UNIVERSITY OF TARTU

Faculty of Science and Technology

Institute of Technology

Sára Babincová

The effect of elevated temperature on stomatal development in *Arabidopsis thaliana*

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Supervisor:

Associate professor, PhD Hanna Hõrak

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Abstract

Stomata are small pores in the leaf epidermis, each surrounded by a pair of guard cells, that mediate gas exchange between the plant and the environment. Their development is greatly influenced by environmental conditions such as light intensity, carbon dioxide (CO_2) levels, and temperature. With global temperatures rising, understanding how stomatal patterning is regulated in the increasingly warmer climate is becoming paramount. This study addressed stomatal developmental response to elevated temperature (ET) in plants deficient in key signalling pathway components involved in stomatal function and development in *Arabidopsis thaliana* to identify potential new actors in this response. The mutant lines were grown at 30°C/26°C day/night (ET) and at 23°C/19°C day/night (control temperature, CT). Analysis of stomatal density (SD), stomatal ratio (SR) and stomatal index (SI) was performed. We found that SD generally decreased in response to ET. SR was mostly not affected by ET. SI increased on the abaxial side in the control line Col-0 and mitogen-activated protein kinases 3 and 6 (MPK3/6) overexpressor lines *MPK3OX* and *MPK6OX* when grown at 30°C, suggesting the role of MPK3/6 in the regulation of the response of stomatal development to ET. The mutant line *raf27-2* initially showed increased SD in response to ET. However, this effect was not seen in a repeat experiment grown under the same conditions on a different shelf of the growth cabinet, suggesting an unknown inconsistency across the growth cabinet. A further investigation of the role of *RAF27* in the temperature response is required.

Keywords

stomata, stomatal development, stomatal density, stomatal ratio, elevated temperature, environmental cues

CERCS: B225 Plant genetics

Institute name: Institute of Technology

Research group: Molecular Plant Physiology lab

Kõrge temperatuuri mõju õhulõhede arengule harilikus müürloogas (*Arabidopsis thaliana*)

Lühikokkuvõte

Õhulõhed on väikesed avad lehe epidermis, mis moodustuvad kahest sulgrakust ja vahendavad gaasivahetust taime ja keskkonna vahel. Õhulõhede arengut mõjutavad suuresti keskkonnatingimused, nagu valguse intensiivsus, süsihappegaasi (CO_2) tase ja temperatuur. Kuna temperatuur globaalselt tõuseb, on üha olulisem mõista, kuidas kujuneb õhulõhede muster soojemas kliimas. See töö keskendus hariliku müürlooga õhulõhede funktsiooni ja arenguga seotud peamiste signaaliradade komponentide rolli uurimisele kõrge temperatuuri mõjul toimuvas õhulõhede arengu regulatsioonis, et tuvastada selle protsessi potentsiaalseid uusi regulaatoreid. Mutantseid taimeliine kasvatati temperatuuril 30°C/26°C (päev/öö, kõrge temperatuur) ja temperatuuril 23°C/19°C (päev/öö, kontrolltemperatuur). Analüüsiti õhulõhede tihedust, õhulõhede tiheduste suhet ja õhulõhede indeksit. Leiti, et õhulõhede tihedus üldiselt väheneb vastusena kõrgele temperatuurile. Kõrge temperatuur õhulõhede tiheduste suhet enamasti ei mõjutanud. Kontrolltaimedes (Col-0) ja mitogeen-aktiveeritud proteiinkinaaside 3 ja 6 (MPK3/6) üleekspressiooni liinides *MPK3OX* ja *MPK6OX* suurenes õhulõhede indeks lehe alaküljel 30 °C juures kasvatatud taimedes, mis viitab MPK3/6 rollile õhulõhede arengu regulatsioonis vastusena kõrgele temperatuurile. Mutantse taimeliini *raf27-2* õhulõhede tihedus suurenes kõrge temperatuuri tingimustes esimeses katses, kuid korduskatses, kus taimi kasvatati samades tingimustes kasvukapi teisel riiulil, sellist tulemust ei leitud, mis viitab võimalikele ebaühtlastele tingimustele kasvukapi erinevates osades. Seega on *RAF27* rolli õhulõhede arengu vastuses kõrgele temperatuurile vaja täiendavalt uurida.

Võtmesõnad:

õhulõhede areng, õhulõhede tihedus, õhulõhede tiheduste suhe, kõrge temperatuur, keskkonnategurid

CERCS: B225 Taimegeneetika

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TERMS, ABBREVIATIONS AND NOTATIONS

ABA - abscisic acid

abaxial - distant from the central axis of the plant, bottom surface in leaves

adaxial - close to the central axis of the plant, upper surface in leaves

ANOVA - analysis of variance

bHLH - basic helix-loop-helix

BHP - BLUE LIGHT-DEPENDENT H^+-ATPASE PHOSPHORYLATION

CA1 - β-carbonic anhydrase βCA1

CA4 - β-carbonic anhydrase βCA4

CO_2 - carbon dioxide

Col-0 - Columbia-0, wild-type Arabidopsis in this study

COP1-SPA - CONSTITUTIVE PHOTOMORPHOGENIC 1- SUPPRESSOR OF PHYA-105

CT - control temperature

EPF - EPIDERMAL PATTERNING FACTOR

ERf - ERECTA family RLKs

ET - elevated temperature

GC - guard cell

GHR1 - GUARD CELL HYDROGEN PEROXIDE RESISTANT 1

GMC - guard mother cell

HIC - HIGH CARBON DIOXIDE

HSP90 - Heat shock protein 90

HT1 - HIGH LEAF TEMPERATURE 1

ICE1 - INDUCER OF CBF EXPRESSION 1

ILK5 - INTEGRIN-LINKED KINASE 5

MAPK - mitogen-activated protein kinase

MMC - meristemoid mother cell

NCED - 9-cis-epoxycarotenoid dioxygenase

PIF - PHYTOCHROME INTERACTING FACTOR

PYL - PYR1-LIKE

PYR/RCAR - PYRABACTIN RESISTANCE/REGULATORY COMPONENTS OF ABA RECEPTOR

RAF27- Raf-like protein kinase (also known as BHP or ILK5)

RLK - receptor-like kinase

SCRM - SCREAM

SCRM2 - SCREAM 2

SD - stomatal density

SDD1 - STOMATAL DENSITY AND DISTRIBUTION 1

SE - standard error

SI - stomatal index

SLGC - stomatal lineage ground cell

SnRK2s - SNF1-related protein kinases

SPCH - SPEECHLESS

SR - stomatal ratio

TF - transcription factor

TMM - TOO MANY MOUTHS

INTRODUCTION

Stomata are microscopic pores in the epidermis of plant leaves, flanked by a pair of guard cells that adjust their turgor pressure to mediate gas exchange. This allows plants to take up carbon dioxide (CO_2) and release oxygen and water vapour into the environment. As stomata ensure the uptake of CO_2 and plant transpiration, they maintain the balance between photosynthesis and water loss (Chaerle et al., 2005).

The rapid changes to the global climate are pushing plants to adapt in order to grow and survive. In addition to controlling stomatal movements to balance the uptake of CO_2 with water loss, plants can respond to prevailing environmental conditions by modulating stomatal density and distribution (S. A. Casson & Hetherington, 2010; S. Casson & Gray, 2008; Driesen et al., 2020). The general trend in *Arabidopsis thaliana* is a decrease in stomatal density in response to high CO_2 levels and elevated temperatures. This is because these environmental cues, through different mechanisms, act upon the developmental pathway of the stomatal lineage. With the global temperature and CO_2 levels predicted to rise in the near future (Adak et al., 2023), it is imperative to understand how these stimuli affect stomatal patterning in plant leaves and which pathways and genes are involved in coordinating stomatal development under these changing conditions.

This study focuses on the regulation of stomatal development responses to elevated temperature by examining several mutant lines of *Arabidopsis thaliana* deficient in genes belonging to various signalling pathways involved in stomatal patterning, light responses and abscisic acid (ABA) and CO_2-mediated stomatal movements to identify potential new regulators of stomatal development under elevated temperature.

LITERATURE REVIEW

1.1 STOMATA AND THEIR FUNCTION

Stomata are pores on the epidermis of vascular plant leaves. They are formed by a pair of guard cells (GC), which have a characteristic kidney shape in dicots and a dumbbell shape in most monocots, such as grasses (Bertolino et al., 2019; Figure 1). Their shape and elasticity are due to the polysaccharide-based polymers in their cell wall and the asymmetrical organisation of cellulose fibres. In dicots, the cell wall is stiffer on the polar ends of the cells, which gives the GC a typical kidney shape (Rui et al., 2018).

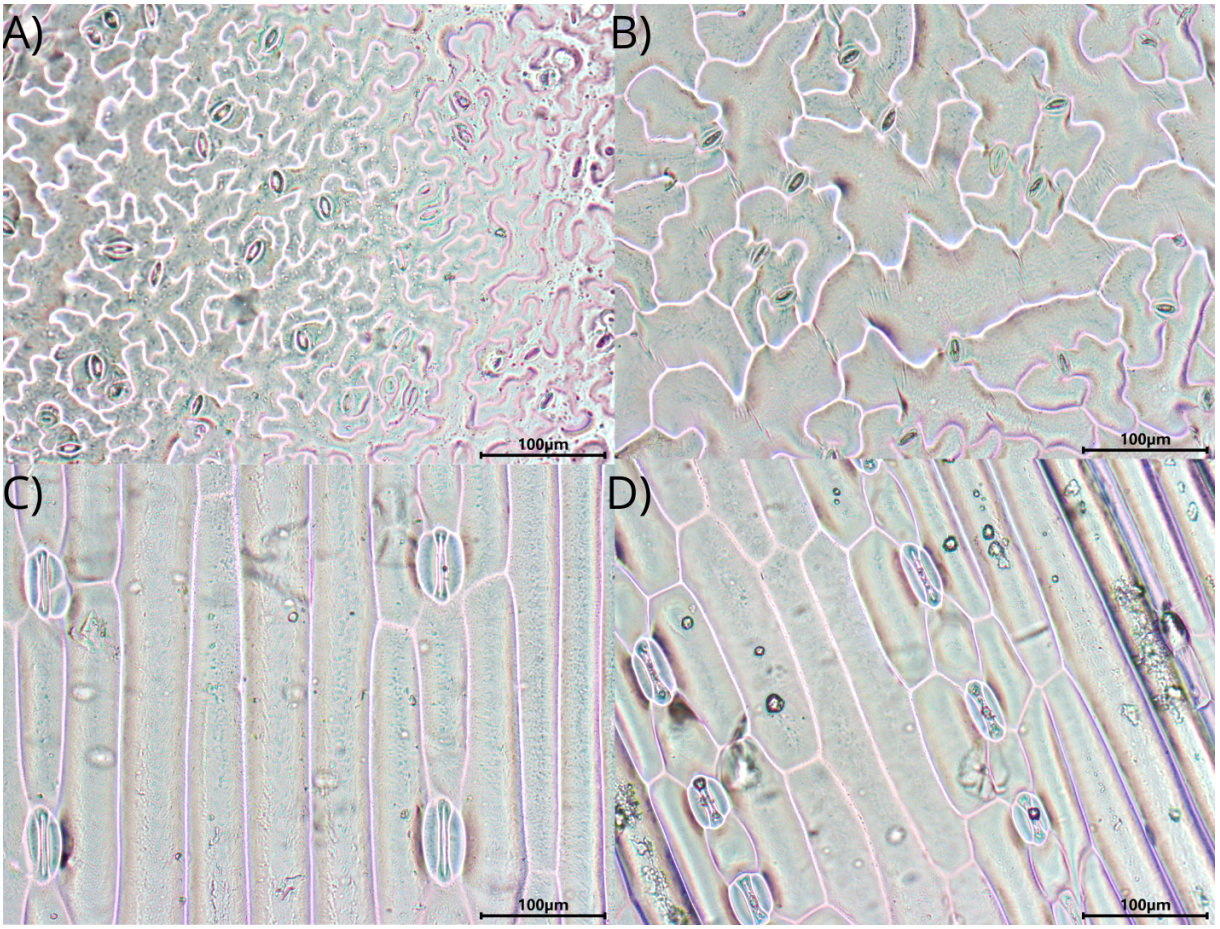

Figure 1. Examples of leaf surfaces of monocots and dicots. A) *Arabidopsis thaliana* abaxial leaf surface; B) *Arabidopsis thaliana* adaxial leaf surface; C) *Hordeum vulgare* (barley) abaxial leaf surface; D) *Hordeum vulgare* adaxial leaf surface.

The leaf surface is covered by a waxy layer called a cuticle that prevents water loss and CO_2 entry; stomata are thus needed to maintain proper gas exchange. The opening and closing of the stomatal pore that facilitates gas exchange between the plant and the environment is mediated by turgor pressure in the GC. Stomata represent a bridge between the outside world and the inner environment of the plant. The delivery of CO_2 to the mesophyll is imperative for

photosynthesis, and the controlled pore width restricts water loss (Nadeau & Sack, 2002). Stomatal opening and closing are mediated by a change in concentration of certain solutes - ions and sugars - that drive the flux of water in and out of the guard cells. Stomata are open when the guard cells fill with water, increasing the turgor pressure, and bow apart. Stomatal closure is mediated by the metabolising or expelling of solutes, which drives water out of the cells, making them more flaccid (Jezek & Blatt, 2017).

1.2 STOMATAL DEVELOPMENT

1.2.1 Stomatal lineage

Stomata arise from a specialised cell lineage (Lau & Bergmann, 2012). Their development starts when a protodermal cell transitions into a meristemoid mother cell (MMC) state. MMC undergoes an asymmetric division, generating a meristemoid and a stomatal lineage ground cell (SLGC). SLGC can differentiate into pavement cells or adopt the MMC fate and produce a second meristemoid in a spacing division. The meristemoid can undergo another asymmetric division (amplifying) for self-renewal, or it can become a guard mother cell (GMC) that divides once symmetrically to finally produce a pair of GC (Figure 2).

Stomatal differentiation is governed by a group of basic helix-loop-helix (bHLH) transcription factors (TF), namely SPEECHLESS (SPCH), MUTE, and FAMA (MacAlister et al., 2007; Pillitteri et al., 2007; Ohashi-Ito & Bergmann, 2006). These TFs are accompanied by two more bHLH-leucine zipper proteins: INDUCER OF CBF EXPRESSION 1 (ICE1), also known as SCRM; and SCRM2 (Kanaoka et al., 2008). They have been shown to partner with SPCH, MUTE, and FAMA to orchestrate stomatal development (Figure 2).

The entry into the stomatal lineage is initiated by SPCH, which allows for MMC formation and the asymmetric division into a meristemoid and SLGC (MacAlister et al., 2007). SPCH is also involved in spacing and amplifying divisions (Lau et al., 2014). The leaf epidermis will be covered only with pavement cells when the function of *SPCH* is lost. Conversely, the excess of stomata is seen when *SPCH* is overexpressed. To continue the developmental process, MUTE terminates asymmetrical division and promotes the differentiation of meristemoids (Pillitteri et al., 2007). The overexpression of *MUTE* is phenotypically similar to the overexpression phenotype of *SPCH*, where stomata cover all of the leaf. The loss-of-function mutant shows no stomata, meristemoids undergo rounds of asymmetric division, unable to transition, and the SLGCs create a spiral, rose-like pattern until eventually arresting. FAMA is responsible for the termination of differentiation and the initiation of the symmetric division of GMCs to GCs (Ohashi-Ito & Bergmann, 2006). When *FAMA* is

ectopically overexpressed, it leads to excess GC formation. FAMA can also convert cells not belonging to the stomatal lineage into GC. The loss-of-function *fama* mutants produce small, accordion-like clusters of GMCs where a regular stoma would be located.

During each step, SCRM and SCRM2 accompany SPCH, MUTE and FAMA and specify the stages of stomatal development by forming heterodimers with these bHLH TFs (Kanaoka et al., 2008). Their loss-of-function mutants replicate the phenotype of *spch, mute* and *fama*. The fewer copies of functional *SCRM* and *SCRM2*, the earlier the stage of arrest; thus, the partial loss-of-function *SCRM*/*ICE1* mutants show similar phenotypes to the loss-of-function *FAMA*, with their GMC clusters, while a complete loss of functional SCRM and SCRM2 leads to a phenotype identical to that of *spch*. This shows how pivotal their presence is in driving the developmental processes.

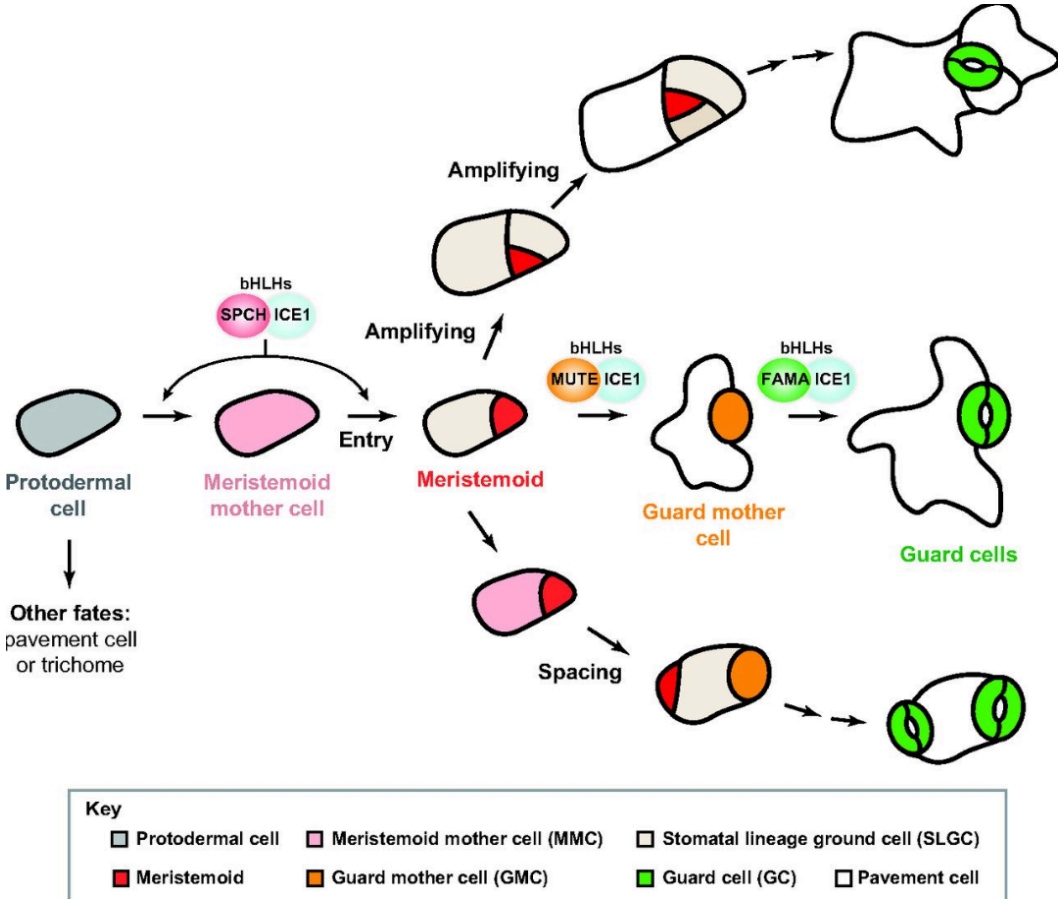

Figure 2. Stages of stomatal development of the stomatal lineage. Once a protodermal cell commits to the stomatal lineage, SPCH assisted by ICE1/SCRM allows for the asymmetric division, creating an SLGC and a meristemoid. At this stage, the SLGC becomes an MMC and divides asymmetrically in a spacing division or adopts a pavement cell fate. The meristemoid can divide again in an amplifying division. With the help of the MUTE/ICE1 complex, the meristemoid transitions into a GMC. FAMA then drives the symmetric division to produce two GCs. bHLH, basic helix-loop-helix; ICE1, INDUCER OF CBF EXPRESSION 1/SCRM; SPCH, SPEECHLESS (modified from Lau & Bergmann, 2012)

1.2.2 Stomatal patterning regulation

The distribution and patterning of stomata are regulated by intercellular communication upstream of stomatal TFs mediated by secreted peptides of the EPIDERMAL PATTERNING FACTOR (EPF) (EPF1/EPF2) family (Hara et al., 2007, 2009), along with their receptor proteins, such as the membrane-bound receptor-like kinases (RLK) of the ERECTA family (ERf) (Shpak et al., 2005) and their co-regulator TOO MANY MOUTHS (TMM) (Yang & Sack, 1995). STOMAGEN/EPFL9, a positive regulator of stomatal development (Kondo et al., 2010; Sugano et al., 2010; Hunt et al., 2010), also binds to TMM (Figure 3).

The secreted peptide EPF1 is responsible for the proper spacing of stomata, regulating asymmetric division (Hara et al., 2007). It is expressed in the cells of the stomatal lineage. It is the ligand to the ERf receptor kinases, such as ERL1, and its activity depends on TMM. Another member of the EPF family, EPF2, is secreted by MMC and their early descendants and regulates the number of stomata by inhibiting the number of cells adopting the MMC fate (Hara et al., 2009). Both of these peptides play an inhibitory role in stomatal development. On the other hand, STOMAGEN/EPFL9, a peptide secreted by mesophyll, promotes stomatal development through competitive binding to ERf members and is an antagonist to EPF2 (Hunt et al., 2010; J. S. Lee et al., 2015; Ohki et al., 2011).

The cleavage of pro-peptides is necessary for the formation of biologically active signalling peptides. The subtilisin-like serine protease STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1) has been proposed as this processing agent (Berger & Altmann, 2000; von Groll et al., 2002). Its loss-of-function mutant exhibits an increase in stomatal density and disturbance to the distribution and patterning, promoting the clustering of stomata. SDD1 has also been shown to be dependent on TMM activity (Figure 3).

TMM is a receptor-like protein that forms complexes with ERf members to modulate the specificity of ERfs to perceive EPF and EPF-like proteins, regulating their activity (Lin et al., 2017). The loss-of-function *tmm* mutant shows stomatal clustering and increased stomatal density. As previously mentioned, the function of multiple proteins involved in the signalling pathway depends on TMM, highlighting its importance in this process.

Mitogen-activated protein kinases (MPK) signalling cascade coupled with RLKs negatively regulate the core heterodimers of SPCH, MUTE, and FAMA, with ICE1 and SCRM2 downstream of the ERf RLK (Figure 3). The MPK cascade is mediated by an MPKKK YODA (Bergmann et al., 2004). Downstream of YODA, two other sets of MPKs, MKK4/5 and MPK3/6, are the following components of this cascade and have been shown to

negatively regulate stomatal development (Wang et al., 2007). One of the final targets of the MPK phosphorylation cascade is SPCH. Through the phosphorylation of SPCH, which leads to the subsequent SPCH degradation, the MPK cascade negatively regulates stomatal development (Lampard et al., 2008).

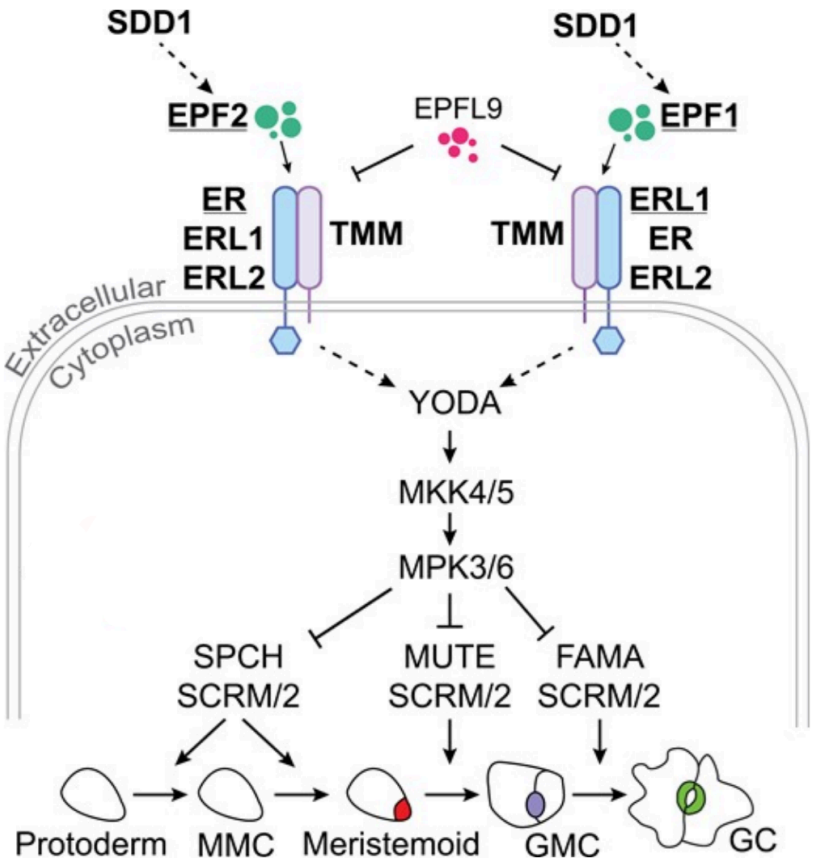

Figure 3. Stomatal patterning signalling pathway. SDD1 has been proposed to cleave pro-peptides, such as EPF1 and EPF2, into active signalling peptides. Binding of EPF1, EPF2 and EPFL9/STOMAGEN to the ERf receptor complex (ER, ERL1, ERL2) and TMM activates mitogen-activated protein kinase (MPK) signalling cascade consisting of YODA, MKK4/5 and MPK3/6, which in turn represses the bHLH TFs (SPCH, MUTE, FAMA) and their partner bHLH proteins SCRM/SCRM2 to reduce the production of stomata. bHLH, basic helix–loop–helix; EPF, EPIDERMAL PATTERNING FACTOR; ER, ERECTA; ERL, ERECTA-LIKE; GC, guard cell; GMC, guard mother cell; MMC, meristemoid mother cell; SCRM, SCREAM; SDD1, STOMATAL DENSITY AND DISTRIBUTION 1; SPCH, SPEECHLESS; TMM, TOO MANY MOUTHS; (modified from Jalakas et al., 2024).

1.3 ENVIRONMENTAL REGULATION OF STOMATAL DEVELOPMENT

1.3.1 Light signalling and stomatal development

In Arabidopsis, three major photoreceptors govern the light signalling and shade avoidance mechanism. Phototropins, blue light photoreceptors such as phot1/2, cause phototropism, a change in stem shape triggered by low light levels (Legris & Boccaccini, 2020). Another group of blue light receptors, cryptochromes such as cry1/2 (Chaves et al., 2011), along with UV receptors and phytochrome family red light receptors, e.g. phyB (Hernando et al., 2021), work as inhibitors to the E3 ligase complex CONSTITUTIVE PHOTOMORPHOGENIC 1-SUPPRESSOR OF PHYA-105 (COP1-SPA) (Hoecker, 2017; Podolec & Ulm, 2018). In the presence of active cryptochromes and phytochromes, PHYTOCHROME INTERACTING FACTORS (PIFs), bHLH TFs promoting photomorphogenesis, are inhibited (Franklin & Whitelam, 2004). When the plant is experiencing shade, the light-activated cry1/2 and phyB do not inhibit the COP1-SPA complex, leading to growth by stabilising PIFs (Ponnu & Hoecker, 2022; Legris, 2023).

Both cryptochromes and phytochromes are also involved in the regulation of stomatal development through their regulation of COP1-SPA, which works as an upstream regulator of the YODA-MPK phosphorylation cascade that results in the degradation of SPCH (Wei et al., 2020). COP1-SPA is also capable of degrading ICE1, downregulating the development of stomata (J.-H. Lee et al., 2017). When light levels are high, COP1-SPA is inhibited, which leads to the stabilisation of ICE1 and the promotion of stomatal development.

1.3.2 Stomatal development under elevated temperature

In response to elevated temperatures, plants change their morphology, with the most common adaptations being petiole elongation, changes in leaf positioning (hyponasty), reduced leaf thickness, and decreased stomatal density, all of which promote the plant's cooling capacity. (Crawford et al., 2012; Legris, 2023)

The responses of stomatal development to ambient temperature can be divided by the duration of exposure. The way Arabidopsis reacts to long-term heat exposure is similar to the mechanism of shade avoidance and involves multiple of the same components. PhyB acts as a temperature sensor, destabilising and transitioning into an inactive state during heat exposure (Hahm et al., 2020; Jung et al., 2016; Legris et al., 2016). Another component of the shade avoidance pathway involved in the temperature response is PIF4. During long-term exposure, the inhibition of SPCH is carried out directly by PIF4, resulting in fewer stomata.

Interestingly, a negative feedback loop has been reported between PIF4 and SPCH, which directly represses the expression of PIF4 (Lau et al., 2018).

Acute heat stress, characterised by short-term exposure to elevated temperatures, triggers a response mediated by the HEAT SHOCK PROTEIN 90 (HSP90) (Vierling, 1991) that phosphorylates YODA, which in turn triggers the phosphorylation cascade that leads to the suppression of SPCH (Samakovli et al., 2020).

1.3.3 Stomatal development under elevated CO_2

In response to increasing CO_2 levels in the atmosphere, multiple plant species adapted by lowering their stomatal density (Woodward, 1987). Only a few genes have been identified that link CO_2 perception with stomatal development, the first of them, *HIGH CARBON DIOXIDE* (*HIC*), is involved in the production of long-chain fatty acids necessary for cell wall wax synthesis (S. A. Casson & Hetherington, 2010; Chua & Lau, 2024; Gray et al., 2000). *HIC* gene mutants exhibit increased stomatal density and index under elevated CO_2, hinting at the role of HIC in stomatal development. However, the exact mechanism remains unknown.

An *A. thaliana* double mutant, *ca1ca4*, with impaired β-carbonic anhydrases βCA1 (CA1) and βCA4 (CA4), also shows an increase in stomatal density under elevated CO_2 as well as changes in the CO_2-mediated stomatal movements (Engineer et al., 2014; Hu et al., 2010). It has been demonstrated in the *ca1ca4* mutant that the induction of EPF2 does not occur under elevated CO_2 levels (Engineer et al., 2014). As a mediator in the CO_2-induced regulation of stomatal development, CO_2 RESPONSE SECRETED PROTEASE (CRSP) has been identified. CRPS is a secreted protease that carries out the cleavage of the pro-peptide EPF2, which is closely tied to the regulation of stomatal patterning. Altogether, CA1 and CA4 anhydrases, CRSP and EPF2, appear to orchestrate the developmental response to increased CO_2 levels, but the exact mechanisms are still unclear (Chua & Lau, 2024; Engineer et al., 2014).

1.3.4 Drought-induced phytohormone ABA and stomatal development

Abscisic acid (ABA) is a phytohormone that mediates stress responses related to low air humidity and soil water content. It is responsible for stomatal closure, which prevents water loss, but also regulates stomatal development. Downstream of ABA, three subclass III SNF1-related protein kinases (SnRK2s): SnRK2.2/SRK2D, SnRK2.3/SRK2I, and SnRK2.6/SRK2E/OPEN STOMATA 1 (OST1) (Fujita et al., 2009) can phosphorylate SPCH in response to low water availability, regulating the number of cells entering the stomatal lineage (X. Yang et al., 2022). Different ABA levels have been shown to affect stomatal

density, with more stomata produced in ABA-deficient plants (Chater et al., 2015; Merilo et al., 2018; Tanaka et al., 2013).

The regulation of stomatal development by environmental cues is summarised in Figure 4.

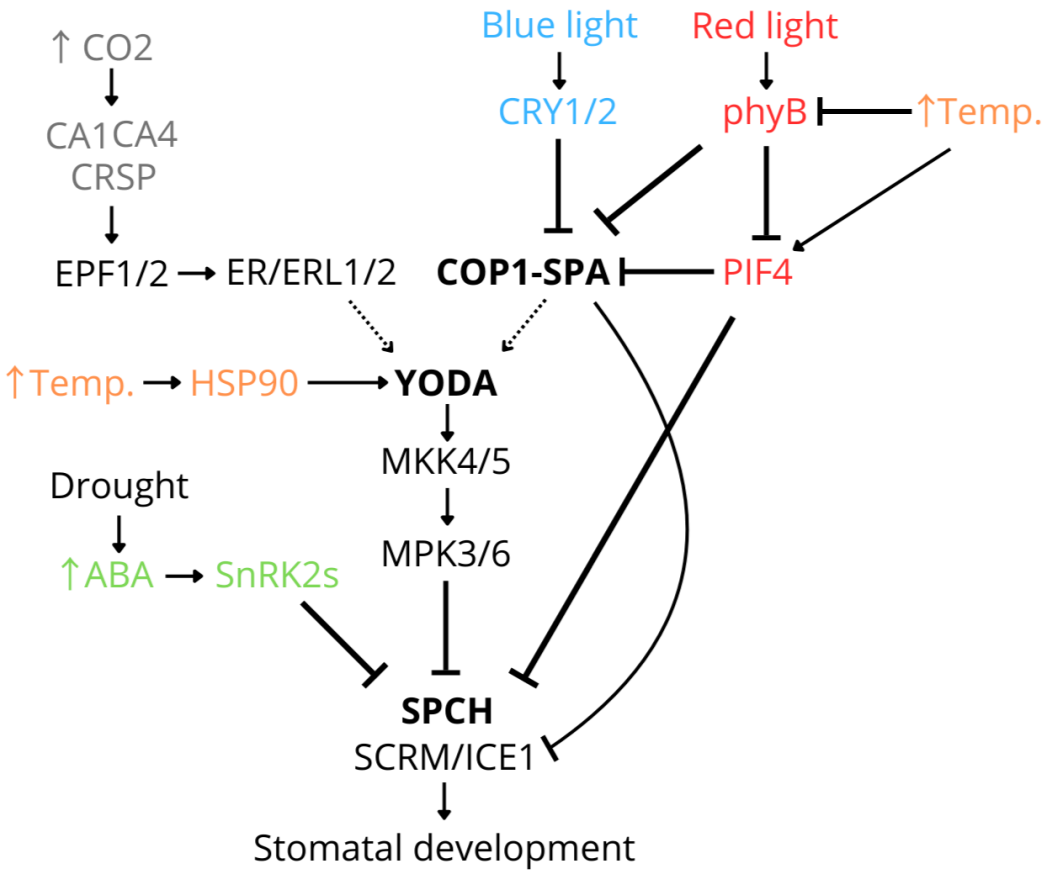

Figure 4. Regulation of stomatal development in response to environmental cues. Blue and red light receptors CRY1/2 and phyB downregulate COP1-SPA, which is one of the regulators of YODA and suppressors of SCRM/ICE1. The red light receptor phyB suppresses a repressor of YODA, PIF4, which also suppresses SPCH directly and is activated by elevated temperatures. The HEAT SHOCK PROTEIN 90 (HSP90) phosphorylates YODA during acute heat stress. Elevated CO_2 levels also activate YODA through the interaction of CA1, CA4 and CRSP with EPF1/2 and the ERf receptor complexes (ER, ERL1/2). The mitogen-activated protein kinase (MPK) signalling cascade, consisting of YODA, MKK4/5 and MPK3/6, acts to suppress SPCH. Drought and low humidity promote the production of abscisic acid (ABA), downstream of which SnRK2s phosphorylate SPCH, directly suppressing stomatal development.

1.4 MAJOR PATHWAYS IN STOMATAL APERTURE REGULATION

1.4.1 Light signalling pathway

Stomata open in response to light. The opening of the stomata is mediated by phot1 and phot2 (Kinoshita et al., 2001) acting on the plasma membrane H^+-ATPase (Figure 5). Downstream of phot1 and phot2, a Raf-like protein kinase, BLUE LIGHT-DEPENDENT H^+-ATPASE PHOSPHORYLATION (BHP), acts as a signalling mediator in the blue-light-mediated stomatal opening (Hayashi et al., 2017). BHP is also known as INTEGRIN-LINKED KINASE 5 (ILK5) and participates in the innate immune response (Kim et al., 2023). In this study, two mutant lines, *raf27-1* and *raf27-2*, are mutants of the *BHP*/*ILK5* gene.

1.4.2 ABA signalling pathway

ABA biosynthesis is closely tied to plant growth and tolerance to water stress. 9-cis-epoxycarotenoid dioxygenase (NCED) is a catalyst of carotenoid cleavage, one of the steps in ABA biosynthesis (Frey et al., 2012). The mutant studied in this work, *nced3nced5,* showed decreased ABA levels, leading to enhanced water loss and dehydration in vegetative tissues. ABA levels are controlled not only by biosynthesis but also by catabolism. In Arabidopsis, ABA 8′-hydroxylase encoded by genes in the *CYP707A* family is responsible for ABA catabolism, and mutants from this family exhibit increased endogenous ABA levels (Okamoto et al., 2006a).

For stomatal closure, the cell needs a flux of ions, for which specialised ion channels are required. There are two types of ion channels in GC, slow and rapid types, which orchestrate stomatal closure (Jalakas et al., 2021). SnRK2s, protein kinases activated by ABA, act on the SLOW ANION CHANNEL 1 (SLAC1) to initiate stomatal closure (Geiger et al., 2009; S. C. Lee et al., 2009). Another protein, GUARD CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1), is a leucine-rich repeat receptor-like kinase that activates SLAC1 to mediate stomatal closure (Sierla et al., 2018).

The *pyr1pyl1pyl2pyl4pyl5pyl8* (*112458*) is a sextuple mutant of six PYRABACTIN RESISTANCE/REGULATORY COMPONENTS OF ABA RECEPTOR (PYR/RCAR) PYR1/PYR1-LIKE (PYL) receptors that are responsible for ABA perception (Gonzalez-Guzman et al., 2012). This mutant has impaired ABA-mediated SnRK2s activation, leading to smaller growth and larger stomatal apertures.

1.4.3 CO_2 - signalling pathway

Plants maintain a balance between CO_2 uptake and water loss by regulating stomatal aperture. Stomata close in response to high ambient CO_2 levels. The regulatory network of stomatal movements triggered by increased CO_2 levels is complex, comprising multiple unique components as well as components previously mentioned in the ABA-mediated closure pathway, such as OST1 (Xue et al., 2011), GHR1 (Hõrak et al., 2016; Hua et al., 2012), PYR/RCAR receptors (Merilo et al., 2013) and the slow-type anion channel SLAC1 (Negi et al., 2008; Vahisalu et al., 2008; Zhang et al., 2018).

Other mediators of the CO_2-mediated stomatal movements are the HIGH LEAF TEMPERATURE 1 (HT1) protein kinase (Hashimoto et al., 2006), β-carbonic anhydrases CA1 and CA4 (Engineer et al., 2014; Hu et al., 2010), and the protein kinases MPK12 and MPK4 (Jakobson et al., 2016; Tõldsepp et al., 2018).

The Raf-like protein kinase HT1, expressed in GCs, is a key regulator in CO_2-induced stomatal closing (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016). Through inhibiting OST1, as well as GHR1, HT1 is able to down-regulate the activity of SLAC1 (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). When CO_2 levels are high, HT1 activity is inhibited, allowing for proper stomatal closure. Furthermore, HT1 is inhibited by the MITOGEN-ACTIVATED PROTEIN KINASE4 (MPK4) and MPK12 (Hõrak et al., 2016; Jakobson et al., 2016; Tõldsepp et al., 2018).

The regulation of stomatal aperture by environmental cues is summarised in Figure 5.

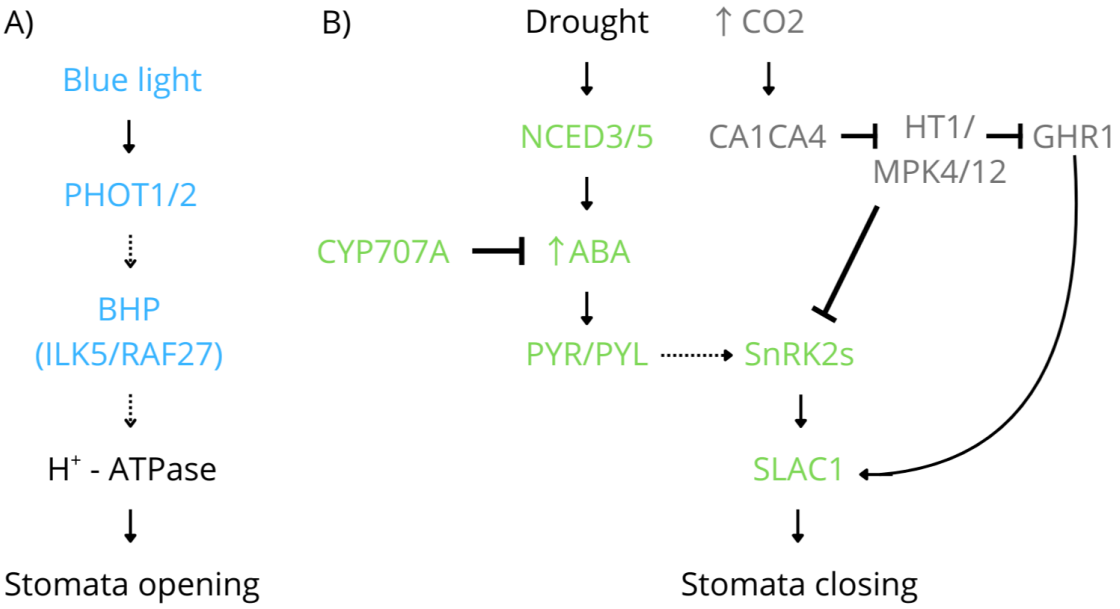

Figure 5. Environmental regulation of stomatal movements. A) Phototropin-mediated stomatal opening. Blue light is captured by the blue light receptors phototropin 1 and 2 (PHOT1/2), their activation triggers a signaling pathway via the Raf-like protein kinase BLUE LIGHT-DEPENDENT H^+-ATPASE PHOSPHORYLATION (BHP), also known as INTEGRIN-LINKED KINASE 5 (ILK5) and RAF27, ultimately leading to the activation of the H^+-ATPase, mediating the influx of H^+ ions and stomatal opening. B) ABA- and CO_2-mediated stomatal closure. Plants experiencing water stress employ 9-cis-epoxycarotenoid dioxygenase (NCED) to catalyse abscisic acid (ABA) biosynthesis. ABA 8′-hydroxylase from the CYP707A family catabolises ABA. ABA is perceived by the PYRABACTIN RESISTANCE/REGULATORY COMPONENTS OF ABA RECEPTOR (PYR) and PYR1/PYR1-LIKE (PYL) receptors. Downstream of PYR/PYL, SnRK2s activate the SLOW ANION CHANNEL 1 (SLAC1) to initiate stomatal closure. Elevated CO_2 levels promote the activity of β-carbonic anhydrases CA1 and CA4 (CA1/CA4). High CO_2 levels via bicarbonate generated by the CA1/CA4 trigger the interaction of MITOGEN-ACTIVATED PROTEIN KINASES 4 and 12 (MPK4/12) with the HIGH LEAF TEMPERATURE 1 (HT1) protein kinase, which is an inhibitor of SnRK2s and the GUARD CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1) kinases, leading to release of inhibition of the kinases and stomatal closure via SLAC1 activation upon elevated CO_2.

1.5 PARAMETERS DESCRIBING STOMATAL PATTERNING

1.5.1 Amphistomaty

The distribution of stomata between upper (adaxial) and lower (abaxial) leaf surfaces is not uniform across different plant species. It is generally thought that most plants are hypostomatous, possessing stomata solely on the abaxial leaf surface, as is the case in most trees and shrubs; whereas *Arabidopsis thaliana*, along with many other herbs and grasses, is amphistomatous, and both sides of the leaf have stomata (Jalakas et al., 2024; Muir, 2015; Salisbury, 1928). Amphistomatous plants have been shown to have increased photosynthesis and a higher stomatal conductance, the measure of the capacity for exchanging CO_2 or water vapour (Beerling & Kelly, 1996; Xiong & Flexas, 2020). A recent study suggests that stomatal patterning is regulated at least partially independently on the adaxial and abaxial leaf surfaces (Jalakas et al., 2024).

Some mutants have been isolated in the host lab that may have different effects on adaxial and abaxial stomatal development. Of these, *spen3* (Mammadzada, 2024), *chr5* and *iqd19* were studied for stomatal development temperature response.

1.5.2 Stomatal density

Stomatal density (SD) is the number of stomata per unit leaf area. Total SD is the sum of the abaxial and adaxial SD. SD and the distribution of stomata are linked to stomatal conductance; SD is used to calculate the theoretical maximum stomatal conductance (Harrison et al., 2020; Ochoa et al., 2024).

1.5.3 Stomatal ratio

The stomatal ratio (SR) is the ratio between adaxial and abaxial SD. This parameter shows whether the plant is hypostomatous, amphistomatous, or, in rare cases, hyperstomatous with stomata only on the adaxial leaf epidermis. Changes in the ratio can hint at the differential patterning regulation between the adaxial and abaxial leaf surfaces (Jalakas et al., 2024).

1.5.4 Stomatal index

The stomatal index (SI) is the proportion of stomata from all epidermal cells, including stomata and pavement cells (PC). It is calculated as (SD/(SD + PCD)). This parameter describes stomatal development patterns and does not depend on leaf size.

2 THE AIMS OF THE THESIS

- Understanding how elevated temperature affects stomatal patterns in leaves.
- Understanding if and how mutations in the stomatal signalling pathways influence the leaf developmental response to high temperature.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Plant lines

Table 1. Pant lines used in this study

Mutants	**T-DNA insertion line name**	**Reference**	**Role/pathway**
Col-0	-	-	control
112458	Q169stop, SALK_054640, GT_2864, SAIL_517_C08, SM3_3493, SAIL_1269_A02	(Gonzalez-Guzman et al., 2012)	ABA receptors
chr5	GK-773A12	isolated in the lab	density mutant
ca1ca4	SALK_106570,WiscDsLox508D11	(Hu et al., 2010)	CO_2
cyp707a1a3	SALK_069127, SALK_101566	(Kushiro et al., 2004; Okamoto et al., 2006b)	ABA catabolism
epf1/2	SALK_137549 and GK_673E01	(Hunt & Gray, 2009)	patterning
ghr1-3	GK_760C07	(Sierla et al., 2018)	ABA, aperture
ht1-2	point mutation leading to partial deletion	(Hashimoto et al., 2006)	CO_2
iqd19-3	SALK_151458C	isolated in the lab	density mutant
mpk12-4	GK_665G12, back-crossed to remove *cas-2* allele	(Jakobson et al., 2016)	CO_2
mpk3	SALK_151594	(Nakagami et al., 2006)	patterning, immunity
MPK3OX	MPK3 overexpression by 35S promoter	seeds provided by M. Brosché	patterning, immunity
mpk6	SALK_073907	(Nakagami et al., 2006)	patterning, immunity
MPK6OX	MPK6 overexpression by 35S promoter	seeds provided by M. Brosché	patterning, immunity
nced3/5	GK-129B08, GK-328D05	(Frey et al., 2012)	ABA, aperture
O276	ES1M5S10350 (EMS mutant)	(Carrère et al., 2024)	patterning
ost1-3	SALK_008068	(Yoshida et al., 2002)	ABA, aperture
phyB	SALK_022035	(Mayfield et al., 2007)	light
raf27-1	WiscDsLox345-348B17	isolated in the lab	light, aperture, immunity
raf27-2	GABI_626D02, back-crossed to remove *mpk12-4* allele	isolated in the lab	light, aperture, immunity
sdd1-1	GK-627D04	(Hara et al., 2007)	patterning
slac1-3	SALK_099139	(Vahisalu et al., 2008)	ABA, aperture
spen3-3	GABI_461F01	(Mammadzada, 2024)	density mutant
tmm	SALK_115723C	(Jalakas et al., 2024)	patterning
triple (*quac1-1slac1-3 slah3-1)*	SALK_099139,GK-371G03,SM_3_38592	(Jalakas et al., 2021)	aperture

3.1.2 Growth conditions

Plants were germinated and grown in growth cabinets (Snijders Microclima Arabidopsis MCA1600-3LP6-E growth cabinets (Snijders Scientific, Tilburg, Netherlands)) at control temperature (CT, see Table 2) for two weeks before half of them were moved to elevated temperature (ET) conditions for the rest of the experiment, to avoid potential selection bias during germination (as per Pérez-Bueno et al., 2022). Other conditions remained the same (see Table 2).

A 2:1 (by volume) mixture of peat (OPM 025 W, Kekkilä Oy, Vantaa, Finland) and vermiculite (Vermikuliit Medium 0–4 mm, TopGreen, Vahi, Estonia) was used. The plants were watered once per week, more often if needed. Nematodes (*S. carpocapsae*; *S. feltiae*, BIOTUS OY, Foressa, Finland) were added as required for pest control. Due to the large number of plants, the experiment was conducted in three batches, planted one week apart. An additional fourth batch was grown after obtaining the results from the previous batches. Each batch contained nine plants of each genotype, including control plants (Col-0).

Table 2. Growth conditions used for this experiment

Growth conditions	**Day**	**Night**
Photoperiod	10 h	14 h
Humidity	60%	80%
CT	23°C	19°C
ET	30°C	26°C
Light intensity at the plant level	~250 µmol $m^{-2}s^{-1}$	Ø

3.1.3 Sample preparation

Leaves were numbered, and the 10th leaf was marked for sampling according to the spiral order of the *Arabidopsis thaliana* growth pattern (Farmer et al., 2013).

Samples were collected during the 7th week for plants grown at ET and the 8th week for plants grown at CT. The 10th leaf was cut along the midvein (Figure 6), and dental silicone was applied (Speedex light body from Coltène/Whaledent AG on the adaxial leaf side and Oranwash L from Zhermack on the abaxial leaf side). When the silicone hardened, clear nail varnish was applied, and the impression was transferred to a microscope slide with clear tape. The area of 0.254 mm^2 from each imprint was recorded with a microscope and camera (Kern OBF 133; ODC 832; Kern & Sohn GmbH) with 200x magnification. SD, SR, and SI were

determined with the ImageJ software (National Institutes of Health, USA; Schneider et al., 2012). The sample size for each genotype was 8 to 9 plants.

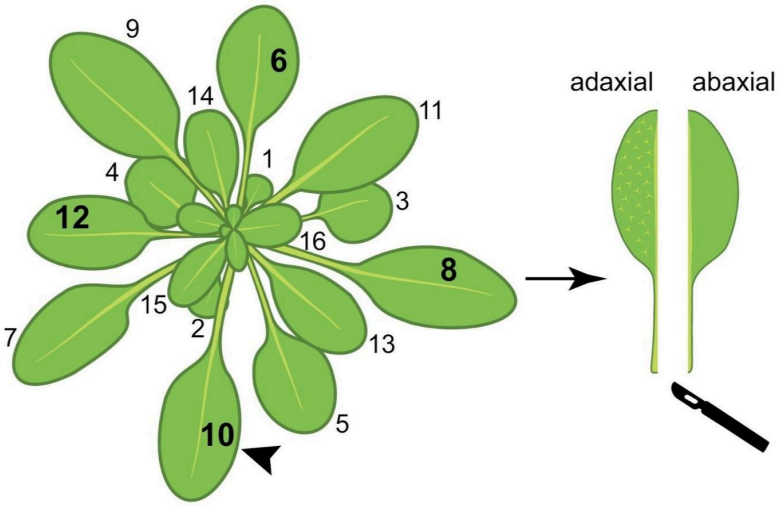

Figure 6. Spiral growth pattern in *Arabidopsis thaliana*. The 10th leaf is indicated with an arrow. The 10th leaf is cut along the midvein to create impressions from both the adaxial (top) and abaxial (bottom) leaf sides. (Modified from Jalakas et al., 2024)

3.1.4 Statistical analysis

The data were analysed using Student's t-test when comparing only two groups and two-way analysis of variance (ANOVA) with a Tukey *post hoc* test for analysing multiple groups done with the STATISTICA 7.0 program. Results were considered significant at p-value < 0.05.

3.2 RESULTS

SD and SI were measured on the adaxial and abaxial leaf surfaces, and total SD and SR were calculated to assess whether ET affects stomatal development. The results are represented as average ± standard error (SE).

First, we pooled data from all experiments and observed the behaviour in our control line (Col-0). Plants grown in ET had significantly lower SD on both leaf surfaces separately and combined (Figure 7. A, B, C; $p < 0.05$; $n = 35\text{-}36$). However, the SR did not change under ET (Figure 7. D; $p = 0.29$).

While ET had no significant changes in SI in Col-0 on the adaxial leaf surface (Figure 8. A), the abaxial side showed an increase in SI (Figure 8. B; $p < 0.05$; $n = 35\text{-}36$).

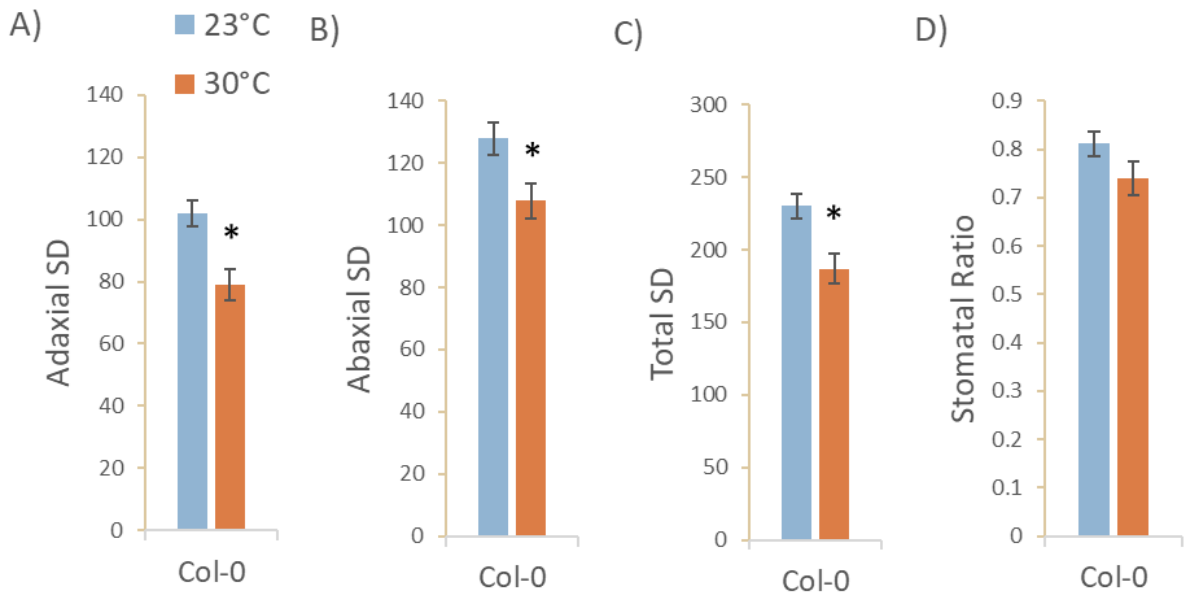

Figure 7. Temperature effects on control plants (Col-0). A) adaxial SD; B) abaxial SD; C) total SD; D) Stomatal ratio. SD overall decreased ($p < 0.05$), while SR did not change significantly (t-test; $p = 0.29$; $n = 35$-36). Significant changes ($p < 0.05$) are indicated with asterisks; data are represented as average ± SE.

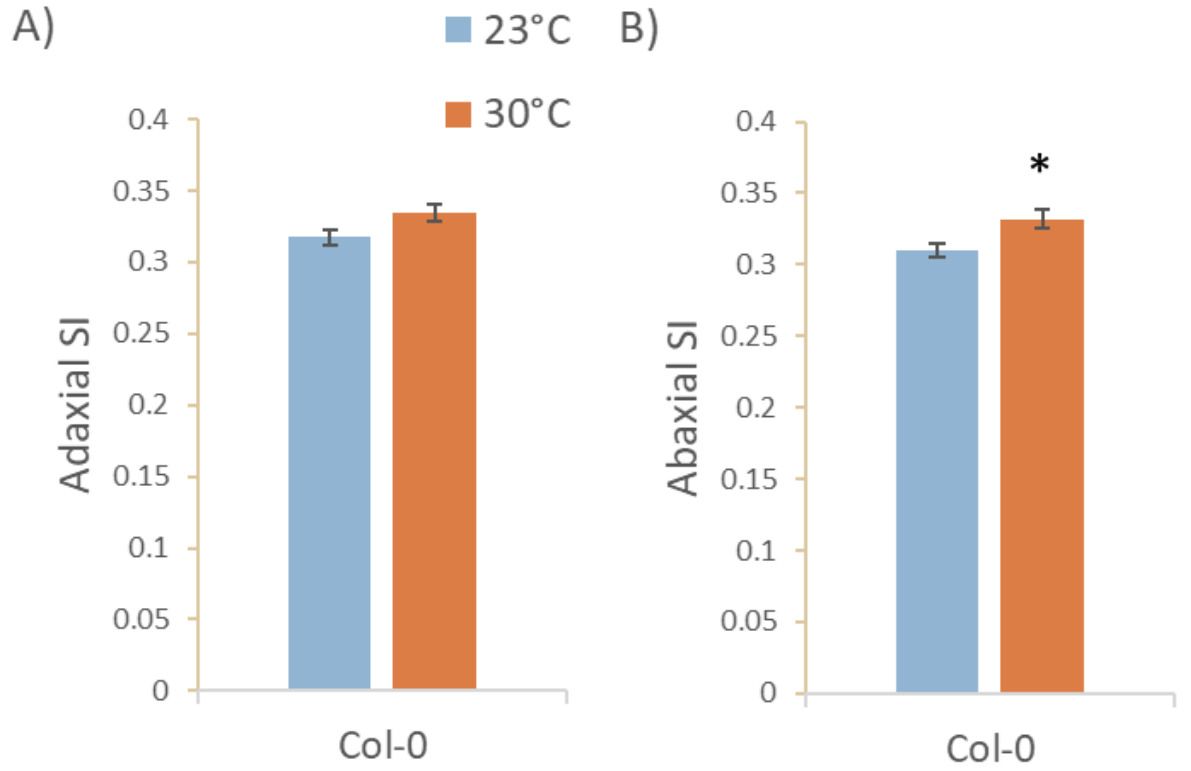

Figure 8. Temperature effects on the stomatal index (SI) in control plants (Col-0). A) adaxial SI; B) abaxial SI. (t-test; $p < 0.05$; $n = 35$-36). Significant changes ($p < 0.05$) are indicated with asterisks; data are represented as average ± SE.

In the first batch, the plant lines used were mutants related to CO_2-mediated regulation of stomatal aperture and light signalling (Figure 9). The most notable genotypes were *raf27-2* and *phyB*. The *raf27-2* had a significant increase in SD on both the adaxial and the abaxial leaf surface and in total SD after growing at ET conditions (Figure 9 A-C; $p < 0.05$; $n = 8$-9). However, there was no significant change in the SR under ET in *raf27-2*. The *phyB* allele showed a strong effect on the abaxial SD, which increased in this mutant under ET (Figure 9. B; $p < 0.05$; $n = 8$-9). The Tukey post hoc test did not reveal any significant changes in the SR in *phyB* under ET (Figure 9. D). There were no significant effects of ET and genotype on SI (Annexe, Figure 2)

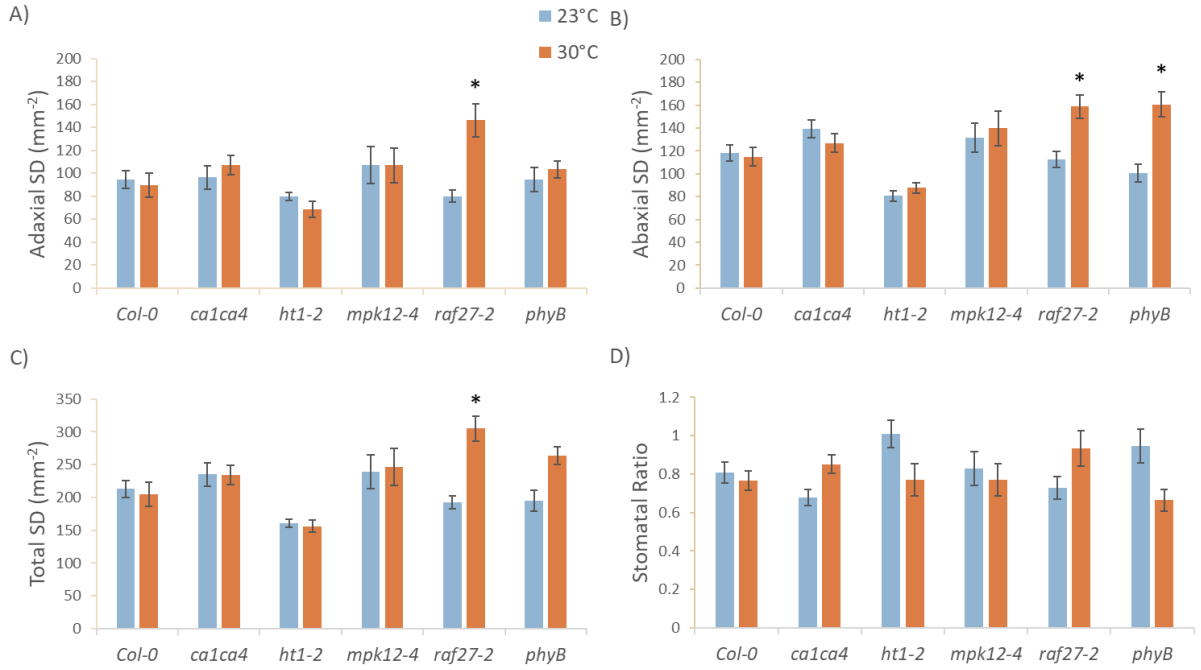

Figure 9. Temperature effects on plants in the first batch. A) adaxial SD; B) abaxial SD; C) total SD; D) stomatal ratio. Statistically significant changes between CT and ET ($p < 0.05$) are indicated with asterisks; data are represented as average ± SE.

The second batch was primarily composed of ABA and stomatal aperture-related mutant lines (Figure 10). While the statistical analyses did not reveal different responses to elevated temperature in different genotypes, a consistent decrease in SD and a slight decrease in SR related to temperature were observed (Figure 10. A-D; two-way ANOVA; significant main effect of temperature). Genotype significantly affected SD, and a *post hoc* test on genotype effects showed that the *112458* mutant line across both leaf surfaces and *nced3/5* on the adaxial surface had a higher SD. There was no significant effect of temperature on SI (Annexe, Figure 3)

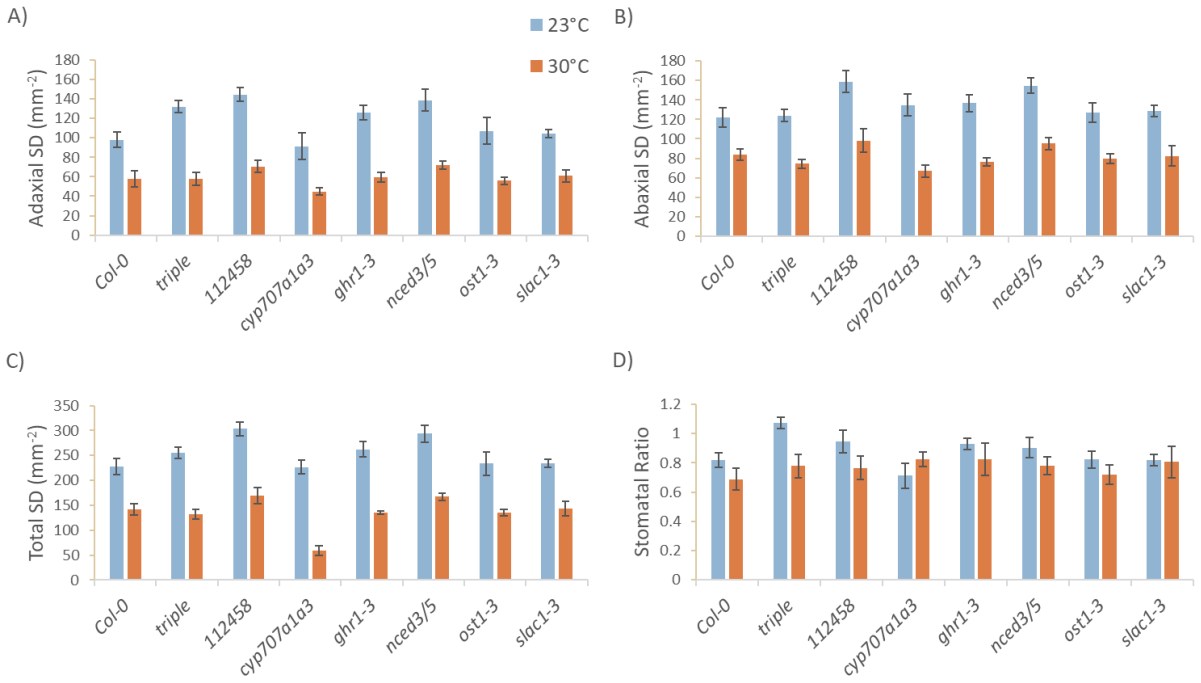

Figure 10. The temperature effect on plants in the second batch. A) adaxial SD; B) abaxial SD; C) total SD; D) SR. Data is represented as average ± SE.

The third batch consisted mostly of mutants with disrupted stomatal patterns and previously observed changes in stomatal density (Figure 11). The high-density mutants *epf1/2*, *O276*, and *sdd1-1* showed a significant drop in SD under elevated temperature on both leaf surfaces, while *tmm* only showed a significant decrease on the abaxial leaf side (Figure 11. A-C; $p < 0.05$; $n = 8$-9). There was no significant effect of elevated temperature on SR (Figure 11. D). Additionally, no effects on SI were seen (Annexe, Figure 4)

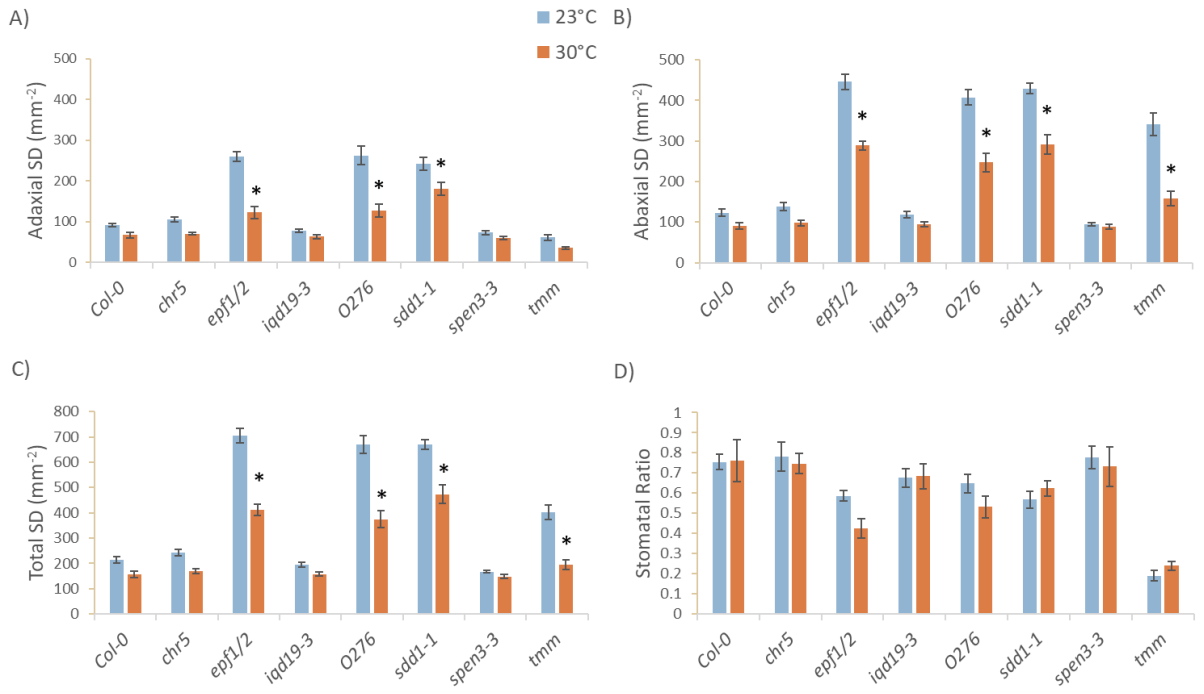

Figure 11. Temperature effects on plants in the third batch. A) adaxial SD; B) abaxial SD; C) total SD; D) SR. Statistically significant changes between CT and ET ($p < 0.05$) are indicated with asterisks. Data is represented as average ± SE.

The last batch was dedicated to MPK3/6 and *raf27* mutants, which are involved in plant immune response and have respective functions in stomatal development and light signalling (Figure 12). Another light-signalling mutant, *phyB*, was also grown. However, the *phyB* plants grown at ET did not grow the necessary number of leaves, only growing leaves 5-8. As leaf size plays a role in stomatal density and distribution, such leaves could not be used. Once again, the interaction of genotype and temperature effects was not significant, but the SD and SR followed a decreasing trend (Figure 12; two-way ANOVA; significant main temperature effect). Genotype affected SD, and the Tukey *post hoc* test revealed lower SD in both *mpk6* and *MPK6OX* mutants on the abaxial leaf surface, while only *mpk6* showed a similar result on the adaxial leaf side, which is also reflected in the total SD. When comparing *raf27-1* and *raf27-2*, there is a striking difference in SD that is unexpected for two mutants of the same gene.

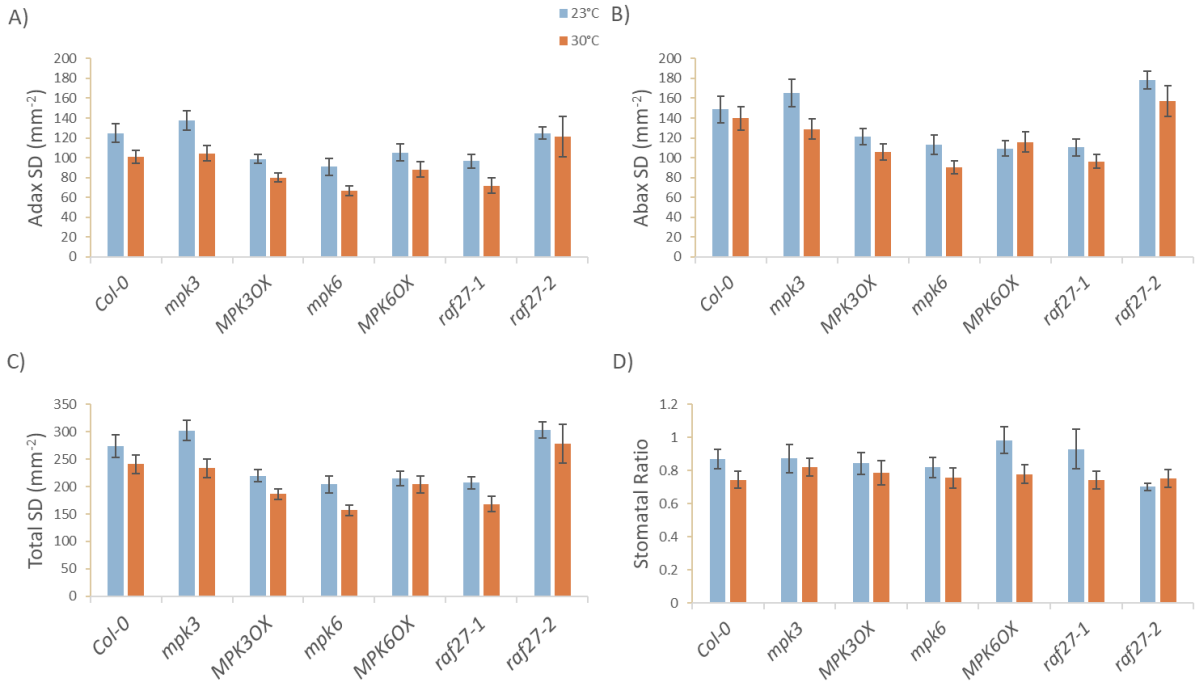

Figure 12. Temperature effects on plants in the fourth batch. A) adaxial SD; B) abaxial SD; C) total SD; D) SR. Data is represented as average ± SE.

Additionally, after analysing the SI for the abaxial and adaxial leaf surfaces, we found that SI in *MPK3OX* and *MPK6OX* increased in response to elevated temperature on the abaxial leaf side but not on the adaxial leaf side (Figure 13; $p < 0.05$, $n = 9$).

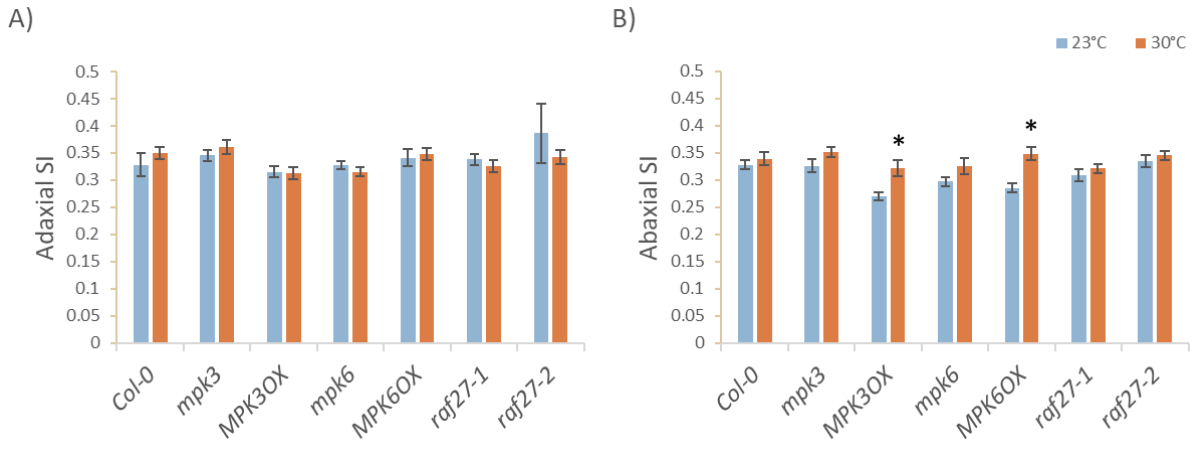

Figure 13. Temperature effects on stomatal index in the fourth batch. A) abaxial SI; B) adaxial SI. Statistically significant changes between CT and ET ($p < 0.05$) are indicated with asterisks. Data is represented as average ± SE.

3.3 DISCUSSION

This study addressed the variations in stomatal patterns of *Arabidopsis thaliana* mutant lines in response to ET. A consistent decrease in SD on both leaf surfaces was observed in most mutant lines and the control line Col-0 (Figure 9-12), as previously shown (Crawford et al., 2012; Vile et al., 2012). Decreased SD under ET could be the result of increased cell expansion which causes stomata to be more dispersed across the leaf surface. Increased cell expansion can be due to elevated auxin levels, which also cause other thermomorphological changes such as hyponasty, as auxin biosynthesis is promoted under ET (Franklin et al., 2011; Legris, 2023). Alternatively, the decrease in SD could be caused by reduced stomatal development in which case SI would be expected to decrease as well. However, SI remained unchanged or increased slightly, suggesting that effects of ET on SD are mostly due to increased cell expansion.

Mutant lines related to the ABA-signalling pathway behaved similar to control plants (Col-0) under ET for all measured parameters, indicating that components of the ABA-signalling pathway are not involved in regulating stomatal development in response to ET (Figure 10).

3.3.1 Effects of elevated temperature on stomatal development in Col-0

The pooled data from all batches of this experiment show a small but significant increase in SI in Col-0 in response to ET, but only on the abaxial leaf surface. This suggests that even though the SD decreases, the plant generally produces more stomata, which might be a mechanism aimed at promoting cooling at ET. Producing relatively more stomata on the abaxial leaf surface might be a strategy to minimise water loss, as the abaxial side is not as exposed to dry air and high light intensities. *Arabidopsis thaliana* has been shown to increase its cooling capacity despite a decrease in SD, both in Col-0 and a low SD mutant *spch-5* (Crawford et al., 2012; Pérez-Bueno et al., 2022). *Arabidopsis thaliana* promotes cooling by developing thinner leaves, elongated petioles, and hyponasty (upward bending), which were all seen in this experiment (Annexe, Figure 1). However, the most used mechanism is transpiration, which is carried out by stomata. Increasing stomatal numbers may thus promote cooling, but may lead to increased water loss.

Moreover, the change in SI in response to ET only on the abaxial epidermis might imply a partially independent coordination of stomatal patterning on the abaxial and adaxial leaf surface. Previous works have recorded different stomatal developmental responses in upper and lower leaf surfaces in mutants related to stomatal patterning and in response to

environmental cues such as relative air humidity, demonstrating differential regulation of abaxial and adaxial stomatal patterns (Jalakas et al., 2024; Tulva et al., 2024, 2025).

3.3.2 Potential differences in growth conditions between growth cabinet shelves

Some mutant lines grown in the first batch examined did not follow the general decreasing trend in SD, namely *ca1ca4, mpk12-4, phyB* and *raf27-2* (Figure 9).

Additionally, Col-0 in the first batch behaved abnormally compared to the other three batches. This is visible in the relatively small decrease of SD on both leaf surfaces in response to higher temperature (Figure 9. A-C) and the plants' appearance (Annexe; Figure 1). This might hint at a difference in the conditions between the shelves of the growth cabinet that was not detected during the initial setup. As the first batch was grown on the top shelf of the growth cabinets, while the second and fourth were grown on the second shelf and the third on the bottom shelf, there is a possibility that the top shelf receives different conditions than the rest of the growth cabinet.

The light-signalling mutant *phyB* showed a significant increase in SD on the abaxial leaf surface in response to ET in the first batch. However, when grown in the fourth batch, it did not achieve the number of leaves necessary for reliable data, most plants growing only between 5-8 leaves. The difference in growing conditions is evident as the plants grown in the first batch managed to consistently grow over 10 leaves. The *phyB* is a red-light receptor known as a temperature sensor in *Arabidopsis* (Figure 4; Hernando et al., 2021; Jung et al., 2016) and is likely to affect stomatal development under ET. For these reasons it should be studied further.

The mutant line *raf27-2,* grown in both the first and the fourth batch, shows perhaps the most drastic difference, exhibiting an increase in the first batch (Figure 9) and a decrease in the fourth (Figure 12). The difference is also apparent in the appearance of the plants, with the plants of the first batch being bigger and greener than the plants of the other batches (Annexe, Figure 1). The inconsistency in conditions could be related to light scattering in a dissimilar fashion across the shelves, as well as fluctuations in air temperature caused by the construction of the cabinet.

The inconsistent growth across the shelves of the grow cabinets were also noted by other members of the host lab with plants grown on the top shelves growing larger and greener than in the rest of the cabinet (P. Jalakas, H. Hõrak, personal communication, April 2025).

3.3.3 O276 and stomatal density mutant lines

The *O276* mutant is a line from the homozygous EMS-mutagenised (HEM) line collection under the HEM name ES1M5S10350 (Carrère et al., 2024). In this experiment, *O276* displayed significant changes in SDs on both leaf surfaces (Figure 11). This line possesses a high-impact mutation at the AT3G01140 locus in chromosome 3 (Carrère et al., 2024; *Locus: AT3G01140*, https://lipm-browsers.toulouse.inra.fr/pub/ATHEM/), in the *MYB106* gene, which codes for a protein involved in the regulation of trichome branch formation and flowering time (Hong et al., 2021; Jakoby et al., 2008). It is also expressed in the guard cells during the MUTE stage of stomatal development, among other plant tissues (Adrian et al., 2015; *Locus: AT3G01140*; The Arabidopsis Information Resource (TAIR), www.arabidopsis.org). Mutation in the *AT3G01140* locus thus might be responsible for the increased SD of *O276*. Analyzing other mutant lines with mutations in this gene could clarify whether this is truly the case.

Other notable SD mutant lines were *epf1/2, sdd* and *tmm*. Under ET, both *epf1/2* and *sdd* showed a significant decrease in SD on both leaf surfaces, and *tmm* on the abaxial leaf epidermis compared to plants grown at CT (Figure 11). In relation to Col-0, they all showed increased SD. An increase in the SD in these mutant lines was already shown in previous works, where the SD in both *epf1/2* and *sdd1* increased in both adaxial and abaxial surfaces, while the SD in *tmm* increased only in the abaxial leaf surface (Jalakas et al., 2024; Vráblová et al., 2017).

3.3.4 Changes in SI in MPK3/6 overexpression lines

The mutant lines *MPK3OX* and *MPK6OX* showed an increase in the SI on the abaxial leaf surface in response to ET (Figure 13. B), which is directly related to stomatal developmental patterns and mirrors the response of Col-0 but appears stronger. This might suggest MPK3/6 are directly involved in the mechanism of stomatal development response to ET. The changes in SI were only observed on the abaxial leaf surface; this might hint at the role of MPK3/6 in the independent regulation of stomatal patterning in the adaxial and abaxial leaf surfaces. The differential regulation of stomatal patterning in the abaxial and adaxial surfaces was shown before (Jalakas et al., 2024; Tulva et al., 2025), and it would be worth investigating the roles of MPK3/6 in these processes.

Additionally, as MPK3/6 are the central regulatory point in stomatal development as as well as in plant immunity (Mao et al., 2011; Meng et al., 2013; Wang et al., 2007; Xu et al., 2016),

the involvement of other components of immune signalling in the stomatal development response to ET could be studied in the future by addressing other immune signalling mutants.

Another interesting detail is that, contrary to expectations, the mutant line *mpk6*, which, similarly to *mpk3*, was expected to achieve higher SD than Col-0, as the double mutant produces numerous stomata (Wang et al., 2007), showed lower densities on both leaf sides (Figure 12. A-C). The lower SD compared to both Col-0 and *mpk3* in *mpk6* might indicate the redundancy of the *MPK3/6* genes, which might make studies of the loss-of-function difficult, as the single mutants likely have no phenotype, and the double mutants are lethal (Wang et al., 2007). Addressing the function of these key regulators of stomatal development should be done in other ways, such as expressing constitutively active versions of the kinases in the mutant background.

3.3.5 The *raf27-2* mutants

The mutant line *raf27-2* is worth further exploration as it displayed interesting patterns in both the first batch and the fourth batch (Figure 9 and Figure 12). The inverted response to ET in the first batch, showing an increased SD in ET, and the large difference between the SDs between *raf27-1* and *raf27-2* are both interesting and unexpected results. While the response in the first batch could point to the potential role played by RAF27 in the developmental response to higher temperature, the differences recorded in the fourth batch might indicate an unknown background mutation within this plant line that is affecting the response to ET instead of the *raf27* mutation.

RAF27, also known as ILK5, is a protein kinase involved in plant immunity (Kim et al., 2023). It acts as one of the activators of the MPK phosphorylation cascade, acting upstream of MPK3/6, key MPKs involved in both plant innate immunity (Mao et al., 2011; Meng et al., 2013; Xu et al., 2016) and stomatal development (Wang et al., 2007). As an increase in SI in the MPK3/6 overexpressor lines could be seen as a sign of the involvement of MPK3/6 in the mechanism affecting stomatal development under ET, the potential interaction of RAF27 and MPK3/6 in the response to ET is worth further study.

SUMMARY

With global temperatures rising, the need to understand how plants adapt to these changing conditions is growing. Stomata, mediators of gas exchange, are some of the plant's most essential structures. Balancing the intake of CO_2 necessary for photosynthesis with water loss, these small pores respond to environmental cues by modifying their numbers and distribution. This study focused on understanding stomatal patterning under elevated temperature (ET) and aimed at identifying potential actors in coordinating stomatal patterning in response to ET.

The leaf epidermis of plant lines used in this work generally displayed decreased stomatal density (SD) on both adaxial and abaxial surfaces in response to ET, mostly without impact on stomatal ratio. While stomatal index (SI) mainly remained unchanged, in the rare cases that SI changed under ET, it only appeared on the abaxial leaf surface, showing that the abaxial and adaxial stomatal patterns are at least partially independently regulated. To deepen our understanding of the responses of plants to changing environmental conditions, it would be highly relevant to address both abaxial and adaxial stomatal development in future studies.

Furthermore, the small yet significant increase in SI in Col-0 grown under ET, but only on the abaxial leaf surface, could hint at a possible mechanism for heat dissipation. The overexpression mutants *MPK3OX* and *MPK6OX* also showed an increase in SI under ET conditions, which points to the potential roles of *MPK3* and *MPK6* in the developmental response to ET and the independent coordination of stomatal patterns in the abaxial and adaxial leaf surfaces. As MPK3/6 are also involved in plant immune signalling, investigating the response of other components of immune signalling to ET would be worth further study.

Another mutant line, *raf27-2*, which initially exhibited an increase in SD under ET, did not show the same response in the repeat experiment. This might be due to dissimilar growth conditions in different shelves of the growth cabinets. Further investigation of the *raf27-2* mutant line in future studies is necessary for understanding the true role of RAF27 in the stomatal development temperature response. RAF27, also known as ILK5, is another element of plant innate immunity possibly related to MPK3/6 and might play a potential role in the stomatal development response to ET.

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ANNEX

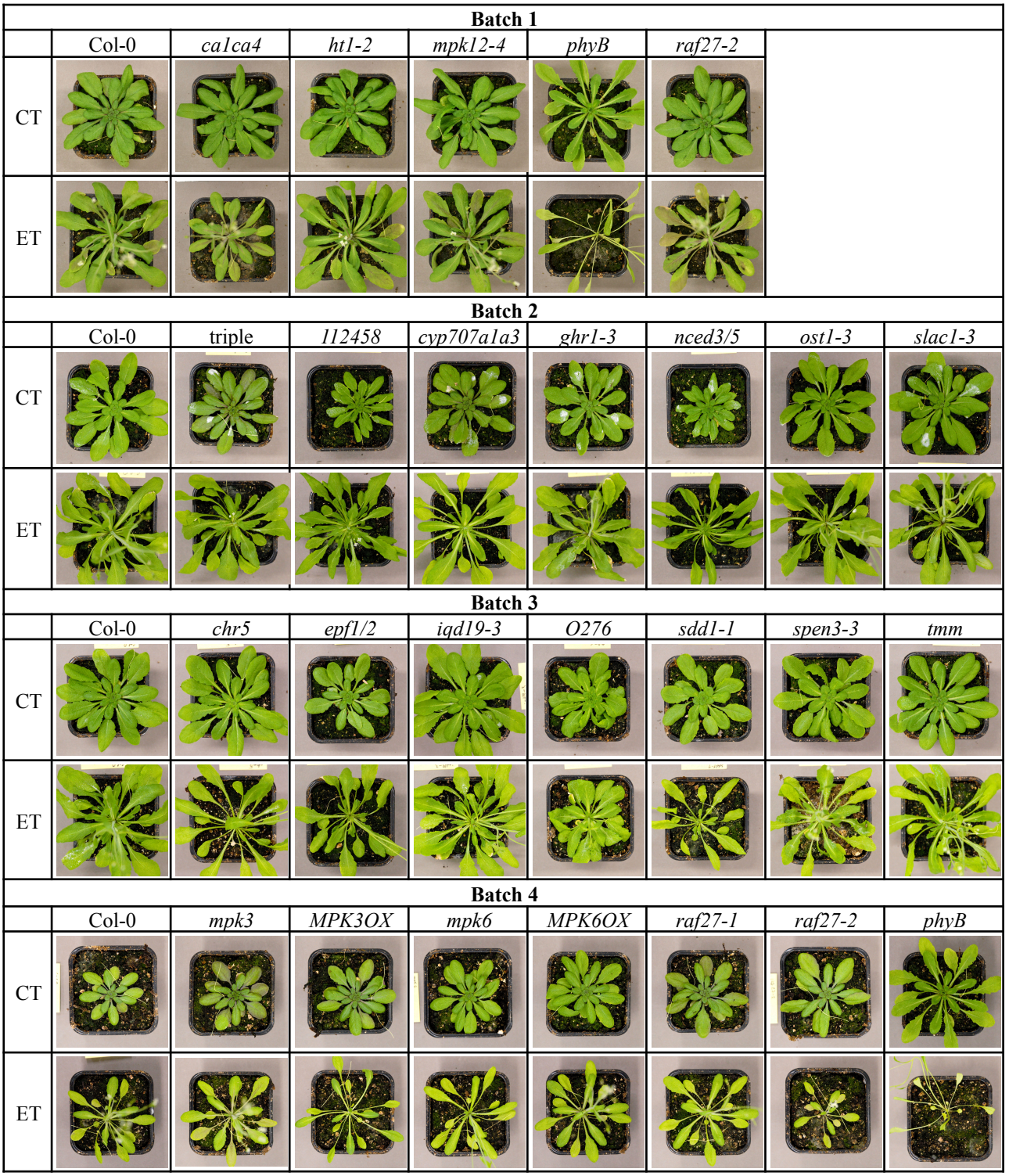

Figure 1. Representative images of plants in this study at week five.

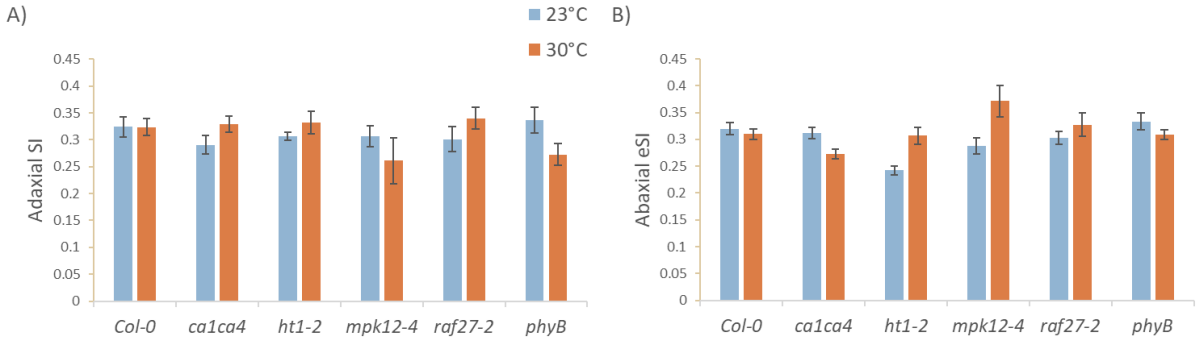

Figure 2. Temperature effects on stomatal index in the first batch. A) adaxial SI; B) abaxial SI. Data is represented as average ± SE

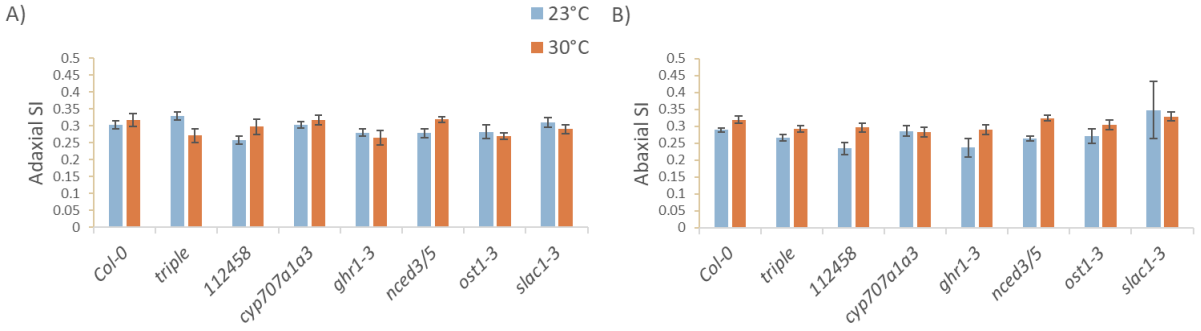

Figure 3. Temperature effects on stomatal index in the second batch. A) adaxial SI; B) abaxial SI. Data is represented as average ± SE

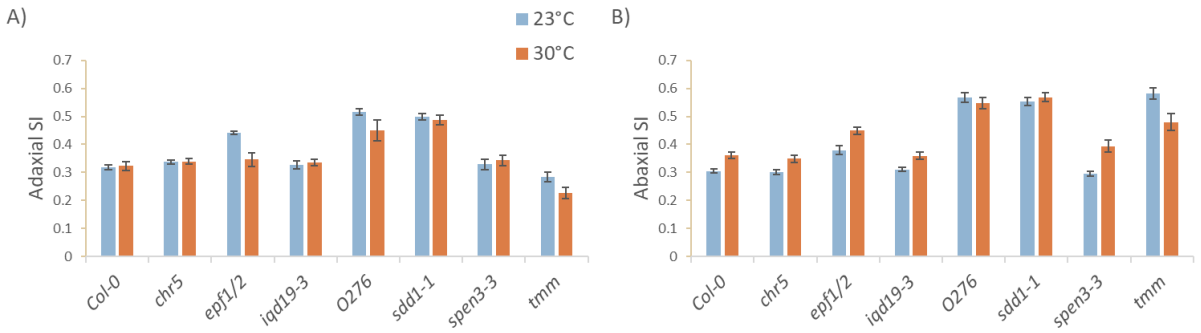

Figure 4. Temperature effects on stomatal index in the third batch. A) adaxial SI; B) abaxial SI. Data is represented as average ± SE

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