*) to see whether these features stack additively and how it affects plant anatomy, growth and physiology in different humidity conditions. Results showed that stomatal density and sensitivity traits combined largely as expected. As anticipated, *epf1 epf2* exhibited higher than WT leaf conductance, whereas *epf1epf2ht1-2* and *epf1epf2cyp707a1/a3* showed reduced conductance relative to *epf1epf2*, while remaining above WT (III, Figure 1A). Thus, introducing a high sensitivity stomatal closing background partially reduced the elevated conductance caused by very high stomatal density, demonstrating an additive stacking effect on stomatal conductance.

Low RH generally reduced leaf conductance and lines with *epf1epf2* were strongly affected (III, Figure 1A). This indicates that increased stomatal density did not prevent down regulation of conductance under low RH in our plants, consistent with reports that some high-stomatal-density genotypes can maintain

strong drought responsiveness (Caine et al., 2023). In contrast, several studies have described impaired stomatal regulation in high-stomatal-density backgrounds (Dow et al., 2014; Lehmann and Or, 2015; Papanatsiou et al., 2016), but those effects were largely attributed to stomatal clustering, where adjacent stomata in direct contact compromise coordinated function.

Stomatal density increased slightly under low RH, with lines containing *epf1epf2* showing a significant increase on the adaxial surface (III, Figure 4A). Reported humidity effects on stomatal density remain inconsistent across studies, with increases reported under high RH (Fanourakis et al., 2013; Laur and Hacke, 2013) as well as decreases (Leuschner, 2002; Aliniaeifard et al., 2014), suggesting strong dependence on species and context (Fanourakis et al., 2020). Growth was independently affected by low RH and stomatal density (III, Table 3), *epf1epf2* lines had reduced dry mass and leaf area, and low RH reduced leaf area and biomass across genotypes, whereas sensitivity mutants, particularly *ht1-2* were least affected by low RH.

Finally, it has been suggested that targeting single limiting traits is unlikely to deliver sufficient gains for future crop performance, and that stacking multiple trait modifications should be pursued (Flexas, 2016). Consistent with this framework and prior modelling predictions (Flexas, 2016), our results show that genetic modifications affecting stomatal density and closing sensitivity can be combined with additive effects on stomatal conductance. In practice, this approach could support improved drought performance by lowering water loss through enhanced stomatal closure sensitivity even in high stomatal density backgrounds. Additionally, similar approach could also be used to generate plants with high stomatal density and conductance, these would theoretically perform well in high humidity and CO_2 fumigated greenhouses. High humidity has been shown to improve WUE (Suzuki et al., 2015) and it also generally lowers transpiration, plants with high SD and highly open CO_2 insensitive stomata could offer increased production if sufficient light can be supplied. Agricultural viability of this approach could be studied by generating *epf1epf2 ht1-8D* tomato plants, this plant line should have high stomatal density with open stomata that are CO_2 insensitive. Although, as very high stomatal density of Arabidopsis *epf1epf2* negatively affects growth (Jalakas et al., 2024), constructing several lines with varying stomatal densities would allow to find optimal stomatal density. In theory, such plants should work well in high humidity greenhouse with increased CO_2 concentrations.

6. CONCLUSIONS

This thesis aimed to deepen our understanding of stomatal CO_2 regulation, molecular mechanisms involved and interactions with effects from elevated VPD. In addition, this research made use of knowledge gained from studying model plants to modify stomatal behaviour in crop plants. To achieve this, experiments were conducted in *Arabidopsis thaliana* and tomato.

Key findings from this thesis are:

- Stomatal CO_2 response kinetics in Arabidopsis differs in sub and above ambient CO_2 levels and stomatal movements in these concentration ranges are controlled by different molecular mechanisms.
- Elevated VPD conditions generally increase the speed of stomatal CO_2 responses in sub and above ambient CO_2 levels.
- Plants deficient in both SLAC1 and QUAC1 have insensitive stomata to CO_2 changes in all concentration ranges.
- MPK4 and MPK12 are key kinases in stomatal CO_2 responses in *Arabidopsis*. These MAPKs act as signalling hub, linking CO_2 sensing to guard cell anion channels.
- Arabidopsis HT1 homologues in tomato are necessary for stomatal CO_2 regulation, suggesting that HT1 is highly conserved and indicating that a broadly shared stomatal CO_2 signalling mechanism is present across species.
- SLHT1 and SLHT2 loss-of-function mutations decreased tomato plant water usage and improved iWUE, indicating potential for agricultural use.
- Fruit parameters of *slht1slht2* plants were comparable or better than WT, exhibiting higher water content and larger fruit volume.
- Dominant SLHT1 and SLHT2 mutations or loss-of-function mutants of MPK12 should be investigated as a strategy to develop plants for high-tech greenhouses with CO_2 fumigation.
- High stomatal density does not hamper ability to close their stomata as plants with *epf1epf2* exhibited strong stomatal closure during low RH conditions. Elevated stomatal density and low RH independently suppressed plant growth. (Thus, the cost of high stomatal numbers is not caused by large stomatal water loss.)
- Mutations affecting stomatal density and stomatal conductance combined independently. Similar strategy could theoretically be feasible for improving plant water use efficiency.

Collectively, these findings advance our understanding of guard cell signal integration under changing CO_2 and humidity conditions. By identifying key components in stomatal CO_2 and VPD responses, this work provides potential targets for improving intrinsic water-use efficiency and drought resilience in plants. Future research can translate these insights into crop breeding to develop climate-resilient plants with good performance under rising CO_2 and elevated VPD conditions.

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SUMMARY IN ESTONIAN

Õhulõhede süsihappegaasi signaalirada ja selle rakendamine tomatitaimedes

Valdav enamus elust Maal on sõltuv taimedest. Taimed loovad sobiliku elukeskkonna ning fotosünteesi käigus süsihappegaasist ja veest valmistatud orgaanilised ühendid on toiduks teistele organismidele. Lisaks võimaldab kõrvalproduktina vabanenud hapnik aeroobset ainevahetust. Inimestele on taimed võtmetähtsusega nii toidu kui ka ehitusmaterjalide, kangaste, ravimite, kütuse ja paljude teiste toodete valmistamisel. Käimasolevad keskkonnamuutused tekitavad põllumajanduslikult mitmeid probleeme, temperatuuri tõus toob kaasa suurenenud taimede veekulu ja kättesaadava vee vähenemise, see põhjustab niisutussüsteemide suuremat veekasutust või selle puudumisel saagikuse langust. Seetõttu on inimkonnale vajalik paremini mõista taimede toimimist ja rakendada neid teadmisi taimede aretamisel, et vähendada nende veekulu ja suurendada stressitaluvust.

Taimed ammutavad fotosünteesiks vajalikku süsihappegaasi atmosfäärist läbi õhulõhede, kuid samal ajal kaotavad nad ka väärtuslikku vett. Õhulõhed paiknevad taimede maapealsete osade pinnal ja koosnevad kahest sulgrakust ning nende vahele moodustuvast õhupilust. Õhulõhede avatust kontrollivad taimed läbi sulgrakkude veesisalduse, suurem veehulk sulgrakkudes muudab nende kuju kaarjaks ja õhupilu suureneb, veehulga vähenemine sulgrakkudes muudab nende kuju sirgemaks ja õhupilu kahaneb. Õhulõhede regulatsioon võimaldab taimedel kontrollida oma gaasivahetust keskkonnaga: õhulõhed avanevad valguse, madala süsihappegaasi taseme ja kõrge õhuniiskuse tingimustes ning sulguvad pimeduses, kõrge süsihappegaasi keskkonnas ja patogeenide ning saasteainete mõjul. Säärane regulatsioon lubab taimedel kiiresti muutuvates keskkonnatingimustes oma gaasivahetust sobivaks kohandada. Sulgrakkude veesisaldust kontrollitakse ioonide rakust välja ja sisse pumpamise abil, ioonide hulga suurenedes liigub rakku ka vesi ja vähenedes liigub vesi rakust välja. Ioonid liiguvad rakust sisse ja välja läbi rakumembraanis paiknevate ioonkanalite, mis on reguleeritud läbi eri signaalradade.

Oma doktoritöös käsitlesin sulgrakkude süsihappegaasi toimelist signaalirada harilikus müürloogas (*Arabidopsis thaliana*), täpsemalt signaalirajas osalevate komponentide uurimist ja erinevate süsihappegaasi tasemete mõju õhulõhede sulgumisreaktsioonidele tavapärases ja madalas õhuniiskuses. Nii leidsime, et mitogeen-aktiveeritud proteiinkinaasid MPK12 ja MPK4 on olulised komponendid taimede süsihappegaasi toimelises õhulõhede regulatsioonis. Mitte toimiv MPK12 valk põhjustas taimedel suurenenud õhulõhede juhtivust ja vähenenud reageerimisvõimet süsihappegaasi muutustele. MPK4 ja MPK12 valkude funktsiooni rikkumisel kaotasid taimede õhulõhed süsihappegaasi regulatsiooni võimekuse. Süsihappegaasi kontsentratsiooni mõju õhulõhede regulatsioonile uurisime mitmete hariliku müürlooga mutantide abil, kus olid rikutud süsihappegaasi signaaliraja või ioonkanalite funktsionaalsus. Leidsime, et õhulõhede vastused erinevad vastavalt kasutatud süsihappegaasi kontsentratsioonile. Madalas kuni

tavalises süsihappegaasi kontsentratsioonis on õhulõhede reaktsioonid aeglasemad aga suurema ulatusega, samas tavalises kuni kõrges kontsentratsiooni vahemikus on reaktsioonid kiiremad ja väiksema amplituudiga. Samuti avastasime, et karboon-anhüdraaside CA1 ja CA4, MPK12 ja SLAC1 anioonkanali funktsiooni rikkumisel olid tugevamalt häiritud õhulõhede vastused madalas kuni tavalises süshappegaasi tasemel. Madal õhuniiskus langetas üldiselt õhulõhede juhtivust ja kiirendas süsihappegaasi toimeliste õhulõhede reaktsioonide toimimist.

Lisaks rakendasime saadud teadmisi tomatis (*Solanum lycopersicum*), eesmärgiga vähendada tomatitaimede veekulu ja suurendada põuataluvust. Kasutasime täppisaretuse meetmeid hariliku müürlooga *HT1* geeni homoloogide funktsiooni rikkumiseks tomatis. Gaasivahetuskatsete tulemusena leidsime, et meie loodud tomati topeltmutandi *slht1 slht2* õhulõhed on tundetud süsihappegaasi taseme muutustele, kuid reageerisid endiselt madalale õhuniiskusele ja abstsiishappele. See näitab, et taimed olid endiselt võimelised õhulõhede sulgemiseks stressitingimustel ja häiritud oli vaid süsihappegaasi tundlikkus. Meie loodud tomatite õhulõhede juhtivus oli üleüldiselt madalam kui metsiktüübil ja seetõttu nägime ka madalamat veekulu ja kõrgemat veekasutusefektiivsust, samuti pidasid muudetud tomatid paremini vastu põuastele tingimustele. Biomassi mõõtmised näitasid metsiktüübiga võrreldavat kasvu ning ka viljad olid metsiktüübiga võrreldavate või isegi paremate omadustega, *slht1 slht2* tomati viljad oli suurema veesisalduse ja ruumalaga. Meie loodud tomatid võiksid praeguste uuringute tulemusena sobida küllaltki hästi avamaa põldudele, kus nende madalam veetarbimine võimaldaks säästa vett niisutuse arvelt ning suurendada ellujäämust põua korral.

Viimase teemana katsetasime õhulõhede tihedust mõjutavate mutatsioonide kombineerimist nende avatust vähendavate mutatsioonidega. Varasemalt on spekuleeritud, et tuleviku taimearetuses ei piisa enam üksikute tunnuste muutmisest, vaid on tarvis omavahel kombineerida muudatusi mitmetes eri tunnustes. Tulemustes väljendus, et tunnused kombineerusid ootuspäraselt, näiteks kombineerides omavahel kõrge õhulõhede tiheduse ja madalama juhtivuse tunnused oli saadud taimede õhulõhede juhtivus madalam kui ainult kõrge õhulõhede tihedusega taimedel. Sarnast lähenemist on võimalik tulevikus kasutada taimede sordiaretuses, näiteks kombineerides omavahel kõrge õhulõhede tiheduse kõrge juhtivusega võiksid saadud taimed teoreetiliselt sobida hästi moodsatesse kasvuhoonetesse, kus õhuniiskus ja vee kättesaadavus on hea ning lisaks kasutatakse tootlikkuse tõstmiseks süsihappegaasi lisamist.

Selles töös käsitlesime õhulõhede süsihappegaasi signaalirada ja selle toimimist eri kontsentratsiooni piirkondades ja niiskustingimustes. Harilik müürlook on hea mudeltaim esmaste avastuste tegemiseks, kuid tal puudub praktiline väärtus põllumajanduses. Teadmiste rakendamiseks valmistasime muudetud õhulõhede juhtivuse ja regulatsiooniga tomatitaimed, mis praeguste tulemuste valguses omavad kasulikke tunnuseid avamaa põldudel kasvatamiseks.

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PUBLICATIONS

CURRICULUM VITAE

Name: Kaspar Koolmeister
Date of Birth: 2nd of April 1995, Palamuse
Citizenship: Estonian
Contact: Nooruse 1, Tartu 50411, Estonia
kaspar.koolmeister@ut.ee

Education

2019–…	University of Tartu, doctoral studies in Engineering and Technology (Environmental Technology)
2017–2019	University of Tartu, MSc (Gene Technology)
2014–2017	University of Tartu, BSc (Gene Technology)
2011–2014	Hugo Treffner Gymnasium

Professional employment

2023–…	Plantinvent Ltd, Sales and Marketing Manager
2020–2025	University of Tartu, Institute of technology/Institute of Bioengineering, Junior Research Fellow in Plant Biology

Professional self-improvement

2020	Seminars for Supervisors of Students Research Papers, University of Tartu, Estonia
2019	"Microscopy Techniques in Agriculture" Workshop, Estonian Crop Research Institute, Estonia

Scientific publications

Tulva, I., **Koolmeister, K**. and Hõrak, H., 2024. Low relative air humidity and increased stomatal density independently hamper growth in young Arabidopsis. *The Plant Journal*, *119*(6), pp. 2718–2736.

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ELULOOKIRJELDUS

Nimi: Kaspar Koolmeister
Sünniaeg: 2. aprill 1995, Palamuse
Kodakondsus: Eesti
Kontakt: Nooruse 1, Tartu 50411, Eesti
kaspar.koolmeister@ut.ee

Hariduskäik

2019–…	Tartu Ülikool, tehnika ja tehnoloogia (keskkonnatehnika eriala) doktoriõpingud
2017–2019	Tartu Ülikool, loodusteaduse magister (Gene Technology)
2014–2017	Tartu Ülikool, loodusteaduse bakalaureus (Gene Technology)
2011–2014	Hugo Treffneri Gümnaasium

Erialane teenistuskäik

2023–…	Plantinvent OÜ, tehnoloogia ja müügi juht
2020–2025	Tartu Ülikool, Tehnoloogia instituut/Bioinseneeria instituut, taimebioloogia nooremteadur

Erialane enesetäiendus

2020	Üliõpilaste uurimistööde juhendajate koolitus, Tartu Ülikool, Eesti
2019	"Microscopy Techniques in Agriculture" töötuba, Eesti Taimekasvatuse Instituut, Eesti

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