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INGMAR TULVA

Causes and consequences of stomatal density in relation to atmospheric humidity

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Causes and consequences of stomatal density in relation to atmospheric humidity

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IV. **Tulva, Ingmar**; Jalakas, Pirko; Ivandi, Elena; Wierzchula, Alexander; Obinwanne, Patricia Kika; Hõrak, Hanna (2025). Low relative air humidity leads to smaller, denser stomata and higher stomatal ratios in Arabidopsis. Preprint https://doi.org/10.1101/2025.01.31.635895

Author's contribution

I. Designed most of the experiments, participated in conducting the experiments, did most of the labwork, analysed and visualised the data, and wrote most of the article.

II. Designed one of the experiments and participated in conducting it; contributed to the final text of the article.

III. Designed and performed part of the experiments, analysed part of the data and contributed to writing the article.

IV. Designed a part of the experiments, participated in conducting the experiments, did part of the labwork, analysed the data and did most of the visualisation.

TERMS AND ABBREVIATIONS

Leaf conductance (g_l): sum of stomatal and cuticular conductance.

Leaf rank: (in this work) a number indicating the order in which leaves appear, starting with the oldest true leaf.

Relative humidity (RH): a measure of air humidity; RH = actual water vapour partial pressure / saturating water vapour partial pressure at given temperature.

Stomatal density (SD): number of stomata per unit of leaf area.

Stomatal index (SI): number of stomata per epidermal cells; SI = number of stomata / (number of stomata + number of pavement cells). In the context of this work, the number of stomata incorporates all epidermal cells in the stomatal lineage, including stomatal precursors.

Stomatal number per leaf: obtained as SD * leaf area.

Stomatal ratio (SR): adaxial to abaxial stomatal density ratio.

Vapour pressure deficit (VPD): a measure of air humidity; VPD = saturating water vapour partial pressure – actual water vapour partial pressure at given temperature.

INTRODUCTION

As living organisms colonised land – canonically in Ordovician-Silurian, but some evidence suggests much earlier, pre-Cambrian dates (Heckman *et al.*, 2001) – they faced the task of maintaining the water environment inside their cells while interacting with the atmosphere. Land plants in particular, using external CO_2 as their carbon source, require good access to the atmosphere while preventing uncontrollable water loss. For this reason, they have evolved to be covered with a largely impermeable cuticle, perforated with closeable gates known as stomata. Stomata maintain the balance between CO_2 access and water loss by closing and opening in response to environmental stimuli.

Stomata generally close to prevent water loss due to decreasing water availability or increasing atmospheric water demand (i.e. lower air humidity), but also to prevent entry of potentially harmful agents such as pollutant gases and pathogens, and open in response to CO_2 scarcity in intercellular spaces. While much of this balancing can be achieved by reacting to CO_2 and letting passive evaporation control stomatal guard cell water status, flowering plants have developed a machinery that speeds up stomatal movements by reacting directly to light environment, and employing a complex signalling network mediated by the stress hormone abscisic acid (ABA).

In addition to relatively instantaneous movements, the abundance of stomata on leaf surface is also of importance for optimisation of plant gas exchange, and is controlled according to the environment of the developing leaf or the whole plant.

1. LITERATURE REVIEW

1.1. Abscisic acid

Abscisic acid (ABA) is a ubiquitous plant stress hormone that controls plant development as well as many instantaneous responses to stress factors. Among other roles, ABA is involved in the control of plant water loss via cuticle synthesis (Macková *et al.*, 2013) and as a mediator of stomatal reactions to air humidity (Zeevaart & Creelman, 1988). It is synthesised in a multi-step chain of reactions from xanthophylls in plastids and cytosol; an important group of enzymes involved in the step of cleaving xanthophylls into xanthoxin is nine-cis-epoxy-carotenoid dioxygenases (NCEDs), at least five of which have been shown to participate in ABA biosynthesis (Schwartz *et al.*, 2003; Frey *et al.*, 2012). Due to redundancy in NCED action, single mutants show weak ABA-deficient phenotypes (Nambara & Marion-Poll, 2005); the Arabidopsis double mutant *nced3-nced5* encountered in this work has been shown to come with severely reduced ABA content (Frey *et al.*, 2012; Merilo *et al.*, 2018) and vastly increased stomatal openness (Merilo *et al.*, 2018), as well as striking shape with excessive number of narrow leaves.

The first step in ABA catabolism is hydroxylation of one of three methyl groups in its ring structure. Of most importance among these is hydroxylation of the C-8' position, catalysed by cytochrome P450, family 707, subfamily A (CYP707A) polypeptides (Saito *et al.*, 2004; Kushiro *et al.*, 2004), of which four are known. Plants deficient in CYP707A hydroxylases display increased ABA levels (Okamoto *et al.*, 2006; Rowe *et al.*, 2023) but there is redundancy between them, which together with the non-C-8' hydroxylation paths to ABA deactivation leads to relatively weak phenotypes of single CYP707A mutants. CYP707A3 is primarily expressed in guard cells and there is evidence that the *cyp707a1cyp707a3* double mutant and *CYP707A* overexpression lines show altered stomatal anatomy or conductance (Umezawa *et al.*, 2006; Okamoto *et al.*, 2009; Jalakas *et al.*, 2018).

1.2. Regulation of stomatal movement

The ultimate driving force behind stomatal movement is the turgor pressure within guard cells. Stomatal opening and closing involve ion transport across the guard cell membranes. During stomatal opening, potassium ions (K^+) are actively pumped into the guard cells, leading to an increase in osmotic pressure, followed by influx of water through aquaporins that causes the guard cells to swell and the stomatal pore to open. Conversely, stomatal closure involves release of K^+ ions and anions out of the guard cells, leading to decrease in osmotic pressure, cell shrinkage, and stomatal closure.

Stomatal openness is influenced by environmental cues, such as light intensity, CO_2 concentration, air humidity, and temperature. These factors are mediated by an interwoven network of signal transduction pathways, which involve the

activation of specific receptors and proteins within guard cells, leading to changes in ion transport, osmotic pressure, and ultimately stomatal aperture.

Stomatal closure is facilitated by ion channels of two general types. R-type channels activate in milliseconds, S-type channels in tens of seconds to minutes (Schroeder & Keller, 1992). An ion channel of central importance in stomatal closure is SLOW ANION CHANNEL 1 (SLAC1; Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Plants with impaired *SLAC1* show diminished or drastically slowed stomatal response to environmental signals as well as application of ABA (Vahisalu *et al.*, 2008). SLAC1 is, in turn, activated by a sucrose non-fermenting 1-related protein kinase SnRK2.6 a.k.a OST1 (OPEN STOMATA 1; Merlot *et al.*, 2002), a kinase of central importance in stomatal closure (Geiger *et al.*, 2009). Via mechanisms hitherto unclear, SLAC1 function is dependent on the pseudokinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1 (GHR1; Sierla *et al.*, 2018).

The signalling network responsible for stomatal closure in response to environmental stimuli can be roughly divided into ABA-dependent and CO_2-dependent systems (Figure 1). In the core ABA signalling pathway, ABA is bound to receptors from the wide and redundant family PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL), causing a conformational change that results in formation of a stable complex with TYPE 2 PROTEIN PHOSPHATASES (PP2C) and inhibiting the activity of the latter (Hsu *et al.*, 2021). The roles of individual PYR/PYL receptors are unclear; various studies have seen evidence of PYR1 (Pri-Tal *et al.*, 2024), PYL1 (Merilo *et al.*, 2013), or PYL2 (Dittrich *et al.*, 2019) as the most important or even sole receptor responsible for stomatal ABA-reaction. Inhibition of PP2C phosphatases activates SNF1 related kinases (SnRKs; Umezawa *et al.*, 2009), as well as Ca^{2+}-dependent kinases (CPK). There are three central SnRK2 kinases involved in ABA signalling. SnRK2.2 and SnRK2.3 redundantly mediate ABA signalling in seed germination and seedling growth (Fujii & Zhu, 2009). SnRK2.6 (a.k.a OST1) is expressed more widely and is the central hub of stomatal ABA sensitivity, but can also partially fulfill the roles of SnRK2.2 and 2.3 (Fujii & Zhu, 2009; Fujita *et al.*, 2009). All these kinases regulate gene expression by phosphorylating ABA-responsive element binding transcription factors (ABFs), while SnRK2.6 also activates (along with CPKs) guard cell anion channels (Vlad *et al.*, 2009; Geiger *et al.*, 2009). The CO_2-dependent part of the signal transduction network ultimately goes through the mitogen activated protein kinase MPK12 (Jakobson *et al.*, 2016; Takahashi *et al.*, 2022) and the protein kinase HIGH LEAF TEMPERATURE 1 (HT1; Hashimoto *et al.*, 2006). The latter is a negative regulator in CO_2 signalling, with its kinase domain recessive mutants (such as *ht1-2* used in this work) incapable of inhibiting *SLAC1* activation and displaying decreased steady-state stomatal openness, being effectively in a permanent high [CO_2] response state (Hashimoto *et al.*, 2006; Hashimoto-Sugimoto *et al.*, 2016). Despite CO_2 signal bypassing OST1 (Takahashi *et al.*, 2022) and not affecting ABA levels (Hsu *et al.*, 2018), there is indication for some involvement of ABA in stomatal CO_2 reaction, or at least ABA-related signalling pathway upstream of OST1 (Merilo *et al.*, 2013; Chater *et al.*, 2015; Movahedi *et al.*, 2021).

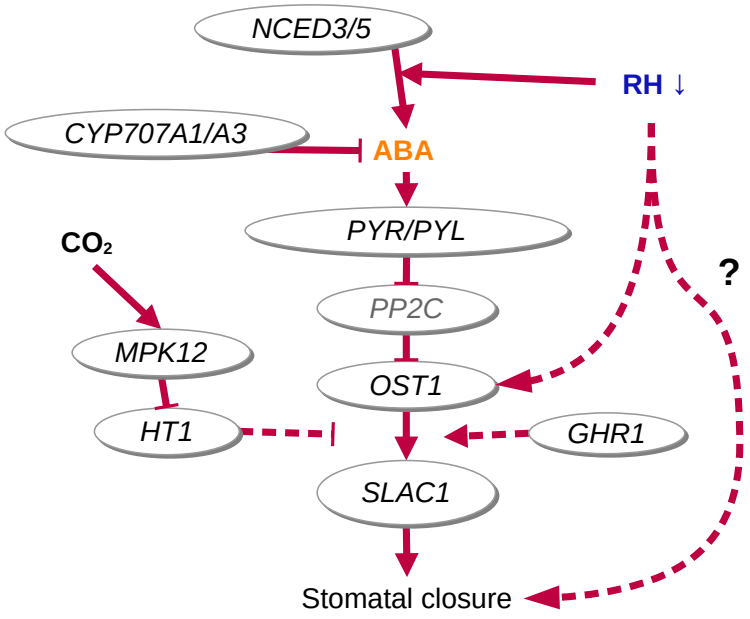

Figure 1. Basic outline of the signalling network controlling stomatal closure in Arabidopsis leaf. Genes or their groups that appear in this thesis shown in ovals. Arrows with sharp heads indicate upregulation, those with blunt heads inhibition. The dashed arrows denote regulatory pathways that are unclear at this time, including ABA-independent activation of *OST1* and the stomatal RH response that bypasses OST1 entirely.

The CO_2-dependent part of the signal transduction network ultimately goes through the mitogen activated protein kinase MPK12 (Jakobson *et al.*, 2016; Takahashi *et al.*, 2022) and the protein kinase HIGH LEAF TEMPERATURE 1 (HT1; Hashimoto *et al.*, 2006). The latter is a negative regulator in CO_2 signalling, with its kinase domain recessive mutants (such as *ht1-2* used in this work) incapable of inhibiting *SLAC1* activation and displaying decreased steady-state stomatal openness, being effectively in a permanent high [CO_2] response state (Hashimoto *et al.*, 2006; Hashimoto-Sugimoto *et al.*, 2016). Despite CO_2 signal bypassing OST1 (Takahashi *et al.*, 2022) and not affecting ABA levels (Hsu *et al.*, 2018), there is indication for some involvement of ABA in stomatal CO_2 reaction, or at least ABA-related signalling pathway upstream of OST1 (Merilo *et al.*, 2013; Chater *et al.*, 2015; Movahedi *et al.*, 2021).

A crucially important environmental cue from the perspective of stomatal function is air humidity, in plant physiology usually expressed as vapour pressure deficit (VPD). Plant stomata react to reduced air humidity (increased VPD) by closing (Grantz, 1990; Buckley, 2005), however, there is indication that even long-term stomatal response to air humidity may be absent unless a threshold VPD increase is reached (Binstock *et al.*, 2024). However, abrupt increase in VPD often causes a transient strong stomatal opening, known as wrong-way response (Darwin, 1898; Mott & Franks, 2001). This is thought to be a result of

the passive reaction of epidermal cells to increased evaporation, where the pavement cells lose turgor more rapidly than the guard cells, relaxing the back-pressure that they apply to the guard cells and allowing a stoma to open before its active closure mechanism via ion channel opening is activated (Mott *et al.*, 1997; Buckley, 2005).

The role of abscisic acid in stomatal VPD response is subject to a lively debate. In theory, stomata can react to changes in air humidity passively, with water potential in guard cells changing in concert with the whole leaf and guard cells closing by simple deflation (Grantz, 1990). It used to be an accepted fact that stomata of ferns and lycopods react to air humidity by purely physical means, which is, indeed, widespread among non-vascular plants and even gymnosperms (Brodribb & McAdam, 2011; McAdam & Brodribb, 2012, 2015). There is, however, growing evidence that ABA-mediated stomatal closure exists in all groups of vascular plants and was probably secondarily lost in some taxa (Hõrak *et al.*, 2017; Clark *et al.*, 2022; Meigas *et al.*, 2024).

In angiosperms, abscisic acid is normally involved in stomatal closure in response to VPD increase (Grantz, 1990; McAdam *et al.*, 2016; McAdam & Brodribb, 2016). There is, however, some indication of partial stomatal closure independently of ABA. Purely passive stomatal reaction to water potential in angiosperms is theoretically problematic due to their epidermal anatomy, where the more sturdily built guard cells should react to water potential change more slowly than surrounding epidermal cells providing backpressure, resulting in stomatal opening in response to water loss and vice versa (McAdam & Brodribb, 2015). Nevertheless, there is some experimental evidence for VPD-related stomatal closure in angiosperms with defunct ABA-signalling (McAdam & Brodribb, 2015; Merilo *et al.*, 2018), likely driven by ABA-independent activation of the ABA-signalling hub OST1 (Yoshida *et al.*, 2006; Katsuta *et al.*, 2020; Jalakas *et al.*, 2021). Purely passive response may not be entirely ruled out, subject to precise pathway of water to guard cells and epidermis, or enabled by decreased epidermal cell turgor (Buckley, 2019).

1.3. Regulation of stomatal formation

1.3.1. Genetic control of stomatal formation

Stomatal formation in Arabidopsis takes place early in leaf development and is part of the formation of epidermis (Figure 2). Protodermal cell, an epidermal cell precursor, may enter stomatal lineage, become a pavement cell, or a trichome. The correct path through the stomatal lineage is driven by a trio of sequentially activated basic helix-loop-helix (bHLH) subgroup Ia transcription factors SPEECHLESS (SPCH), MUTE, and FAMA (Ohashi-Ito & Bergmann, 2006; MacAlister *et al.*, 2007; Pillitteri *et al.*, 2007, 2008; Pillitteri & Torii, 2012). These are heterodimerically paired with a bHLH subgroup IIIb transcription factors SCREAM (SCRM, a.k.a ICE1) or SCRM2 (Kanaoka *et al.*, 2008), each

of which may also form heterodimers with multiple other transcription factors unrelated to stomatal formation.

SPCH, expressed at low levels in protodermal cells, binds to the promoters of itself and genes encoding its heterodimers SCRM and SCRM2, activating a loop that directs the cell to stomatal lineage entry division (Lau *et al.*, 2014; Horst *et al.*, 2015), first resulting in cell transition into the meristemoid mother cell, and then asymmetric entry division where a smaller meristemoid cell separates from the stomatal lineage ground cell (at this stage *SPCH* is only expressed in MMC). The meristemoid mother cell may (and usually does) undergo additional asymmetric divisions under the influence of SPCH, resulting in a spiralling pattern of multiple, successively smaller ground cells. The stomatal lineage ground cell may also divide asymmetrically a second time, now giving a second meristemoid at the opposite end to the first one (Nadeau & Sack, 2003).

Eventually, the second bHLH transcription factor MUTE activates and transfers the meristemoid into the stage of guard mother cell, putting an end to the successive asymmetric divisions (Pillitteri *et al.*, 2007; Han *et al.*, 2018b). In the last step, the third transcription factor FAMA is responsible for the correct symmetric division of the guard mother cell and transition of the daughter cells into terminal guard cells, and, when bound to RETINOBLASTOMA-RELATED, maintains guard cell identity (Ohashi-Ito & Bergmann, 2006; Matos *et al.*, 2014).

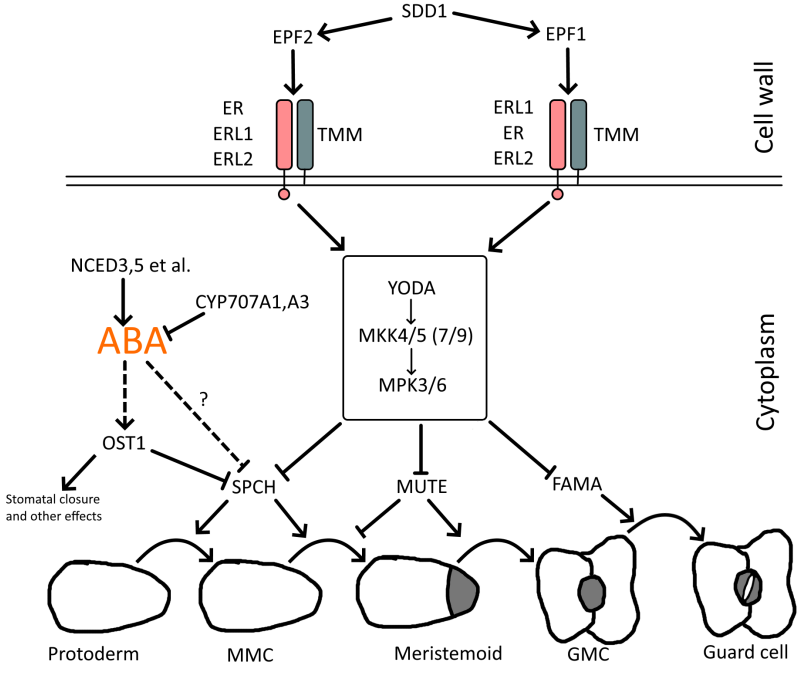

Figure 2. Arabidopsis leaf stomatal lineage and major components of the signalling network that control it. Stomatal lineage stages shown at the bottom (MMC, meristemoid mother cell; GMC, guard mother cell). Epidermal patterning factors, occurring in apoplast and binding to the ERx-TMM complex at the cell membrane, activate the map kinase cascade (in the box) that ultimately controls transcription factors. Also shown is a part of the abscisic acid signalling pathway relevant to this work.

In order to produce an unclustered distribution of stomata and to prevent an excessive proportion of epidermal cells from becoming stomata, the above process is coordinated between cells via release of epidermal patterning factors (Hara *et al.*, 2009). These are small closely related cysteine-rich peptides secreted by plant cells that are sensed by other neighbouring cells. In total 11 of them are known, of which four (EPF2, EPF1, EPFL6, and EPFL9) have unambiguously been shown to have a role in stomatal development. EPF2 and EPF1 are negative regulators of stomatal formation; they are secreted by cells in the stomatal lineage – EPF2 by cells in early stomatal lineage up to meristemoid stage (Hunt & Gray, 2009). EPF1 is secreted by cells all the way to late stomatal lineage, including guard cells (Hara *et al.*, 2007, 2009; Qi *et al.*, 2017). Epidermal patterning factors are sensed by dedicated receptors in epidermal cell plasma membrane, consisting of one of the leucine-rich receptor-like kinases ERECTA, ERECTA-LIKE1, or ERECTA-LIKE 2 (ER, ERL1, ERL2 resp) (Masle *et al.*, 2005; Lee *et al.*, 2012; Shpak, 2013), and paired with the leucine-rich receptor-like protein co-receptor TOO MANY MOUTHS (TMM; Geisler *et al.*, 1998; Lee *et al.*, 2012). Binding of EPF2 or EPF1 to this complex initiates a MAP kinase cascade in the cell, involving YODA, MKK4/5, and MPK3/6, which ultimately leads to phosphorylation of a MAPK target domain in SPCH, resulting in its rapid degradation (Lampard *et al.*, 2008). Neither MUTE nor FAMA possess such a domain and it is not quite clear how exactly MPK3 or MPK6 inhibit their action (Guo *et al.*, 2019).

STOMAGEN (EPFL9) is an EPF-like peptide secreted by mesophyll cells, which promotes stomatal formation by competing for the receptor of EPF2 and EPF1, binding to the ER(-like) and TMM complex without triggering the downstream suppressive cascade (Berger & Altmann, 2000; Hronková *et al.*, 2015). CHALLAH (EPFL6) is the fourth patterning factor affecting stomatal formation; it acts similarly to EPF1/2 but is synthesised in inner vascular tissues of stems and hypocotyls, affects stem and hypocotyl stomata, and binds to ER-like kinases without requiring TMM (Rychel *et al.*, 2010).

SDD1 is a protease probably located in the apoplast and possibly involved in cleavage of at least some epidermal patterning factors (Berger & Altmann, 2000; von Groll *et al.*, 2002; Schaller *et al.*, 2018). Its precise role is still not known; there are results contradicting its interaction with EPF1 (Hara *et al.*, 2007) or with EPF2 (Hunt & Gray, 2009), the latter corroborated by the fact that the expression of SDD1 is promoted by MUTE (which simultaneously suppresses EPF2) but not SPCH (Han *et al.*, 2018b).

SPCH directly binds to the promoters of *EPF2*, *TMM*, *ERECTA-LIKE* genes, activating the signal network that provides the coordination between cells in early stomatal lineage (Matos *et al.*, 2014; Horst *et al.*, 2015). MUTE notably represses EPF2 expression and promotes expression of ERL1 (the primary receptor of EPF1), but also initiates FAMA expression, leaving the MUTE-controlled phase in stomatal lineage cells rather short (Hara *et al.*, 2007; Han *et al.*, 2018b).

1.3.2. Hormonal control of stomatal formation

Many plant hormones have been shown to affect stomatal formation one way or the other. Arguably most important of them are brassinosteroids (BR), promoters of cell division and expansion, which suppress stomatal development in leaves and cotyledons while promoting it in hypocotyls (Gudesblat *et al.*, 2012; Park *et al.*, 2025), resembling the action of CHALLAH (Wei *et al.*, 2021). Disruption in BR signal transduction in leaves and cotyledons leads to excess stomatal production and clustering much like disruption of the core pathway kinases (Serna, 2014). The mechanism of BR action involves the key branching point in BR regulation BRASSINOSTEROID INSENSITIVE 2 (BIN2), itself down-regulated by BR presence (via the membrane-bound brassinosteroid receptor BRI1), inhibiting YDA or MPKK4/5 step in the core pathway (Gudesblat *et al.*, 2012; Serna, 2014; Qi & Torii, 2018).

Jasmonates suppress stomatal development by inhibiting *SPCH*, *MUTE*, and *FAMA* expression independent of the EPF signalling (Han *et al.*, 2018a). Auxins are generally suppressors of stomatal development (Saibo *et al.*, 2003; Balcerowicz *et al.*, 2014) but may play a role in stomatal development by promoting the amplifying asymmetric division in early stomatal development (Qi & Torii, 2018). Auxin concentration in stomatal lineage cells decreases over time and reaches near zero in guard mother cells (Le *et al.*, 2014).

Abscisic acid, the central actor in the stomatal closure regulation, also affects stomatal development. ABA levels correlate negatively with stomatal production: ABA biosynthesis mutants *aba2*, *aba3*, *nced3nced5* consistently show increased SD, while *cyp707a1/a3* double mutation leading to ABA accumulation has been shown to come with decreased stomatal production (Tanaka *et al.*, 2013; Chater *et al.*, 2015; Jalakas *et al.*, 2018).

ABA prevents entry into stomatal lineage (Tanaka *et al.*, 2013; Wei *et al.*, 2021). The big picture of ABA action on stomatal formation is still forming; one identified mechanism involves three SnRK2 kinases (2.2, 2.3, and 2.6 a.k.a OST1), which act redundantly, directly phosphorylating two serines in SPCH MAPKTD region leading to its degradation (Yang *et al.*, 2022). Unlike in stomatal movement regulation, single knockout of OST1 has no stomatal anatomy phenotype (Jalakas *et al.*, 2018). ABA also activates the core stomatal development suppression cascade via AIK1 (alias MKKK20)-MKK5-MPK6, bypassing YODA but still leading to suppression of the stomatal development driving transcription factors (Serna, 2014; Li *et al.*, 2017).

The signalling downstream of SnRK2s, as well as ABA receptors other than PYR/PYL are also involved in ABA-related growth regulation, including root development (Finkelstein *et al.*, 2002; Raghavendra *et al.*, 2010; Hong *et al.*, 2013).

1.3.3. Environmental control of stomatal formation

Environmental effects on stomatal formation are still not thoroughly understood. While stomatal formation takes place in young developing leaves, these leaves are themselves quite insensitive to their environment and their development instead depends on the signals received from older, already fully formed leaves (Lake *et al.*, 2001). Hence, a long-distance signalling between different plant parts must take place.

Growth under elevated CO_2 tends to result in somewhat reduced stomatal density (Woodward & Bazzaz, 1988; Woodward & Kelly, 1995; Engineer *et al.*, 2014) and size (Haworth *et al.*, 2023), but the relationship is not strict and even increased stomatal density under elevated CO_2 is not uncommon (Hetherington & Woodward, 2003; Ainsworth & Long, 2005). The mechanism behind stomatal density decrease could in theory involve differential expansion growth, but at least in some cases, stomatal index has been reported to change in the same direction (Gray *et al.*, 2000). A wax biosynthesis-related gene *HIC* has been identified as a mediator to this regulation (Gray *et al.*, 2000). Again, a functional abscisic acid signalling network may be crucial for such response (Chater *et al.*, 2015). Under elevated CO_2, leaves induce EPF2 via a mechanism that involves β-carbonic anhydrases (Engineer *et al.*, 2014). Indeed, intact EPF signalling may be required for stomatal density reaction to altered atmospheric CO_2 conditions (Doheny-Adams *et al.*, 2012).

Light intensity during plant development correlates positively with stomatal density. Part of this is due to decreased leaf expansion, but the stomatal index also increases somewhat under high light. Known mechanisms include auxin-mediated repression of cotyledon stomata production in darkness (Balcerowicz *et al.*, 2014) and COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) which degrades ICE1 but is inhibited by light, resulting in positive light-stomatal production relationship (Lee *et al.*, 2017). The red end of the spectrum affects stomatal development via phytochrome sensing (mediated by the transcription factor PIF4) (Casson *et al.*, 2009). Other possible links between light and stomatal production include EPFL9 synthesis in mesophyll, where COP1 degradation leads to accumulation ef ELONGATED HYPOCOTYL 5 (HY5) and promotes EPFL9 synthesis (Wang *et al.*, 2021), or decreasing CO_2 concentration in intercellular space (Hronková *et al.*, 2015).

Much like in the case of stomatal movement, the particular mechanisms of how water potential in the air or in the plant affects stomatal development are not well known. Reduction of stomatal production under soil water stress is common, but the effect is confounded by major change in leaf size (Quarrie & Jones, 1977; Kumari *et al.*, 2014) – indeed, in some experiments growth RH has merely affected cell expansion with constant stomatal index (van de Sanden & Veen, 1992). Nevertheless, low air humidity can lead to methylation and resulting partial inactivation of SPCH and FAMA (Tricker *et al.*, 2012). Abscisic acid is an inevitable mediator between stomatal development and water stress (Quarrie & Jones, 1977), including air humidity changes; other than that, active control mechanisms are not well known (Qi & Torii, 2018).

1.3.4. Distribution of stomata between leaf sides

In most angiosperms, stomata can be found on the lower (abaxial) leaf side, and many of them do indeed have no stomata on the upper (adaxial) side, a phenomenon known as hypostomaty (Muir, 2015). There is, however, a large number of amphistomatous plant species that have stomata on both leaf sides (hyperstomaty is rare but somewhat common among grasses; Muir 2015). Often only the lower (abaxial) leaf side is studied, leading to scarce knowledge on stomata on the upper (adaxial) side. *Arabidopsis thaliana* is an amphistomatous species that has a substantial number of its stomata on the upper leaf surface (Dow *et al.*, 2014).

Having stomata on both leaf surfaces has obvious advantages. Amphistomaty reduces overall diffusion distance and thereby resistance in the intercellular spaces (Parkhurst & Mott, 1990) and correlates well with photosynthesis and stomatal conductance (Xiong & Flexas, 2020). As a general trend, the longer-lived a plant group, the more it tends towards hypostomaty, but there are exceptions and amphistomatous species are not uncommon even among trees (Muir, 2015). Among plants of similar growth form, light-demanding species generally tend to be more amphistomatous (Muir, 2018). This latter phenomenon is reflected in individual plants' acclimatisation: stomatal formation on leaf sides appear to react differently to light environment, with clear increase, with increasing growth light, in stomatal formation on the adaxial and much less on the abaxial side (Hronková *et al.*, 2015).

There are some documented differences in how the genetic pathways directing stomatal formation work in abaxial and adaxial epidermis, but so far nothing conclusive. Mutants in *SDD1*, having generally higher stomatal density on both leaf sides, have been shown to allocate stomata primarily on their abaxial side (Berger & Altmann, 2000; Vráblová *et al.*, 2017), but the extent of this difference may depend on growth light and can be a side-effect of general epidermal cell density (Hronková *et al.*, 2015). TMM, a receptor for epidermal patterning factors, has a particularly striking effect on the abaxial/adaxial balance, with the *tmm* mutant's stomata mostly on the abaxial side (Geisler *et al.*, 1998; Vráblová *et al.*, 2017). A striking Arabidopsis line with silenced SPCH exists which has lost abaxial stomata almost completely while retaining wild-type-like stomatal production on its adaxial side, but the nature of this phenomenon is still not well known (Dow *et al.*, 2014). A *SPCH* repressing transcription factor IDD16 was shown to preferentially reduce stomatal density on the abaxial side (Qi *et al.*, 2019), and modifications of EPFs may also lead to preferential alteration of the abaxial stomatal density (Franks *et al.*, 2015).

1.4. Long-term effects of air humidity on plants

Growing under reduced air humidity affects plants in multiple ways. While atmospheric water vapour demand increase would lead to transpirational water loss, this is at least partially counteracted by closing stomata, which in turn can lead to decreased photosynthetic carbon assimilation (Grange & Hand, 1987; Buckley, 2005). Typically, changes in stomatal conductance are not enough to entirely counteract evaporative demand neither in short nor in long term, and decreased air humidity leads to increase in evaporative water loss (Grantz, 1990; López *et al.*, 2021) while stomatal limitation to photosynthesis remains modest (Grange & Hand, 1987).

Across species, decreased growth RH (increased VPD) is associated with reduced stomatal conductance, increased leaf mass to area ratio (van de Sanden & Veen, 1992; López *et al.*, 2021), reduced above-ground growth rate and altered nutrient uptake, while root growth is more variable but tends to increase (López *et al.*, 2021; Novick *et al.*, 2024). While vapour pressure deficit above 1 kPa correlates negatively with plant growth rate, extent of the response varies between species (Ford & Thorne, 1974) and VPD lower than 1 kPa (more humid air) comes with no significant improvement (Grange & Hand, 1987; Grantz, 1990). In trees, expansion growth depends strongly on the very low VPD (<0.4 kPa) that occurs at night (Cabon *et al.*, 2020; Novick *et al.*, 2024). Very high humidities (RH>90%) suppress transpiration and may result in malfunctioning stomata (Fanourakis *et al.*, 2020), which can have detrimental effect on nutrient uptake (Gale & Hagan, 1966), hampers temperature control (Lysenko *et al.*, 2023) and greatly increases pathogen load (Grange & Hand, 1987).

2. AIMS

- To find out whether it is possible to freely combine mutations leading to increased stomatal density and those resulting in altered stomatal sensitivity, without significant interference.
- To look for ways to obtain plant lines with enhanced photosynthetic capacity via excess stomatal production, hopefully resulting in enhanced growth potential.
- Anticipating increased susceptibility to water stress due to excess stomatal density, to study whether combining increased stomatal density with less open stomata leads to plant lines with optimal balance of photosynthetic capacity and water loss, presumably subject to growth air humidity.
- To dig into the mechanisms of stomatal reaction to atmospheric water vapour, and to understand the long-term effects of disturbed stomatal reactivity at various water availability during plant growth.
- To explore the mechanisms governing abaxial vs adaxial stomatal development, and the effect of leaf rank on stomatal development on both leaf sides.

3. MATERIALS AND METHODS

This thesis deals with results of four experiments with various Arabidopsis mutant lines. In article I, we explored stomatal properties, gas exchange, and growth at different air humidities of lines which combined increased stomatal density and reduced stomatal openness. In article II, we explored stomatal development, with special attention to adaxial/abaxial balance, of a wide variety of plant lines with disturbed stomatal formation, and stomatal anatomy of different rosette leaves. Article III studies the nature of stomatal reactiveness to air humidity without a functional OST1, and its effect on growth under different air humidities like in Article I. Also added is Preprint IV, where we conducted experiments similar to those in Article I but this time combined plant lines with increased stomatal openness and density, and grew them for longer, almost to bolting. The details of these experiments are described in respective articles and the preprint.

For Articles I and III, as well as Preprint IV, growth experiments were performed, where we subjected the plants to differential air humidities (Table 1) while applying a watering regime aimed to avoid different soil moistures in the two treatments. For Articles I and III, we grew plants for 28 days and sampled from each plant leaf #6 for stomatal anatomy; for Preprint IV, we went for sparser arrangement of the plants, grew them for 39 days, and sampled leaf #12 for stomatal anatomy.

Table 1. Air humidity in "Control" and "Dry" treatments in the growth experiments conducted in Article I and Preprint IV. The growth experiment in Article III was done at 5% lower RH except for nighttime control which was 80% RH as in other experiments.

Treatment	Daytime (t=23 °C)	Nighttime (t=19 °C)
Control	RH=60%, VPD=1.1 kPa	RH=80%, VPD=0.4 kPa
Dry air	RH=40%, VPD=1.7 kPa	RH=50%, VPD=1.1 kPa

Table 2. List of mutants studied in the articles that form the basis of this work. All mutant lines are in the background of Col-0.

Mutant	T-DNA insertion lines	Role	Articles
Col-0	–	–	I, II, III, IV
epf1	SALK_147549	epidermal patterning	I, II, IV
epf2	GK-673E01	epidermal patterning	I, II, IV
cyp707a1 and *a3*	SALK_137549 and SALK_101566	ABA catabolism	I, II
ht1-2	not a T-DNA mutant	CO_2 sensing	I
mpk12-4	not a T-DNA mutant	CO_2 sensing	IV
ost1-3	SALK_008068	ABA signalling	III, IV
slac1-3	SALK_099139	anion channel	IV
ghr1-3	GK_760C07	ABA signalling	IV
tmm	SALK_115723C	EPF co-receptor	II
sdd1	GK-627D04 or GK-693D08	somehow involved in EPF signalling	II
er	GK-182D08 or GK-364C05	EPF receptor	II
erl1	GK-109G04	EPF receptor	II
erl2	SALK_015275C or GK-486E03	EPF receptor	II
nced3 and *nced5*	GK-129B08 and GK-328D05	ABA synthesis	II
pyr1 pyl1, 2, 4, 5, 8	Q169stop, SALK_054640, GT_2864, SAIL_517_ C08, SM3_3493, and SAIL_1269_A0	ABA receptors	II

4. RESULTS AND DISCUSSION

4.1. Stomatal anatomy

In two experiments (Article I and Preprint IV), we combined Arabidopsis lines with increased stomatal density (due to defunct EPF2, EPF1, or their combination) with altered stomatal openness. For the latter, lines with differing stomatal behaviour were chosen. The *ht1-2* mutant is known for its impaired CO_2 reaction and generally reduced steady-state stomatal conductance, but fully functional response to ABA and notably air humidity (Hashimoto *et al.*, 2006; Hõrak *et al.*, 2016). The double mutant *cyp707a1/a3* typically shows increased ABA concentrations in leaf tissue and reduced stomatal conductance while remaining responsive to stomatal closure-inducing stimuli (Jalakas *et al.*, 2018). The increased stomatal conductance due to extra stomata in the *epf1* or *epf2* lines would be expected to lead to some enhancement in photosynthetic capacity at the expense of increased sensitivity to water loss, which could be partially counteracted by the *ht1-2* or *cyp707a1/a3* mutations, assuming that none of the mutations hamper stomatal responsiveness to humidity. Anticipating different air humidity optima for different stomatal trait combinations, we grew the plants at two RH regimes while carefully maintaining soil water content. The mutations leading to impaired stomatal closure used in Preprint IV were chosen for their large steady-state stomatal conductance unrelated to stomatal density, to more clearly separate the effects related to increased SD from those potentially stemming from water loss due to increased conductance.

Mutations affecting stomatal density and openness did not interact markedly. In both Article I and Preprint IV, we saw large SD increase in the combined *epf1/2* mutants (Figure 3), and in Article I, a lesser but still significant increase in plants with *epf2* and *epf1* mutations separately (Article I Figure 4). Mutations affecting stomatal openness (i.e. *ht1-2*, *cyp707a1/a3*, *ost1-3*, *mpk12-4*) had little, if any, effect on SD. While there is no known reason to expect *ht1-2* or *mpk12-4* to directly affect stomatal development, and OST1 is known to work redundantly with other SnRK2 kinases and *ost1-3* single mutants do not have stomatal development phenotype, one would expect somewhat suppressed stomatal development in the *cyp707a1/a3* mutant, a consequence of the higher ABA level in these plants (Tanaka *et al.*, 2013; Jalakas *et al.*, 2018). However, we didn't observe clear difference in SD due to the *cyp707a1/a3* mutation in Article I; in Article II, the *cyp707a1/a3* double mutant also showed only slight decrease in SD in leaf #8 in one sub-experiment (Article II Figure 2) and was otherwise wild-type-like in stomatal anatomy. Based on these results, decreased stomatal formation may not always be present in *cyp707a1/a3* mutants. As ABA breakdown is not completely abolished in the *cyp707a* mutants, it is possible that ABA accumulation in given leaves in our conditions was relatively mild and didn't lead to significant perturbation of stomatal development.

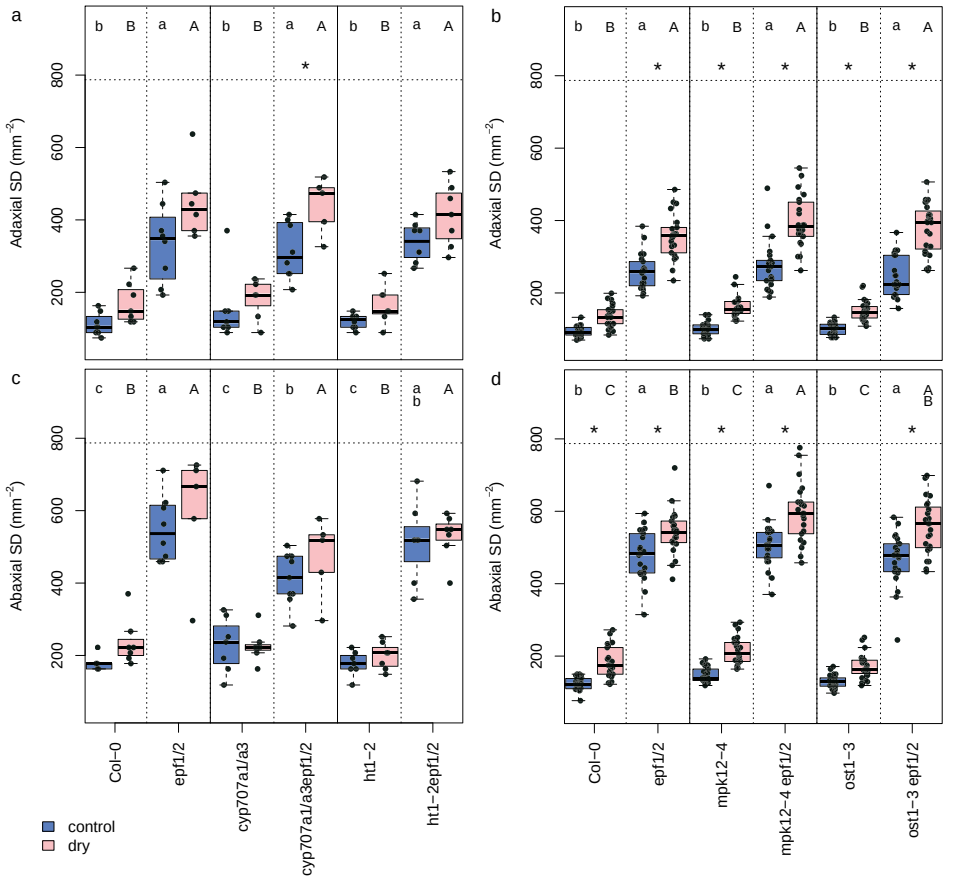

Figure 3. Stomatal densities in growth experiments were affected by the presence of *epf1/2* mutations and growth RH. In Article I (a,c), leaf #6 was sampled at four-week-old plants; in Preprint IV (b,d), leaf #12 was sampled in six-week-old plants. Shown are medians, lower and upper quartile (as a box) and non-outlier range (as the whiskers) as well as individual data points. Asterisk above a pair of boxes indicates a significant difference within that genotype according to Tukey's HSD (ANOVA involving all 12 treatment and genotype combinations). Shared lowercase letters above the control RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only control RH treatment); shared capital letters above the low RH boxes likewise for that treatment.

In contrast to *cyp707a1/a3*, mutations leading to ABA synthesis (*nced3/5*) or perception (*pyr/pyl112458*) impairment result in clear increase in stomatal density (Article II Figures 2 and 3), adding to the body of works showing the importance of ABA in the control of stomatal formation (Tanaka *et al.*, 2013; Chater *et al.*, 2015; Merilo *et al.*, 2018; Jalakas *et al.*, 2018). Mostly, this increase results from stunted growth typical for both of these plant lines – we didn't find a statistically significant difference in stomatal index between *nced3/5* and Col-0 in Article II. Nevertheless, increased stomatal index in ABA-deficient plants has been documented (Tanaka *et al.*, 2013; Merilo *et al.*, 2018) and our results are broadly

consistent with them. There is some indication of an interaction between *epf1/2* on one hand and *nced3/5* on the other hand: the quadruple mutant *epf1/2nced3/5* far exceeds abaxial stomatal production of the *epf1/2* double mutant (Article II Figure 3).

In both Article I and Preprint IV, we saw a clear increase in stomatal density under reduced growth RH (Figure 3). Optimality considerations would suggest otherwise, and indeed decreased SD under decreased growth RH is not unheard of (Bakker, 1991; Fanourakis *et al.*, 2013). However, growth under lower RH leads to both increased cell density and reduced cell size (Carins Murphy *et al.*, 2014), and the expansion growth reduction commonly results in denser stomata under low RH (López *et al.*, 2021), in keeping with the general trend of negative leaf area vs stomatal density relationship known for a century (Salisbury, 1928). Stomatal index, a measure that is less sensitive to expansion, was in our experiments much less affected by growth RH (Preprint IV Figure 4), consistent with the general trend of zero to slightly negative correlation with growth RH (López *et al.*, 2021).

4.1.1. Adaxial/abaxial stomatal ratio is affected by growth conditions and some mutations

While the overall effect of reduced growth RH on stomatal density was positive on both leaf surfaces, there were consistent differences that resulted in increased adaxial/abaxial ratio under reduced RH (Figure 4). Based on Preprint IV, this shift was clearly due to greater difference on the adaxial leaf surface (Preprint IV Figure 3A,B), consistent with greater flexibility of the adaxial leaf side (Muir *et al.*, 2023). Stomatal index, although quite constant compared to SD, showed an overall slight shift under low RH (manifested as significant main effect, Preprint IV Table 3), decreasing on the abaxial but even increasing on the adaxial surfaces (Preprint IV Figure 4). Similar opposite directions in RH reactions were observable in total stomatal number analysed in Article I.

Stomatal development on the abaxial and adaxial side was differently affected by some mutants of the development pathway. As expected based on prior works (Geisler *et al.*, 1998; Vráblová *et al.*, 2017), stomatal index of the *tmm* mutants was twofold increased (compared to the wild-type) on the abaxial side with no difference on the adaxial side (Article II Figure 3), indicating a major difference in the role of that co-receptor between leaf sides. TMM is known to have contrasting roles in mature leaves and hypocotyl stems (Geisler *et al.*, 1998; de Marcos *et al.*, 2015) and Vráblová *et al.* (2017) observed even significant reduction in stomatal density on the adaxial side in the *tmm-1* mutant. While *tmm* mutants end up with excessive number of stomata in mature leaves, their stomatal development is slightly delayed, suggesting that even in leaves, TMM might play another role in addition to being a receptor for EPFs (de Marcos *et al.*, 2016). The contrasting roles of TMM in cylindrical and flat organs are attributable to its antagonistic relationship with CHALLAH that suppresses stomatal initiation much like EPF2, but is itself inhibited by TMM (and doesn't require TMM to bind on ER family receptors). Mostly, CHALLAH is only expressed in veins of

cylindrical organs, but it has been shown to appear briefly in the midribs of leaf primordia (Abrash & Bergmann, 2010). This brief flash of CHALLAH (or indeed some other EPFL peptide) in early leaf development could provide a possible link between the *tmm* mutation and its vastly different impact on the two leaf surfaces, but with the data collected in our experiments, any explanation remains speculative.

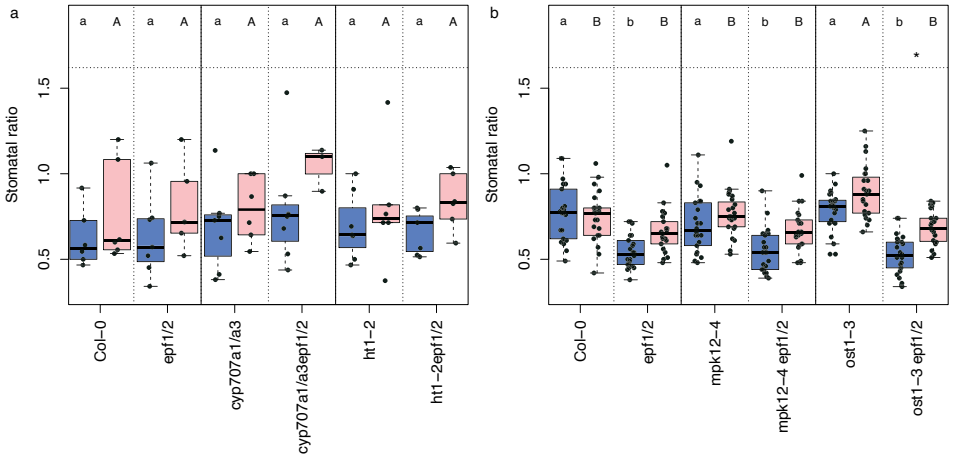

Figure 4. Stomatal ratio (adaxial/abaxial) in Article I and Preprint IV. a, leaf #6 of 4-week-old plants in Article I; b, leaf #12 of 6-week-old plants in Preprint IV. Shown are medians, lower and upper quartile (as a box) and non-outlier range (as the whiskers) as well as individual data points. Asterisk above a pair of boxes indicates a significant difference within that genotype according to Tukey's HSD (ANOVA involving all 12 treatment and genotype combinations). Shared lowercase letters above the control RH boxes indicate no significant difference according to Tukey HSD test (ANOVA involving only control RH treatment); shared capital letters above the low RH boxes likewise for that treatment. Stomatal ratio was increased under low RH (RH main effect in both articles).

We also observed a tendency for increased adaxial/abaxial stomatal ratio in ABA synthesis and receptor mutants (Article II Figure 2 and Table 2), but the results of different experiments varied (Article II Figure 3). The *epf1/2* mutation also sometimes resulted in altered stomatal ratio, but the pattern was surprisingly inconsistent. In Article II as well as Preprint IV, *epf1/2* mutations resulted in decreased stomatal ratio, owing to greater increase of SD on the abaxial side. This was corroborated by a similar change in one of the two *sdd1* mutants in Article II, which could be expected if SDD1 has a key role in activating EPFs (Berger & Altmann, 2000). However, in Article I, the observed pattern was more complicated, with both *epf1* and *epf2* single mutations coming with slight increase in stomatal ratio but the two EPFs combined not adding up to a correspondingly larger stomatal ratio increase (Article I Table 3 and Figure 7).

The leaf analysed in Article I for stomatal anatomy was earlier than in other studies (leaf #6 as opposed to leaf #8 or 12). To my knowledge there are no studies exploring the interplay between stomatal development perturbations, leaf

sides, and leaf rank. In our own experiment published in Article II, we saw no abrupt changes in stomatal patterning between leaf #6 and #8 (Article II Figure 1) but this does not necessarily rule out such a change in the *epf1/2* mutants that were not studied in that experiment. Another difference between these experiments that should be pointed out is different growth cabinets used, which might introduce growth environment differences to the plants that are hard to control or even know about. In any case, some confounding factor is at work, indicating that leaf sides react to the environment somewhat differently, and epidermal patterning factors mediate that difference. The different temporal pattern of stomatal formation between adaxial and abaxial surfaces shown at different parts of Arabidopsis (Geisler *et al.*, 1998; Geisler & Sack, 2002) may be one process behind the difference: the abaxial stomatal initiation lasting longer would naturally lead to disproportionally more stomata on the abaxial side for the *epf1/2* lines. This logic should actually apply to all plant lines with hampered control of stomatal initiation, but not necessarily those of impaired stomatal differentiation. This would predict *er* mutants exhibiting a different stomatal ratio compared to *erl1* or *erl2*, for it is the former that is mainly associated with sensing of EPF2 involved in the control of stomatal initiation (Lee *et al.*, 2012), but we didn't find a clear difference between these mutants, even if the observed trend is in the expected direction (Article II Figure 3E). There was a definite difference between mutants in ER vs mutants in ERL2 in their adaxial stomatal production – interpretation of this is, however, hard because the relative role of the latter between stomatal initiation and differentiation is not well established.

4.1.2. Stomatal size vs density relationship

Often, a negative relationship between stomatal density and size is observed, raising concerns that the change in size may negate the density effects (Dittberner *et al.*, 2018; Haworth *et al.*, 2023). Bigger stomata are required for maintaining constant maximum stomatal conductance if SD is reduced (Franks *et al.*, 2009). Across species, the relationship is rather strong (Franks & Beerling, 2009); also, growth under increased atmospheric CO_2 triggers opposite changes in stomatal density and size but only in evolutionarily younger plant groups (Haworth *et al.*, 2023). In addition to geometric constraint (to pack more stomata on limited leaf surface, they might need to be smaller) and expansion growth (stretching a leaf with existing stomata results in bigger cells and smaller SD), adaptive significance has been proposed (Haworth *et al.*, 2023) but to my knowledge possible mechanisms have not been explored.

We observed only weak negative to nonexistent relationships between stomatal density and size across Arabidopsis lines (Figure 5), significant on the adaxial side in one humidity experiment (Article I Figure S3) and on the abaxial sides in the stomatal patterning experiment (Article II Figure 4). Notably, even when present, the variation of stomatal size remains far smaller than that in stomatal density: few percents vs about twofold respectively in our experiments, while Muir et al. (2023) show about 10-fold variation in stomatal size vs 100–1000-fold in density across species. In one study (Lunn *et al.*, 2024), stomatal

conductance changed relatively less than density, and the discrepancy was primarily due to the variable stomatal aperture rather than the fixed stomatal size. Our results, too, seem to imply that noteworthy compensation in steady-state conductance by this mechanism should be ruled out at least in our selection of plant lines, and suggest that density, along with openness, remains the dominating factor determining gas conductance through stomata, corroborating a similar conclusion by (Ochoa *et al.*, 2024). In our experiments (Article I), stomatal size correlated very weakly with gas phase conductance and stomatal density, while the relationship between conductance and density was convincingly positive – supporting the general pattern that stomatal size has little effect in determining gas phase resistances.

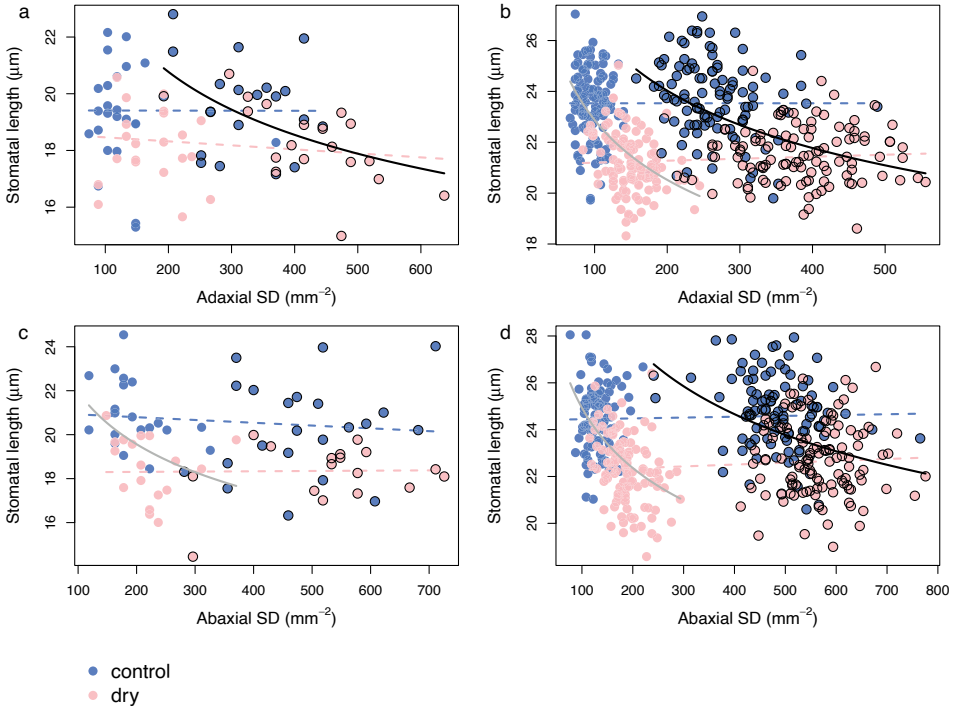

Figure 5. Relationship between stomatal size vs density is significant within, but not between plants with different EPF1/2 states. a and c denote data from Article I (leaf # 6 of 4 weeks old plants), b and d from Preprint IV (Leaf #12 of six weeks old plants). Each dot is one plant, with plants carrying the *epf1/2* mutations marked with black circles, mutations affecting stomatal openness not distinguished. Stomatal length was assessed as the median length of five stomata measured from the same imprint view as density. Straight lines illustrate linear regressions calculated from all plants within each treatment; the slope never significantly differed from zero. Curved lines are power regressions across plants of the same *EPF1/2* state (grey for intact EPFs, black for *epf1/2* mutants), with both treatment levels included (coefficients shown in Table 3). Power regressions for the adaxial side for the intact EPF plants and abaxial side of the *epf1/2* plants in Article I data didn't differ significantly from constant and are not shown.

Based on our results, geometric constraints don't seem to limit stomatal size: in Article I, it was the abaxial leaf side with higher density and slightly bigger stomata that showed (stronger) negative size-to-density correlation, and in Article II, the *epf1/2nced3/5* mutant stands out with particularly high densities of big stomata on its lower side. In our experiments the *epf1/2* double mutants did not come with the stomatal size reduction that could be expected from their SD increase. Instead, there was a negative relationship between SD and stomatal size within plants with intact EPFs, and the *epf1/2* mutants came with their own SD vs size relationship at higher SD values, with no smaller stomata (Figure 5). The expected negative relationship between stomatal size and density did appear within each EPF mutation state, with the data from plants grown under high and low RH apparently falling into the same curve (Figure 5). Mere variable stretching of a whole leaf surface would result in power regression between SD and stomatal length with exponent of –0.5; the exponent being around –0.15 (Table 3) indicates that simple leaf expansion differences don't fully account for the negative SD vs stomatal length relationship, and the smaller stomatal complexes in leaves with larger stomatal densities still occupy larger share of leaf surface, as shown in other studies (Franks *et al.*, 2009; Haworth *et al.*, 2021).

Table 3. Coefficients for the linear regression ln(SL) and ln(SD) and the resulting power equation, as illustrated in Figure 5. SL, stomatal length. The last column gives explicit equations with means only, or "n.s." where the regression slope (i.e. power equation's exponent) didn't differ significantly from zero.

Source	Leaf side	EPF state	Intercept ± SE	Slope ± SE	Equation
Article I	adaxial	wt	3.19 ± 0.19	–0.053 ± 0.038	n.s.
		epf1/2	3.90 ± 0.26	–0.163 ± 0.044	$SL = 49.3 * SD^{-0.163}$
	abaxial	wt	3.85 ± 0.32	–0.166 ± 0.060	$SL = 47.0 * SD^{-0.166}$
		epf1/2	2.62 ± 0.44	0.054 ± 0.071	n.s.
Preprint IV	adaxial	wt	3.85 ± 0.07	–0.157 ± 0.014	$SL = 47.1 * SD^{-0.157}$
		epf1/2	3.93 ± 0.09	–0.142 ± 0.016	$SL = 51.1 * SD^{-0.142}$
	abaxial	wt	3.94 ± 0.08	–0.157 ± 0.017	$SL = 51.4 * SD^{-0.157}$
		epf1/2	4.19 ± 0.19	–0.164 ± 0.030	$SL = 65.8 * SD^{-0.164}$

4.2. Stomatal conductance and density modulating plant gas exchange

Increased stomatal density always resulted in a large increase in steady-state conductance (Article I Figure 1a; Preprint IV Figure 1). We observed expected behaviour of stomatal conductance in the background of altered stomatal density: *ht1-2* mutation resulted in slightly reduced conductance compared to respective lines with intact HT1, while *cyp707a1/a3* mutants generally did not differ from their respective wild-type lines (even then, the trend was in the expected direction, so mild stomatal closure cannot be ruled out). All combinations studied in Article I also retained responsiveness to air humidity (Article I Figures S1 and S2). However, reduced stomatal openness mutations did not do well to ameliorate the increased water loss due to greater SD – indeed, it was the *epf1/2* line that retained better stomatal responsiveness to increased growth VPD compared to *cyp70a1/a3 epf1/2* or *ht1-2 epf1/2*. We can therefore state that simultaneous manipulation of stomatal density and responsiveness by combined mutants in respective genetic pathways is feasible, but with this experiment we failed to showcase a clear benefit of such combination.

With stomata functioning as entry gateways for atmospheric CO_2, a positive relationship between steady-state stomatal conductance and photosynthesis could be anticipated. In Article I, we observed a slight increase in steady-state assimilation associated with the *epf1/2* mutations (Article I Figure 1b), and indeed a positive correlation between stomatal density and photosynthesis, while the effect on A_{net} resulting from reduced stomatal openness due to *ht1-2* or *cyp707a1/a3* mutations was mild at best. While in that study the closed-stomata genotypes chosen had only modest stomatal phenotype, this was not the case in Article III, where the *ost1-3* line showed 2–3-fold increase in g_s compared to wild-type. This was still not associated with significant change in photosynthesis (Article III Figure 1 and Table 3). Likewise, in Preprint IV, conductance differences between plant lines did not amount to significant changes in net photosynthesis (Preprint IV Figure 1). Overall, stomatal density appears to have somewhat stronger effect on A_{net} than stomatal conductance does, suggesting acclimatisation of photosynthetic apparatus to increased SD. Indeed, the increase in photosynthetic capacity may be attributable to increased LMA in the *epf1/2* lines (Article I Figure 8 and Table 3), much like photosynthetic capacity of leaves grown in differing light environments does (Evans & Poorter, 2001). Previous similar studies have reported varied results. Tanaka et al. (2013) achieved significant photosynthetic stimulation (even V_{cmax}) by over-expressing STOMAGEN, but at the cost of even bigger increase in transpiration, overall resulting in no significant change in plant biomass. Mutant lines in SDD1 have been shown to come with stimulated photosynthesis in specific conditions in at least one study (Schlüter *et al.*, 2003) and with significant increase in stomatal conductance but not notable effect on CO_2 assimilation in another (Vráblová *et al.*, 2017).

The closed-stomata lines in Article I showed a slight but consistent increase in intrinsic water use efficiency compared to the respective lines with intact HT1

and CYP707A proteins, while WUE of the *epf1/2* lines in Article I, as well as the *ost1-3* line studied in Article III was significantly reduced compared to wild-type. Nevertheless, these differences in WUE didn't result in notable changes in plant growth. We didn't encounter a clear effect of air humidity on steady-state CO_2 assimilation: in Article I, growth RH had no significant effect on photosynthesis, while in Article III, a mild overall negative VPD-to-A_{net} relationship cannot be ruled out, but it only proved significant for the *ost1-3* plants grown in water deficit (Article III Figure 1C). Growth under reduced RH resulted in reduction of g_l far larger than the difference in A_{net}, leading to clear increase in WUE (Article I Figure 2a). Clearly, stomatal conductance in these experiments remained high enough to cause only little stomatal limitation to photosynthesis even in the *ht1-2* and *cyp707a1/a3* lines. Despite the used incident light flux of 250 μmol m^{-2} s^{-1}, which is above typical used in Arabidopsis experiments, we may have still operated at light limitation, so the results may not be freely extrapolatable to other species and conditions.

4.2.1. Role of OST1 in regulating stomatal VPD reactions

OST1 (SnRK2.6) is a key part in stomatal reaction to air humidity (Yoshida *et al.*, 2006; Xie *et al.*, 2006). Plants lacking functional OST1 exhibit around twice the steady-state stomatal conductance compared to wild-type and their stomata are relatively unresponsive to changes in air humidity, leading to increased water loss and drought sensitivity (Merilo *et al.*, 2018). Open questions in this regard include the mediating role of ABA in such reactions, as well as the extent to which stomata react to air humidity independently of OST1. In our experiments, well-watered *ost1-3* mutants lacked significant RH reaction, consistent with earlier results where such reaction was strongly impaired or lacking (Merilo *et al.*, 2013, 2018; Jalakas *et al.*, 2021), but gained a VPD-reactability comparable to wild-type when grown in soil water deficit (Article III Figure 1). The source of such reactability could be either an alternative active mechanism that bypasses OST1, or a hydropassive mechanism. The former remains a possibility, and indeed the wide array of calcium-dependent protein kinases (CPK), alternative participants in ABA reaction, is an obvious suspect – while OST1-independent anion channel activation via CPKs has been documented (Geiger *et al.*, 2010), so far no clear role of CPKs in stomatal VPD reaction has been found (Hsu *et al.*, 2021).

The mere possibility of passive stomatal closure in angiosperms is a controversial issue. Canonically, stomata of angiosperms should not close in response to turgor loss, for water loss would affect the neighbouring epidermal cells more, leading to stomatal opening rather than closing – a phenomenon that indeed occurs in response to a drop in air humidity or severing of leaf water supply, but that is then quickly (in minutes) overcome by ABA-controlled active stomatal closure. Nevertheless, the possibility of passive stomatal reaction is not entirely ruled out in angiosperms. In particular, it is feasible that already flaccid pavement cells exert little back-pressure on the guard cells and their additional water loss makes little difference (Buckley 2019). In our experiment, we saw somewhat

decreased overall leaf water potential in the *ost1-3* plants grown in water deficit, which further dropped in dry air (Article III Figure 2), ticking the boxes for a possible hydropassive stomatal closure. The verdict is by no means clear, but the lower initial water potential of the dry-grown *ost1-3* plants leaves a possibility that their epiderm was just flaccid enough to not counteract passive closure in those plants.

4.3. Plant growth, stomatal traits, and air humidity

In our experiments, both higher stomatal density and lower growth RH were associated with reduced above-ground growth across plant lines, with no interaction between these factors (Figure 6a,b, Article I Figure 10a, Article II Figure 6). A negative relationship between stomatal density and plant or leaf size has been observed often (Franks & Farquhar, 2007; Xu & Zhou, 2008; Bouvier & Kelly, 2025). While adaptive considerations have been proposed (Chua & Lau, 2024), a simple alternative explanation could be that stomata, formed in the young leaf, simply move apart during subsequent leaf expansion, naturally resulting in an inverse relationship between leaf size and stomatal density. To control for such backwards influence of expansion to SD, we also studied the dependence of plant size on the estimated number of stomata per leaf, a measure less dependent on leaf expansion growth than SD (stomatal index could not be estimated in Article I due to bad imprint quality). In Article I, number of stomata correlated with plant size very weakly (Figure 6c; Article I Figure 10b and Table S2), suggesting that indeed there may have been little direct influence of stomatal development on plant size. In Preprint IV, however, both number of stomata per leaf and stomatal index remained significantly negatively correlated with plant mass (Figure 6d,e), indicating that the process of stomatal development influences plant size.

The most natural cause for such growth reduction would be water loss due to increased stomatal conductance and therefore transpiration (Bouvier & Kelly, 2025). Moderately reducing air humidity and thereby increasing water loss through transpiration resulted in a clear reduction in plant growth (Figure 6; Article I Figures 9 and 10). This reduction, however, was largely uniform across plant lines with vastly different steady-state stomatal conductances, while one would expect the plants with lower stomatal conductance (whether it comes through fewer or more closed stomata) to cope better with increased evaporative demand.

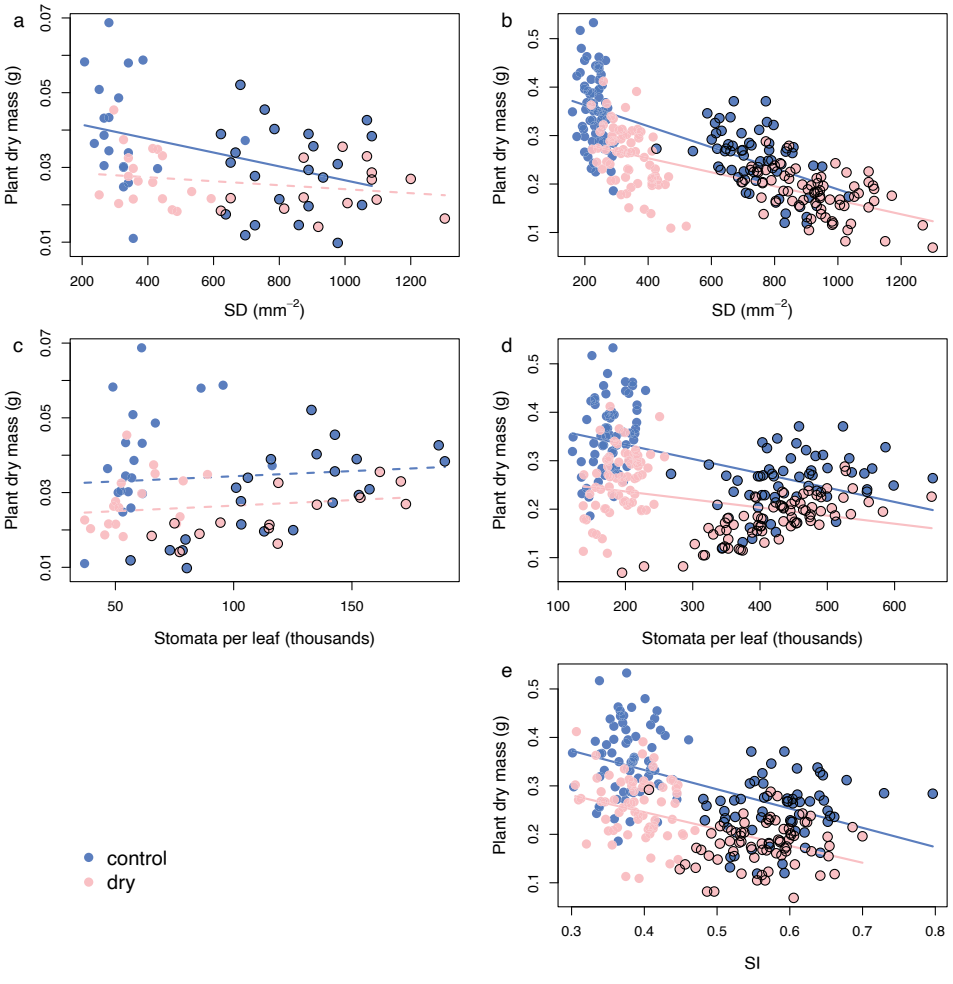

Figure 6. Plant dry mass at harvest time correlates negatively with stomatal density and is reduced under lower growth humidity. a, c based on Article I (4 weeks old plants; mutants in single EPF omitted; SI could not be assessed); b, d, e based on Preprint IV (6 weeks old plants). Each point denotes one plant, circled dots show plants with *epf1/2* mutations (with or without other mutations). Linear regression lines for each growth RH are shown. For dotted lines, regression slope did not differ significantly from zero (α=0.05).

An even more compelling refutation to the water loss driven growth reduction scenario is the fact that stomatal conductance, as long as it was not due to altered stomatal density, did not correlate well with growth. Neither did the ht1-2 and cyp707a1/a3 mutants grow faster than respective lines with intact HT1 and CYP707A genes in the growth experiment in either growth air humidity regimes (Article I Figure 9), nor did the ost1-3 mutant lag behind Col-0 in its growth (Article III Figure 4). In Preprint IV, we didn't see any growth reduction in the plant lines with hampered stomatal closure (Preprint IV Figure 6a).

The growth reduction in above-ground parts specifically associated with excess stomatal production (even in plant lines that maintain good control over stomatal closure, or come with less open stomata to begin with) suggests that it is the process of stomatal development itself that slows down the growth. The size differences appear very early and are more or less proportionally maintained throughout vegetative growth phase (Article I Figure S7 and Table S3). Thicker leaves evidenced as an increased leaf mass/area ratio is a significant development cost (Mizokami *et al.*, 2022). The *epf1/2* mutant lines came with only slightly increased leaf mass/area ratio in one of our studies (Article I Figure 8) and none in the other (Preprint IV Figure S5); this measure, however, is based on the final harvest and may not reflect the processes in leaf primordia well. The cell division process associated with stomatal formation is a likely cost that plants with high SD must pay. Indeed, formation of each stoma normally causes extra divisions of the meristemoid mother cell and eventual rearrangement (with extra cells produced) of the mesophyll (Dow *et al.*, 2017). It has also been shown that stomatal density correlates positively with below-ground allocation (Hepworth *et al.*, 2016), still leaving the question of the mechanism – the proposed water stress due to excess transpiration in the *epf1/2* lines does not seem to work based on lack of similar effects on plant size in lines with hampered stomatal closure (Article III Figure 4; Preprint IV Figure 6).

The plant line strongly impaired in stomatal control in response to all environmental clues apart from CO_2 concentration, *ost1-3* (Merilo *et al.*, 2013) showed no growth impairment compared to wild-type. In Article III, this line actually entered the treatment period (at two weeks of age) bigger than Col-0, and while the relative size difference between wild-type and *ost1-3* perhaps closed up during the subsequent two weeks, this was only evident in the low-RH setting and even then the *ost1-3* plants were bigger at four weeks of age (Article III Figure 4). Likewise in Preprint IV, both *ost1-3* and *mpk12-4* (along with *ghr1-3* and *slac1-3* not shown in this summary) were not significantly smaller from wild-type at the end of the experiment in either growth RH, and indeed *ost1-3* again entered the treatment time at two weeks old with biggest plants (data not shown). Clearly, the conditions used in these experiments (RH at minimum 40–50% and sufficient soil water) do not pose significant water stress even to plants with severely impaired stomatal control.

OST1 is known to have multiple functions besides stomatal control. There could be hundreds of possible targets of the kinase OST1 (Wang *et al.*, 2020); its functions include regulation of cold response (Ding *et al.*, 2015) and it controls guard cell aquaporins (Grondin *et al.*, 2015). The full spectrum of consequences of these functions (or their impairment in the *ost1-3* mutant) is far from fully known. OST1 is not required for ABA-related regulation of seedling growth, a role fulfilled by the closely related SnRK2.2 and 2.3, but can replace them (Fujita *et al.*, 2009), leaving open the option that it operates at low level in normal conditions in seedlings and that in optimal conditions, defunct OST1 may result in small but significant boost in seedling growth. In the core ABA signalling pathway, RAF22 (and likely other RAF kinases) are inhibited via phosphorylation by OST1 (Kamiyama *et al.*, 2021; Sun *et al.*, 2022); RAF kinases have a role in

ABA-dependent growth regulation in seedlings (Wang *et al.*, 2018). SnRK2s enhance phloem loading and thereby root growth in stress conditions (Chen *et al.*, 2022) – diminished root growth could temporarily enhance early shoot growth, which would be consistent with our results with the *ost1-3* mutant. However, it is doubtful that we posed a notable stress on the plants in our growth conditions, nor did we observe any indirect adverse effects of possible perturbed root/shoot ratio in the *ost1-3* mutants.

The growth reduction under reduced RH conforms with generally observed trends (López *et al.*, 2021), but it may not have a single mechanism behind it. Such growth reduction is usually attributed to either source limitation in the form of reduced photosynthesis due to stomatal closure, sink limitation in the form of reduced turgor pressure, or resource re-allocation between plant parts (Novick *et al.*, 2024). It should be noted that we specifically designed our experiments to avoid the coupling between air humidity and soil moisture that commonly plagues air humidity experiments, so we believe soil moisture should not have been a significant factor.

In the one experiment where we directly studied photosynthesis under differential growth conditions, we didn't find clear differences in CO_2 assimilation (Article I Figure 1). This one study is by no means enough to conclusively reject source limitation as a factor in reduced growth, however, indirect evidence points in the same direction. Stomatal limitation to photosynthesis drops off quickly as stomatal conductance increases (Farquhar & Sharkey, 1982); even our wild-type lines exhibited conductances around 200–300 mmol m^{-2} s^{-1}, and the lines with abnormally high conductances, including those with strongly impaired stomatal closure, still showed the same range of growth retardation under reduced RH.

Sink limitation comes off as a possible culprit for the growth reduction under reduced RH. The low RH regime that we applied was far from extreme (Table 1). It has been shown that night-time VPD exceeding 0.4 kPa results in growth reduction in trees (Zweifel *et al.*, 2021) but not necessarily in herbaceous plants such as Arabidopsis (Christman *et al.*, 2009). We did not directly study leaf water potential in our growth experiments, however, leaf water content at harvest time was consistently reduced by about 1% in all growth experiments (e.g. Article I Figure 8b), suggesting that leaf turgor may have indeed been reduced under the mild growth RH reduction.

Preferential allocation to roots under reduced RH is a possibility that was beyond our capabilities to test. Data on root growth under altered RH is variable. López et al. (2021) show a slight but non-significant trend for root production to increase under high VPD (as opposed to clear decrease in aboveground growth). Often, root/shoot ratio remains little affected by growth RH (Zhang *et al.*, 2014; Lysenko *et al.*, 2023) or even raises under increased RH (Hunter *et al.*, 1985; Lõhmus *et al.*, 2019) – in these last examples, however, air humidity may have reached extremely high values at least occasionally. These results are from wildly different plant species and sometimes explore extremely high humidities, but serve to show that root growth as related to air humidity is a subject to explore with no trivial answers.

5. CONCLUSIONS

We demonstrated that it is possible to freely combine mutations that result in excess stomatal production (at least those due to defunct epidermal patterning factors EPF2 and EPF1) with mutations that affect stomatal openness, without marked interaction between these signalling pathways. Increased stomatal density resulted in significantly higher stomatal conductance in all backgrounds, and plants with genetic disruptions to stomatal control maintained expected higher or lower conductance in all *epf* mutant backgrounds.

Altered stomatal density somewhat affected photosynthetic capacity, with higher density correlating with higher net photosynthesis. This effect was rather mild, and our experiments with altered stomatal openness did not show a clear relationship between stomatal conductance and net photosynthesis.

We demonstrated a negative effect of reduced growth air humidity on above-ground plant growth, which was quite uniform across plant lines of different stomatal characteristics.

We showed that increased stomatal density results in reduced above-ground growth under the conditions used, while increased stomatal conductance *per se* does not cause such growth reduction. This suggests that the effect is not related to water loss but could be a result of strain put on resources as developing leaves produce excess leaf cells.

We managed to get stomata of Arabidopsis plants deficient in the OST1 kinase to react to step increase in VPD by growing them in lower soil moisture. The result may point to possible hydropassive stomatal closure in angiosperms when leaf water potential is sufficiently low.

We demonstrated partial decoupling of adaxial vs abaxial stomatal development. Growth RH affected stomatal development on leaf surfaces differently and shifted the balance towards more adaxial stomata. Among the wide array of mutants with perturbated stomatal development tested, *tmm* remained the only one with severely altered stomatal production specifically on its abaxial side, mechanism of which remains still unknown but could possibly involve interactions with other epidermal patterning factors with overlooked roles in leaves. We also show that *epf1/2* mutants have altered adaxial/abaxial stomatal ratios, but this effect, possibly even its direction, appears to depend on leaf age.

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SUMMARY IN ESTONIAN

Põhjused ja tagajärjed: õhulõhed erinevate õhuniiskuste juures

Õhulõhed on maismaataimedele võtmetähtsusega organid, mis võimaldavad tasakaalustada isolatsiooni kuivast atmosfäärist ja juurdepääsu seal leiduvale süsihappegaasile. Lisaks õhulõhede suhteliselt kiiresti muudetavale avatusele on taime pinna gaasijuhtivuse määramisel olulisel kohal õhulõhede tihedus, nende arvukus pinnaühikul. Suurema tihedusega, nagu avatusega, kaasneb suurem potentsiaal fotosünteesiks süsihappegaasi vastu võtta, aga ühtlasi võib suureneda ka taime tundlikkus veekaotusele. Tüüpilistel õistaimedel on õhulõhed lehe alaküljel, aga osal neist, sh. selles töös uuritud harilikul müürloogal, lisaks ka ülaküljel. Mehhanismid, mis juhivad õhulõhede arengut, on üldjoontes teada ja need toimivad mõlemal lehe küljel; üks õhulõheteaduse lahendamata küsimus on, millised mehhanismid põhjustavad lehe külgede erinevat arengut ja miks on mõnedel taimeliikidel erinevused drastilised, teistel aga tagasihoidlikud.

Nii õhulõhede tiheduse kui tundlikkuse manipuleerimiseks on olemas hulk teadaolevaid mutatsioone. Tihedust ja tundlikkust muutvate mutatsioonide omavahel kombineerimist on pakutud potentsiaalika meetodina taimede gaasivahetuse optimeerimiseks.

Üks selle töö eesmärk oli katsetada suurenenud õhulõhede tihedust põhjustavate mutatsioonide kombineerimist õhulõhede avatust vähendavate mutatsioonidega. Küsimused selle lähenemise juures olid, kas niisugune kombineerimine annab taimed, milles vanemliinide omadused on segamatult liitunud või ilmnevad interaktsioonid õhulõhede tihedust ja avatust määravate signaaliradade vahel, ja kas mingites kasvutingimustes võiks saadud taimeliinid saavutada kasvujõudluse, mis metsiktüüpi ületab. Selleks ristasime müürlooga EPF mutante (millel on häiritud õhulõhede arengu kontrolli tõttu ülemäärane õhulõhede produktsioon) eri signaaliradade mutantidega, millel vähenenud õhulõhede avatus, ja kasvatasime saadud ristandliine kahel erineval õhuniiskuse režiimil. Lisaks taime üldisele kasvule uurisime detailselt ka õhulõhede anatoomiat ja funktsiooni. Leidsime, et mutatsioonide kombineerimine andis fenotüüpide lihtsa kombineerumise, kus avatust mõjutavad mutatsioonid ei mõjutanud tuntavalt tiheduse kujunemist ja vastupidi. Taimede maapealsete suuruste võrdluses ilmnes, et madalamas õhuniiskuses kasvasid kõik taimeliinid aeglasemalt ja et kõrgema õhulõhede tihedusega kaasnes alati aeglasem kasv.

Järgnev sarnane eksperiment, kus kombineerisime müürloogas suuri õhulõhede tihedusi mutatsioonidega, mis pärsivad õhulõhede sulgumist, andis samalaadse tulemuse: õhulõhede avatust ja tihedust juhtivad signaalirajad kombineerusid aditiivselt, teineteist segamata, ja suurema õhulõhede tihedusega kaasnes aeglasem kasv mõlemal õhuniiskuse režiimil. Sellest eksperimendist nähtus kindlalt, et suure õhulõhede tihedusega kaasnev kasvu aeglustumine ei olnud seotud veekaotusega, kuna iseäranis suure õhulõhede avatusega ei kaasnenud mingit mahajäämust kasvus. Tõenäolisim mehhanism näib olevat ülemäärase

õhulõhede tootmisega kaasnev energiakulu taime varases elujärgus, võimalik, et juba seemikuna.

Töö teine eesmärk oli uurida lehe ülemise ja alumise külje õhulõhede kujunemise mehhanisme, võrreldes erinevaid müürlooga mutante ja uurides kasvutingimuste mõju õhulõhede arengule. Lisaks eelkirjeldatud eksperimentidele viisime selleks läbi vaatluse, kus uurisime suurt hulka taimeliine, millel õhulõhede areng erinevate mutatsioonide tõttu häiritud. Lisaks *tmm* mutandile (mille järsult kontrastne ülа- ja alakülje erinevus ammu teada) täheldasime väikest ülа- ja alakülje õhulõhede tiheduste suhte hälvet *epf1* ja *epf2* topeltmutandil ja viimasega eeldatavasti funktsionaalselt seotud *sdd1* mutandil. Ka kasvukatsetes ilmnes *epf* mutatsioonidega seotud õhulõhede suhte kõrvalekaldeid, aga huvitaval kombel eri eksperimentides eri suundades, mis võib olla põhjendatav neis katsetes vaadeldud erineva järjekorranumbriga lehtedega.

Kasvukatsetes kasutatud mitmesuguse õhulõhede tihedusega liinid võimaldasid meil kaevuda sügavamalt õhulõhede suuruse ja tiheduse vahekorda. Üldiselt on teada, et suurema tihedusega kaasnevad väiksemad õhulõhed, ja ka meie andmetes on see seos nähtav, aga ainult siis, kui kõrvutada ühesuguse õhulõhede arengu genotüübiga taimi omavahel: madalamas õhuniiskuses kaasneb lehe vähema kasvuga suurem õhulõhede tihedus ja veidi väiksemad õhulõhed. Selgub aga, et see negatiivne seos ei kandu edasi erinevate õhulõhede arengu mutantide võrdlusse: *epf* mutantide ligi kahekordselt suurem õhulõhede tihedus võrreldes funktsionaalsete EPF peptiididega taimedega ei kaasanud märgatavat erinevust õhulõhede suuruses, miska suuruse/tiheduse tavaliselt täheldatav kompromiss ei ole ilmselt seotud ruumipiiranguga.

Kolmanda teemana uurisime tugevasti pärsitud õhulõhede sulgumisraja mutandi *ost1* reaktsiooni õhuniiskusele ja kasvamist eri keskkonnatingimustes. See kinaas vahendab tervetes taimedes õhulõhede sulgumist abstsiishappe toimel, sh. reaktsiooni madalale õhuniiskusele, ja jätkuvalt on ebaselge, kas õistaimede õhulõhed õhuniiskusele ilma abstsiishappe vahenduseta üldse reageerivadki. Demonstreerisime oma eksperimentidega, et kuivemal mullal kasvanud *ost1* müürloogad omandasid võime õhuniiskuse langetamisele õhulõhede sulgumisega vastata, mis tõenäoliselt toimus passiivse reaktsioonina, võimaldatuna epidermi turgorrõhu languse poolt. Näitasime ka, kuidas OST1 kinaas saab toimida õhuniiskuse signaali vahendajana ja põhjustada õhulõhede terve taime ulatuses sulgumise isegi abstsiishappe osaluseta, aga normaalsest kordades aeglasemalt.

Hästituntud ja kergestikäsitsetava müürloogaga katsetatakse sageli esmasid ideid ja tehnikaid, mis võiksid ehk tulevikus põllule jõuda. Selles töös proovisime meetodit õhulõhede suurema tiheduse kombineerimiseks muudetud avatusega ja näitasime, et see on vabalt teostatav, aga me demonstreerisime ka, et see lähenemine ei näi taimekasvu stimuleerimiseks perspektiivikana. Õhulõhede lehe ülaküljel leidumise mõistatusele veel selget lahendust ei leidnud, aga lisasime pilti olulisi infokilde, nagu ka õhulõhede atmosfääriniiskusele reageerimise mehhanismidesse.

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My late mother, who should have been a scientist herself, would certainly have been proud to witness this. Inger, my dear big sister, I'm still not becoming a professor but I suppose you are going to call me one anyway. And Ain, you are literally awesome – thank you for life, the Universe, and everything!

PUBLICATIONS

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