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The effect of loss-of-function of adaxially or abaxially enriched genes on stomatal patterning in *Arabidopsis thaliana*

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ABSTRACT

Stomatal pores regulate CO_2 uptake for photosynthesis and water loss via transpiration. In amphistomatous species such as *Arabidopsis thaliana*, stomata occur on both lower and upper leaf surfaces, yet the mechanisms that control their distribution between leaf surfaces and potential surface-specific functions remain unclear. Here, I analysed stomatal patterning and conductance in loss-of-function mutants of genes enriched in the stomatal lineage cells and preferentially expressed in specific leaf surfaces. Most mutants did not differ from wild type plants in stomatal density or stomatal ratio. However, stomatal index was increased in *el19y-2*, *prx34*, and *el21x-1* on the abaxial surface, and in *el19y-2* on the adaxial surface too, indicating effects of respective genes on stomatal lineage progression. In a subsequent experiment the *scrm* mutant showed reduced stomatal density on both leaf surfaces. Despite this, *scrm* exhibited increased adaxial stomatal conductance and higher conductance per individual stoma on both leaf surfaces, suggesting more open stomata. These results show that the studied genes have limited effects on stomatal distribution, but some of them can influence stomatal development. This work supports partially independent regulation of stomatal traits between leaf surfaces.

Keywords

Stomata, adaxial, abaxial, stomatal patterning, stomatal density, stomatal ratio, stomatal conductance, Arabidopsis, stomatal index

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Õhulõhede mustrid lehe ühel küljel avalduvate geenide funktsiooni häirega hariliku müürlooga (*Arabidopsis thaliana*) mutantides

Lühikokkuvõte

Õhulõhed reguleerivad taimede süsihappegaasi omastamist ja veekadu. Amfistomaatsetes taimeliikides, sh mudeltaim harilik müürlook (*Arabidopsis thaliana*), esinevad õhulõhed nii

lehtede ülemisel kui alumisel küljel. Tänini pole selge, kuidas reguleeritakse taimedes õhulõhede jaotumist lehepindade vahel ja millised on õhulõhede funktsioonid lehe eri külgedel. Selles töös uurisin õhulõhede paiknemise mustreid ja õhulõhede juhtivust taimeliinides, kus oli häiritud mõne geeni funktsioon, mis eelistatult avaldub vaid ühel lehe küljel ja on kirjanduse andmeil aktiivne ka õhulõhede arenguliinis. Enamikul uuritud mutantidel oli metsiktüüpi taimedele sarnane õhulõhede tihedus ja lehepindade vaheline jaotus. Lehe alumise pinna õhulõhede indeks oli metsiktüübist oluliselt kõrgem *el19y-2*, *prx34* ja *el21x-1* mutantides ja ülemise pinna õhulõhede indeks oli metsiktüübist kõrgem vaid *el19y-2* taimedes, viidates vastavate geenide rollile õhulõhede arengus. Lisaks selgus, et *scrm* mutandil oli õhulõhede tihedus metsiktüüpi taimedest väiksem mõlemal lehel küljel, kuid sellest hoolimata oli lehe ülakülje õhulõhede juhtivus nii lehepinna ühiku kui õhulõhe kohta *scrm* mutantides suurem kui metsiktüüpi taimedes, mis viitab selle taimeliini avatumatele õhulõhedele. Töö tulemused näitasid, et uuritud geenidel on õhulõhede lehepindade vahelisele jaotusele piiratud mõju, kuid mõned uuritud geenid võivad mõjutada õhulõhede arengut. Töö toetab hüpoteesi, et õhulõhetunnused on lehe ülemisel ja alumisel küljel osaliselt sõltumatult reguleeritud.

Võtmesõnad:

õhulõhed, adaksiaalne, abaksiaalne, õhulõhede mustrid, õhulõhede tihedus, õhulõhede tiheduste suhe, õhulõhede juhtivus, harilik müürlook, õhulõhede indeks

CERCS: B225, Taimegeneetika

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ABBREVIATIONS

Abax - abaxial (lower in case of leaf surface)

ACO4 - ethylene forming enzyme

Adax - adaxial (upper in case of leaf surface)

bHLH - basic helix-loop-helix

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

EL19Y - ribosomal protein L19e family protein

EL21X - ribosomal protein EL21X

EPF1 - EPIDERMAL PATTERNING FACTOR 1

EPF2 - EPIDERMAL PATTERNING FACTOR 2

EPFL8 - EPIDERMAL PATTERNING FACTOR-LIKE 8

EPFL9/STOMAGEN - EPIDERMAL PATTERNING FACTOR-LIKE 9

EPPs - EPF-PROCESSING PROTEINASES

ER - ERECTA receptor-like kinase

ERf - ERECTA family

ERL1 - ERECTA-LIKE 1

ERL2 - ERECTA-LIKE 2

FAMA - basic helix-loop-helix transcription factor

GCs - guard cells

GMC - guard mother cell

ICE1 - INDUCER OF CBF EXPRESSION 1, basic helix-loop-helix transcription factor

YDA - MAPK kinase kinase YODA

KMD3 - KISS ME DEADLY 3, F-box protein

LRR-RLKs - leucine-rich repeat receptor-like kinases

MAPK - mitogen-activated protein kinase

MKK4 - MAPK kinase 4

MKK5 - MAPK kinase 5

MMC - meristemoid mother cell

MPK3 - MAPK 3

MPK6 - MAPK 6

MUTE - basic helix-loop-helix transcription factor

NPF - nitrate and peptide transporter family

NPF2.10 - nitrate peptide transporter family glucosinolate-specific transporter protein

PD - stomatal precursor cell density

POX - class III plant peroxidase

PRX34 - PEROXIDASE 34

PVD - pavement cell density

ROS - reactive oxygen species

SCRM - SCREAM, basic helix-loop-helix transcription factor

SCRM2 - SCREAM 2, basic helix-loop-helix transcription factor

SD - stomatal density

SEM - standard error of the mean

SI - stomatal index

SLGC - stomatal lineage ground cell

SR - stomatal ratio

TMM - TOO MANY MOUTHS, receptor protein

TUA6 - tubulin alpha-6

WT - wild type

INTRODUCTION

Plants are fundamental to life on Earth, forming the basis of most ecosystems and sustaining global biogeochemical cycles. Through photosynthesis, plants convert atmospheric CO_2 into organic carbon while releasing oxygen, thereby supporting both food webs and atmospheric regulation (Furbank et al., 2023; Kambach et al., 2024). In addition to their ecological importance, plants are indispensable to agriculture and global food security, making the understanding of their growth, development, and responses to environmental conditions highly relevant (Chen et al., 2024; Long et al., 2025).

A key interface between plants and their environment is the leaf epidermis, where stomata regulate the exchange of gases between the plant and the atmosphere. Stomata enable CO_2 uptake for photosynthesis while controlling water loss through transpiration, thereby playing a central role in balancing carbon gain with water-use efficiency (Lawson & Matthews, 2020). Their ability to rapidly respond to environmental and endogenous signals makes them critical for plant performance under changing conditions (Kollist et al., 2014).

Stomatal development is a tightly regulated process that ensures proper formation, spacing, and distribution of stomata within the epidermis. In Arabidopsis thaliana, this process is controlled by a well-characterized genetic network involving master regulatory transcription factors and intercellular signalling pathways, which coordinate the initiation, proliferation, and differentiation of stomatal lineage cells (Kanaoka et al., 2008; MacAlister et al., 2007; Ohashi-Ito & Bergmann, 2006; Pillitteri et al., 2007).

An additional layer of complexity in stomatal anatomy and physiology arises from differences between the two leaf surfaces, which can vary in stomatal number, development, and function. While most species are hypostomatous, the model plant Arabidopsis thaliana is amphistomatous, producing stomata on both leaf surfaces (Hõrak, 2026; Muir, 2015). Increasing evidence suggests that stomatal development is at least partly independently regulated between these surfaces, as genetic and environmental factors can differentially influence stomatal traits (Hronková et al., 2015; Jalakas et al., 2024; Tulva et al., 2024, 2025). However, most studies have focused on the lower leaf surface, leaving the upper surface stomatal development less well understood (Hõrak, 2026).

1. LITERATURE REVIEW

1.1. Stomata

Stomata are microscopic pores each formed by a pair of guard cells (GCs). They are found in the epidermis on the aerial parts of most plants. Stomata have been recorded in the fossil record as far back as 400 million years (Edwards et al., 1998). Gas exchange occurs almost entirely through stomata, as the leaf surface is covered by a waxy cuticle, which has very low permeability for gas (González-Valenzuela et al., 2023). In fact, 95% of all gas exchange occurs through stomata, while only 0.3-0.5% of leaf surface is typically occupied by stomata (Lawson & Matthews, 2020). Plants can actively adjust the turgor pressure of guard cells, thus adjusting the shape of the guard cells, which results in the opening and closing of the stomatal pore. The openness of stomata is called stomatal aperture. By changing the stomatal aperture, the plant is able to moderate gas exchange rate between the atmosphere and the internal leaf space (Kollist et al., 2014). The increase of guard cell turgor pressure results in increased stomatal aperture, leading to elevated rate of CO_2 uptake and elevated water loss through transpiration. Conversely, the reduction of turgor pressure in GCs will result in the decrease of stomatal aperture, leading to reduced CO_2 assimilation and water loss. Stomatal conductance refers to the conductance of the stomatal pore to water vapour, and is a determinant of the CO_2 uptake capacity of the plant due to the coupled nature of gas exchange through stomata (Lawson & Matthews, 2020). The opening and closing of stomata occurs rapidly in response to environmental conditions. Stomatal opening is promoted by increased light intensity, while darkness, elevated CO_2 levels, dry air, and low soil water content trigger closure. This rapid responsiveness to environmental conditions is critical for plant stress tolerance, growth, and yield (Lawson & Matthews, 2020). In addition to responding to external environmental cues, stomata also respond to endogenous signals, such as phytohormones, nitric oxide, hydrogen peroxide and other metabolites (Lawson & Matthews, 2020). Transpiration not only serves to maintain optimal leaf temperature via evaporative cooling (Urban et al., 2017), but also drives the xylem stream required for nutrient uptake from the soil (Hopmans & Bristow, 2002), however open stomata also heighten plant susceptibility to abiotic and biotic stresses by increasing water loss and providing entry sites for microorganisms (Melotto et al., 2006).

1.2. The stomatal lineage

The dicotyledonous plant Arabidopsis (*Arabidopsis thaliana*) produces stomata through a series of cell divisions employing various mechanisms determining cell fate (Fig. 1). Cell divisions begin with undifferentiated epidermal cells called protodermal cells. These cells enter the stomatal lineage by obtaining the meristemoid mother cell (MMC) identity. This is followed by an asymmetric division of the MMC producing a meristemoid cell and a larger sister cell - stomatal lineage ground cell (SLGC) (Fig. 1). The meristemoid cell can then differentiate into a guard mother cell (GMC) or do additional rounds of asymmetric amplification divisions in order to produce more SLGCs and renew itself. SLGCs perform spacing divisions, which produce a satellite meristemoid cell or differentiate into a pavement cell. Lastly, GMC undergoes a symmetric division leading to a pair of guard cells (GCs) ultimately forming the stoma (Fig. 1) (Endo & Torii, 2019; Geisler et al., 2000).

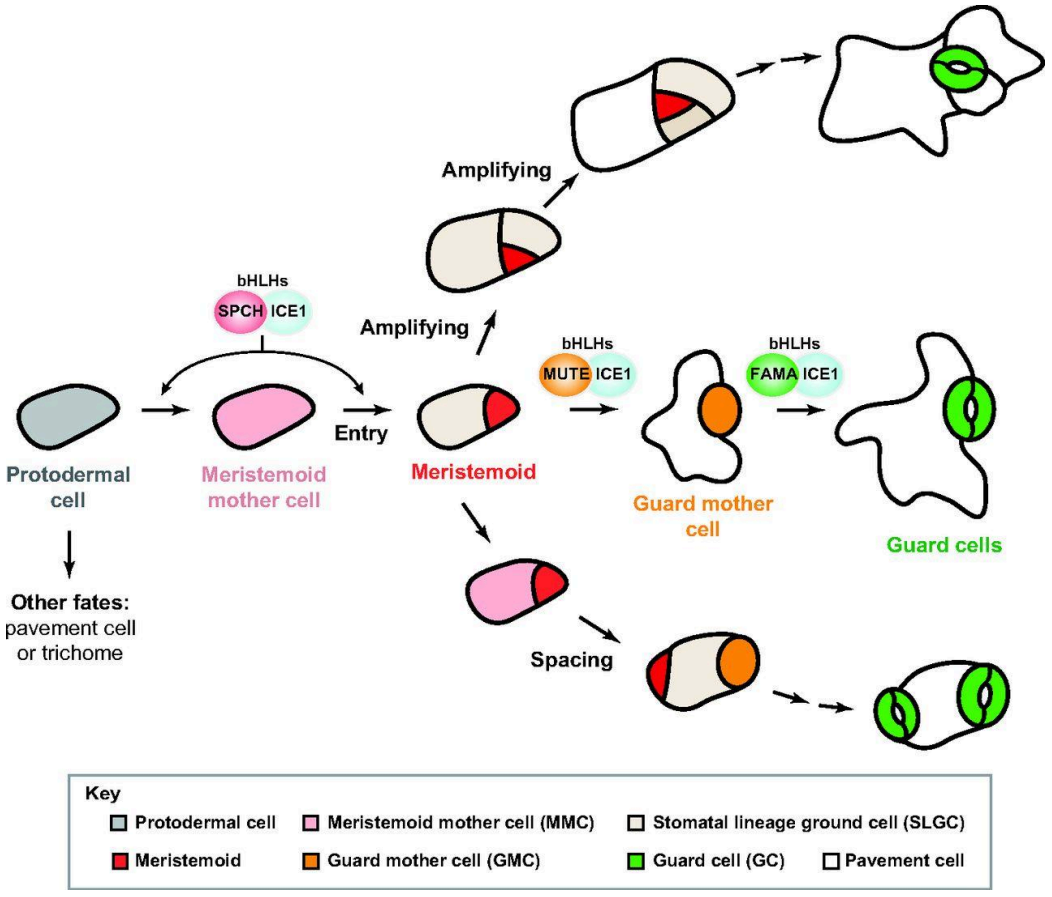

Fig. 1. Cell fate transitions and divisions during Arabidopsis stomatal development. Master regulator basic helix-loop-helix (bHLH) transcription factors SPEECHLESS (SPCH), MUTE and FAMA form heterodimers with partner bHLH proteins ICE1 (INDUCER OF CBF EXPRESSION 1 (ICE1) and SCREAM 2 (SCRM2; not shown), facilitating series of cell

divisions and formation of mature stomata. SPCH initiates the stomatal lineage by assigning the protodermal cell the meristemoid mother cell identity. SPCH further facilitates entry division forming meristemoid cell and stomatal lineage ground cell (SLGC). MUTE facilitates differentiation of meristemoids into guard mother cells (GMC), and FAMA completes GMC differentiation into a pair of guard cells (GCs). All aforementioned transcription factors are involved in spacing and amplifying divisions, but not shown for simplicity. Schematic from (Lau & Bergmann, 2012).

1.3. Main regulatory mechanisms of stomatal development

The stomatal lineage cell divisions in Arabidopsis are governed by the activation of master regulator basic helix-loop-helix (bHLH) transcription factors SPEECHLESS (SPCH), MUTE and FAMA (Fig. 1) (MacAlister et al., 2007; Ohashi-Ito & Bergmann, 2006; Pillitteri et al., 2007). Each transcription factor forms a heterodimer with partner bHLH proteins, SCREAM (SCRM; also known as INDUCER OF CBF EXPRESSION 1 (ICE1)) or SCREAM2 (SCRM2) (Kanaoka et al., 2008). SPCH is responsible for specifying the initiation of stomatal lineage cells - protodermal cells obtain MMC identity and subsequent entry into asymmetric division is facilitated (Fig. 1), making a meristemoid cell and stomatal lineage ground cell. SPCH additionally stimulates the amplifying asymmetric divisions (MacAlister et al., 2007). MUTE terminates the asymmetric division of meristemoid cells and facilitates their differentiation into a GMC (Pillitteri et al., 2007). Finally, FAMA triggers the division of GMCs and facilitates their differentiation into GCs (Fig. 1), and ensures no further divisions occur (Ohashi-Ito & Bergmann, 2006). Mutant phenotypes and expression patterns are congruent with these roles. SPCH is expressed only in MMCs and meristemoids. The loss-of-function mutant *spch* fails to initiate the stomatal lineage cells and no stomatal precursor cells are formed. This results in a phenotype where the epidermis consists exclusively of pavement cells (MacAlister et al., 2007). Similarly, the loss-of-function *mute* mutant also fails to develop any stomata, however meristemoid cells continue dividing, forming clusters of unequally divided cells (Pillitteri et al., 2007). Conversely, the overexpression of MUTE results in an epidermis completely packed with stomata. MUTE is unable to produce GCs without FAMA, however it is able to bypass the need for SPCH (MacAlister et al., 2007). FAMA loss-of-function mutant continues GMC divisions, but fails to differentiate the GCs, resulting in cell tumors (Ohashi-Ito & Bergmann, 2006).

SCRM and SCRM2 act as core partners of SPCH, MUTE, and FAMA to regulate stomatal development in Arabidopsis (Fig. 1). The semidominant mutant *scrm*-D drives constitutive stomatal differentiation: heterozygotes show increased divisions and stomatal clustering, while homozygotes form an epidermis composed almost entirely of stomata (Kanaoka et al., 2008). However, in *scrm*-D *mute* and *scrm*-D *fama* backgrounds, the epidermis accumulates large numbers of meristemoids and GMC-like cells, respectively, amplifying the corresponding mutant phenotypes. This indicates that MUTE and FAMA are required for progression to mature stomata, even when *scrm*-D is constitutively active. Genetic interactions with *spch* show a dosage-dependent effect on the *scrm*-D phenotype. Loss-of-function analyses reveal partial redundancy between SCRM and SCRM2. Single mutants show weak phenotypes, whereas the *scrm-2 scrm2-1* double mutant fails to initiate the stomatal lineage and produces only pavement cells. Heterozygotes indicate that both *SCRM* and *SCRM2* genes are required for the meristemoid-to-GMC transition, while SCRM also contributes to proper GMC division and guard cell differentiation (Kanaoka et al., 2008).

1.4. Stomatal patterning

The patterning of stomata is crucial for optimal gas exchange, and stomata are typically separated by a single non-stomatal epidermal cell. This phenomenon is known as the one-cell-spacing rule (Geisler et al., 2000). The number and placement of stomata within the epidermis are mainly regulated by cell-to-cell signalling and are in part determined by the specific asymmetric and symmetric cell divisions that occur throughout the stomatal lineage (L. R. Lee & Bergmann, 2019). The main differentiation pathway in this lineage is controlled by intercellular communication mediated by secreted peptides of the EPIDERMAL PATTERNING FACTOR (EPF) family (Fig. 2). EPF1 and EPF2 act as negative regulators of stomatal formation, but they function at distinct stages of the stomatal lineage (Hunt & Gray, 2009). EPF2 is expressed in MMCs, meristemoids, and early stomatal lineage precursor cells, and is detected earlier than EPF1, which is expressed in late meristemoids and GMCs. EPF2 primarily restricts stomatal entry divisions, whereas EPF1 reinforces the one-cell-spacing rule by preventing neighbouring cells from adopting stomatal fate (Hara et al., 2007, 2009; Hunt & Gray, 2009). In contrast, the EPIDERMAL PATTERNING FACTOR-LIKE 9 (EPFL9)/STOMAGEN (Fig. 2) peptide promotes stomatal lineage proliferation via competitive binding to the same receptors as EPF1/EPF2. EPFL9 is expressed in immature mesophyll cells and positively regulates stomatal development (Hunt et al., 2010; J. S. Lee et

al., 2015). For EPF proteins to become biologically active, proteolytic processing of their propeptides is required. This processing is facilitated by EPPs (EPF-PROCESSING PROTEINASES)(Fig. 2), a group of subtilisin-like serine proteinases (Meng et al., 2026).

When EPF1 and EPF2 signalling is high, stomatal production in the leaf epidermis is reduced or completely inhibited, whereas elevated EPFL9 signalling promotes increased stomatal production and can lead to stomatal clustering (Hara et al., 2007; Hunt & Gray, 2009; Sugano et al., 2010). Conversely, loss-of-function mutants *epf1* and *epf2* lead to distinct stomatal developmental defects in *Arabidopsis*. The *epf2* mutants show increased stomatal density due to excessive entry of epidermal cells into the stomatal lineage, whereas *epf1* mutants display frequent stomatal clustering as the normal one-cell-spacing rule is violated (Hara et al., 2007; Hunt & Gray, 2009). Loss-of-function of *EPFL9* results in reduced stomatal density on the leaf epidermis, reflecting its role as a positive regulator of stomatal development (Hunt et al., 2010). Overall, these studies underscore that stomatal density and spacing are governed by a delicate balance of inhibitory and promotive peptide signals, with EPF1/EPF2 restricting and EPFL9 enhancing stomatal development. These peptides are perceived by leucine-rich repeat receptor-like kinases (LRR-RLKs) of the ERECTA (ERf) family, comprising ERECTA (ER), ERECTA-LIKE1 (ERL1), and ERL2 (Shpak et al., 2005), which function together with the receptor-like protein TOO MANY MOUTHS (TMM) (Lin et al., 2017; Yang & Sack, 1995).

Downstream of EPF–ERECTA signalling, intracellular signalling cascades further regulate stomatal development (Fig. 2). In particular, a mitogen-activated protein kinase (MAPK) cascade acts downstream of EPF–ERf signalling. This cascade consists of the MAPK kinase kinase YODA (YDA), MAPK kinases 4 and 5 (MKK4 and MKK5), and MAPKs 3 and 6 (MPK3 and MPK6) (Wang et al., 2007). Activation of this cascade leads to phosphorylation and subsequent downregulation of the transcription factor SPCH (Lampard et al., 2008).

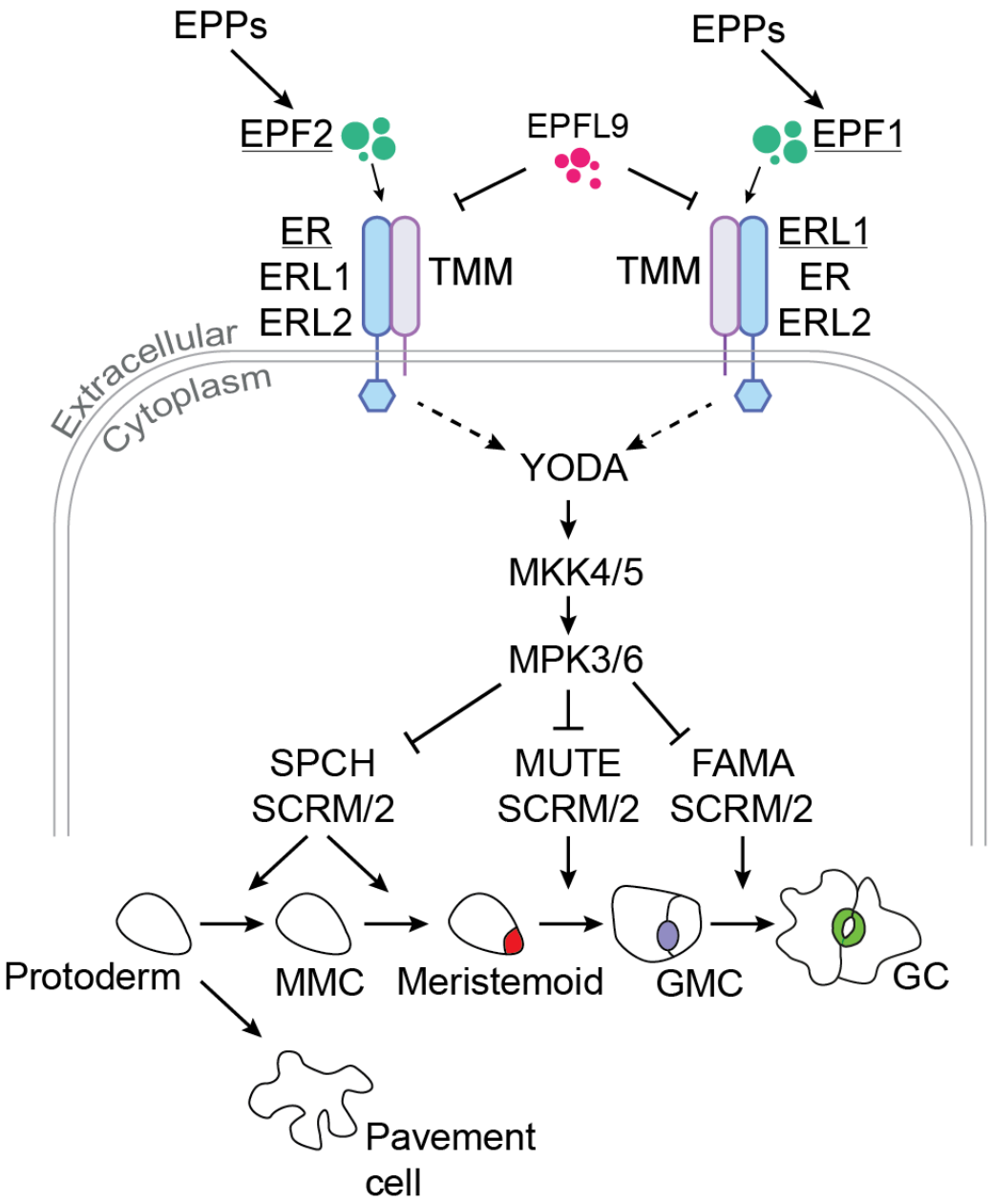

Fig. 2. Modified scheme of stomatal development in Arabidopsis from (Jalakas et al., 2024). SPCH, SPEECHLESS; SCRM, SCREAM; SCRM2, SCREAM 2; EPF, epidermal patterning factor; EPFL, EPIDERMAL PATTERNING FACTOR-LIKE; MKK, mitogen-activated protein kinase kinase; MPK, mitogen-activated protein kinase; EPPs, EPF-PROCESSING PROTEINASES; TMM, TOO MANY MOUTHS; ER, ERECTA; ERL, ERECTA-LIKE; MMC, meristemoid mother cell; GMC, guard mother cell; GC, guard cell.

1.5. Stomatal distribution

Stomata may be present on one or both leaf surfaces, which are referred to as the adaxial (upper) and abaxial (lower) epidermis. Most plants have stomata confined only to the lower leaf surface (hypostomaty), however there are a number of plants, including the model plant Arabidopsis, that have stomata on both leaf surfaces (amphistomaty) (Hõrak, 2026). Most studies focus exclusively on the lower (abaxial) leaf surface, while studies about stomata of the upper (adaxial) leaf surface remain few and far between (Hõrak, 2026).

Stomatal density (SD), stomatal ratio (SR), and stomatal index (SI) are widely used quantitative measures for examining stomatal development and patterning and are particularly informative for studies comparing the adaxial and abaxial leaf surfaces. SD reflects the number of stomata per unit area on each surface, while SR (adaxial/abaxial SD) captures their relative distribution between the adaxial and abaxial epidermis. SI, defined as the proportion of stomata relative to all epidermal cells, provides a developmentally meaningful measure that is less influenced by differences in cell expansion and surface-specific growth. Together, these metrics allow for the assessment of whether stomatal patterning on the adaxial and abaxial surfaces is normal and whether it is differentially regulated in leaf surfaces. Moreover, because stomatal number and distribution influences the potential maximum stomatal conductance, integrating developmental parameters with physiological measurements of conductance offers insight into how surface-specific stomatal development contributes to leaf gas exchange and whole-plant function (Hõrak, 2026; Salisbury, 1928; Wall et al., 2023).

1.6. Abaxial and adaxial stomata

Although much is known about stomatal development on the abaxial leaf surface, comparatively little attention has been given to adaxial stomata, leaving open questions about how their development is regulated and whether adaxial and abaxial stomatal patterning are controlled by distinct or independent genetic pathways (Hõrak, 2026).

Most land plants are hypostomatous and produce stomata only on the bottom or abaxial surface of the leaf (Muir, 2015). The model plant Arabidopsis is amphistomatous and produces a significant amount of stomata on the upper or adaxial surface of the leaf (Fig 3). Most prior studies have focused only on the abaxial stomata (Hõrak, 2026).

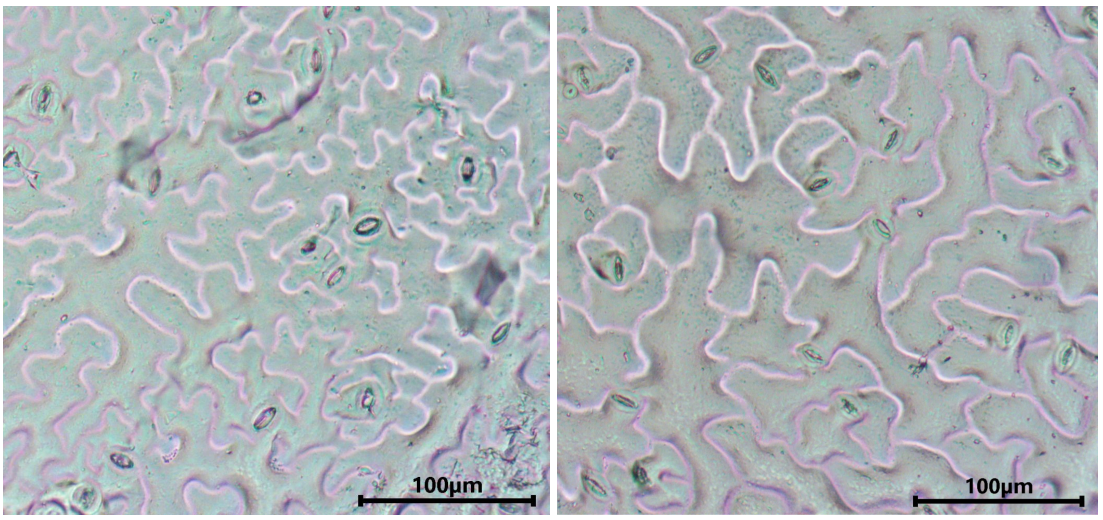

Fig. 3. Microscopy images of epidermal impressions of wild type Arabidopsis abaxial (left) and adaxial (right) leaf surfaces at 200x magnification. The scale bar is 100 µm.

Evidence for partly independent regulation of adaxial and abaxial stomatal development comes from stomatal patterning mutants, which often show different effects on the two leaf surfaces, including changes in stomatal density, stomatal index, or the stomatal ratio. In some well-characterized stomatal development mutants, arrested stomatal precursors are typically confined to the abaxial epidermis, indicating that stomatal differentiation can be regulated in a leaf-side-specific manner (Jalakas et al., 2024).

Silencing expression of the stomatal initiation transcription factor SPCH in Arabidopsis resulted in the loss of abaxial stomata while adaxial stomata remained present (Dow et al., 2014), whereas in transgenic lines of different variants of the MUTE transcription factor, arrested stomatal lineages were more common in adaxial cotyledon epidermis, and stomatal clusters more common in abaxial cotyledon epidermis (Illescas‐Miranda et al., 2025). The loss-of-function allele *erl2* deficient in *ERL2* from the ERECTA family generates increased stomatal index in the adaxial, but not in the abaxial leaf surface (Jalakas et al., 2024). Loss-of-function mutations in key regulators, such as *TMM*, *EPF1* and *EPF2*, have consistently been observed to increase abaxial stomatal density more strongly than adaxial, resulting in reduced SR, further supporting differential regulation of stomatal development between the two leaf surfaces (Jalakas et al., 2024).

The difference of stomatal development and regulation between leaf surfaces can be observed as soon as the embryonic stage. In wild type Arabidopsis embryos, more stomatal precursors were found on the adaxial surface, and in cotyledons, stomatal precursor cells also emerged earlier on the adaxial surface (He et al., 2025). *TMM* might differentially regulate cotyledon

leaf surfaces by preferentially supporting stomatal precursor formation on the abaxial surface. Absence of *TMM* leads to a stronger reduction of stomatal precursors on the abaxial surface and a relatively higher retention of precursors on the adaxial side (He et al., 2025). Authors suggest differential *EPFL8* (EPIDERMAL PATTERNING FACTOR-LIKE 8) expression between leaf surfaces may be responsible, as the *epfl8* knockout mutants generated significantly more precursor cells abaxially in cotyledons (He et al., 2025). This may suggest that *TMM* functions differently in cotyledons and true leaves, given that *TMM* is a stomatal repressor in the abaxial surface (Jalakas et al., 2024). Additional evidence for stomatal development occurring differently in cotyledons and true leaves is found in *epf1* and similar developmental mutants, showing reduced stomatal clustering in true leaves, compared with cotyledons (Dow et al., 2014; Hara et al., 2007).

Adaxial and abaxial stomatal development also respond differently to environmental conditions; for instance, in Arabidopsis, growth under low relative air humidity increased SR, accumulating relatively more stomata on the adaxial leaf surface. Dry air conditions further increase adaxial SI, while decreasing abaxial SI (Tulva et al., 2025). Responses to light follow this trend with increases in adaxial SI occurring upon increased light intensity, while abaxial SI remains largely unchanged (Hronková et al., 2015). A difference between leaf surfaces was also observed in sunflower leaves where stomatal patterning was more responsive to increased light on the abaxial surface, with stronger light-induced increase in SD compared with adaxial surface (Pleva et al., 2025).

1.7. Stomatal conductance

The main function of regulating stomatal aperture is to balance CO_2 uptake for photosynthesis and water loss occurring via transpiration. The exchange of gases between the plant and the atmosphere mainly is dependent on stomatal conductance (Lawson & Matthews, 2020). Stomatal conductance is affected by both stomatal density and stomatal aperture. As increasingly more studies reveal how stomatal development and regulation occurs partly independently between the leaf surfaces, it is reasonable to expect differential and perhaps independent stomatal conductance between the two surfaces. Indeed, stomatal conductance was shown to increase significantly more on the abaxial surface compared to adaxial surface under increasing light (Wei et al., 2025). However, under complete suppression of adaxial stomatal conductance, abaxial stomatal conductance underwent compensatory increase (Mott & Peak, 2018).

These surface-specific differences in conductance are closely linked to anatomical asymmetries within the leaf. The abaxial surface often has higher stomatal density and a more humid microenvironment, whereas the adaxial surface typically has a thicker cuticle and greater exposure to irradiance and evaporative demand, resulting in lower conductance. Mesophyll structure further influences CO_2 diffusion, and in amphistomatous leaves, the presence of stomata on both surfaces can reduce diffusion resistance and enhance photosynthesis (Mott & O'Leary, 1984; Parkhurst, 1978). However, this may increase water loss, particularly from the adaxial surface, requiring tighter regulation. Boundary layer differences also play a role, as the thinner adaxial boundary layer can enhance gas exchange but increase evaporative demand (Parkhurst & Mott, 1990).

2. THE AIMS OF THE THESIS

In this thesis I study how stomata are distributed between the upper and lower leaf surface, and whether the patterning and development of stomata are affected in these two leaf surfaces by loss-of-function mutations in a set of genes. I pose the following aims:

1. Test if selected genes have an effect on stomatal patterns in the leaves of *A. thaliana*

2. Test if selected genes have differential effect on abaxial and adaxial leaf stomatal development

3. Test if selected genes have an effect on stomatal conductance in abaxial and adaxial leaf surfaces

3. EXPERIMENTAL PART

3.1. MATERIALS AND METHODS

3.1.1. Plant lines used in experiments

Experiments were conducted with *Arabidopsis thaliana* accession Col-0 (wild type) and all mutant lines analyzed were in the same genetic background; details of the mutants are provided in Table 1. T-DNA insertion mutants were from the SALK (Alonso et al., 2003) and SAIL (Sessions et al., 2002) collections; ordered from Nottingham Arabidopsis Stock Centre (Scholl et al., 2000). Ten candidate genes were selected by the research group based on single cell transcriptomics studies (Lopez-Anido et al., 2021; Tenorio Berrío et al., 2022) on the basis of preferential expression in one leaf surface, as well as the expression in the stomatal lineage cells. A mutant in the *SCRM* gene was obtained at a later time and included in experiments for phenotypic characterization.

Table 1. List of gene loss-of-function mutants and corresponding T-DNA inserts. T-DNA insert locations based on T-DNA express database: http://signal.salk.edu/cgi-bin/tdnaexpress

Gene ID	**SALK number**	**Gene name**	**Mutant ID**	**T-DNA insert location**
AT4G02230	SALK_060410	*EL19Y*	*el19y-2*	exon
AT1G05010	SALK_064286	*ACO4*	*aco4*	intron
AT3G47960	SAIL_801_603	*NPF2.10*	*npf2.10-1*	intron
AT3G47960	SAIL_169_F11	*NPF2.10*	*npf2.10-2*	exon
AT3G49120	SALK_112466C	*PRX34*	*prx34*	intron
AT2G44130	SALK_143644	*KMD3*	*kmd3*	5'UTR
AT4G14960	SALK_091096C	*TUA6*	*tua6-1*	promoter
AT4G14960	SALK_042870C	*TUA6*	*tua6-2*	3'UTR
AT1G57660	SALK_015274	*EL21X*	*el21x-1*	exon #1

AT1G57660	SALK_094255C	*EL21X*	*el21x-2*	exon #2
AT3G26744	SALK_003155	*SCRM/ICE1*	*scrm/ice1*	exon

Table 2. List of genes used in the experiments and their known or predicted functions.

Gene name	**Known or predicted function**	**Reference**
EL19Y	Encodes a ribosomal protein L19e family protein.	(Cheng et al., 2017)
ACO4	Encodes 1-aminocyclopropane-1-carboxylate oxidase.	(Cheng et al., 2017)
NPF2.10	Encodes a glucosinolate-specific transporter. May be involved in stamen development.	(Nour-Eldin et al., 2012)
PRX34	Class III peroxidase. Involved in reactive oxygen species generation. Component of disease resistance.	(Zhao et al., 2019)
KMD3	Encodes a member of a family of F-box proteins, called the KISS ME DEADLY (KMD) family. KMD family genes may play a role in root growth under streptomyces volatiles exposure.	(Cheng et al., 2017; Dotson et al., 2026)
TUA6	Encodes an alpha-tubulin isoform required for right handed helical growth.	(Cheng et al., 2017)
EL21X	Large ribosomal subunit protein. Translation protein SH3-like family protein.	(Cheng et al., 2017)
SCRM	ICE1/SCRM is an essential bHLH partner that heterodimerizes with SPCH, MUTE, and FAMA to coordinate the sequential cell-state transitions of the stomatal lineage.	(Kanaoka et al., 2008)

3.1.2. Experiment 1: stomatal density, index, and ratio of leaf ten in loss-of-function mutants of stomatal lineage enriched genes

Plants were grown in growth cabinets (Percival AR-22 L, Percival Scientific, IA, USA) under light/dark photoperiod of 10h/14h at 250 μmol m^{-2} s^{-1}, and relative air humidity of 60%/80% daytime/nighttime, and with daytime/nighttime temperature of 23°C/19°C. Seeds were sown into 260 ml pots with a peat (OPM 025W, Kekkilä Oy, Vantaa, Finland):vermiculite (Vermikuliit Medium 0-4 mm, TopGreen, Vahi, Estonia):water mix at 4:2:3 ratio by volume. For the first week the trays were covered with a transparent plastic film to maintain high humidity during seed germination. Individual pots of all genotypes were shuffled in trays for randomization at the beginning of the experiment. Trays were shuffled in the growth cabinet every few days in order to homogenize growth cabinet conditions for all plants. Plants were watered every 4 days and were treated with water+nematode mixture weekly, containing nematodes *S. feltiae* and *S. carpocapsae* (Biotus, Finland) to combat the larvae of the fungus gnat, which eat the leaves of Arabidopsis. Leaf ten was selected for sampling based on the numbering protocol established by (Farmer et al., 2013). Leaves were collected from 7-week-old plants that had started bolting. For the analysis of stomatal patterning, sampled leaves were cut along the midvein and epidermal impressions were collected from both the adaxial and abaxial surfaces, one from each half of the leaf. Impressions were made using dental silicone (Speedex Light Body, Coltène/Whaledent AG, Switzerland, for the adaxial surface; Oranwash L, Zhermack, Italy, for the abaxial surface) following the method described by (Casson et al., 2009). Secondary nail varnish imprints were prepared from the silicone moulds and transferred onto microscope slides using transparent adhesive tape.

Images were captured from an area of 0.286 mm^2 located in the central region of the leaf (midpoint between the leaf base and tip and between the midvein and the leaf margin) using a light microscope (Kern OBF 133, Kern & Sohn GmbH) at ×200 magnification. The number of mature stomata, stomatal precursors and pavement cells were determined from the images using ImageJ (Schneider et al., 2012). SD (stomata mm^{-2}), SR (adax SD/abax SD), PD (precursor cells mm^{-2}), PVD (pavement cells mm^{-2}), and SI (SD/(SD+PD+PVD)) were calculated. Pavement cell density was calculated using an area of approximately 0.2 mm^2. This area size was chosen to allow determination of whether individual pavement cells occupied more than 50% of their total area within the selected region. Only cells with more than half of their area inside the counting region were included in the density calculation, while cells with less than 50% of their area within the region were excluded. Each genotype

and wild type control was represented by 8-9 plants. This experiment was performed for a second time with a separate independent batch of plants. Data from both experimental batches were pooled.

3.1.3. Experiment 2: Stomatal conductance and stomatal density in an undefined healthy leaf of a subset of mutants used in experiment 1

Plants were grown in the same growth cabinet, under the same conditions as in experiment 1, except for pot size, which was 110 ml. The mutants *el19y-2*, *aco4*, *npf2.10-1*, *npf2.10-2*, and additionally *scrm* were grown alongside wild type controls. The *scrm* mutant was included here as its homozygous line was obtained after experiment 1, but it had not been characterised before for adaxial stomatal conductance and patterning. Twenty plants of each mutant line and control were grown. At 5-weeks old, ten biggest plants were selected from each plant line and moved to a separate growth room for acclimatization for two hours under the same daytime light, temperature, and relative air humidity conditions. Stomatal conductance was measured for each plant for one of its leaves from both leaf surfaces using a porometer (LI-600 (Li-Cor)). Afterwards, the same leaf was collected and surface impressions were made the same way as was done in experiment 1. SD and SR were calculated for *el19y-2*, *aco4*, *npf2.10-1*, *npf2.10-2* lines, however for *scrm* and wild type lines, SI and stomatal lineage cell index ((SD+PD)/(SD+PD+PVD)) were calculated too. Stomatal conductance ratio (adax conductance/abax conductance) was also calculated. Conductance per stoma (stomatal conductance/SD) was calculated for all mutants and wild type control to determine individual conductance of stomata.

3.1.4. Statistical analyses

Data from both batches of experiment 1 were pooled. Statistical analyses were carried out by STATISTICA (version 7.1, StatSoft, Inc.). One-way ANOVA analysis was performed for all calculated metrics with Dunnett's T3 post-hoc test being performed for parameters with significant model main effect. All effects were considered significant at $P<0.05$. Standard error of the mean (SEM) was calculated for error bars.

One-way ANOVA analysis with Dunnett's T3 post-hoc test was performed in the same manner for experiment 2. Unpaired Student's t-test was performed for SI and stomatal lineage

cell index data to compare Col-0 and *scrm*. All effects were considered significant at $P<0.05$, and SEM was calculated for error bars.

3.1.5. Use of artificial intelligence

A large language model (OpenAI. (2026), ChatGPT. https://chat.openai.com/) was used to aid in locating topic-relevant sources and edit the language of my own text following the guidelines of ethical and transparent use of artificial intelligence put forth by The University of Tartu.

3.2. RESULTS

3.2.1. Variation in stomatal index, but not stomatal density or ratio, among studied mutants

Many studies examining stomatal density in true leaves (i.e., excluding cotyledons) base their analyses on a single fully expanded leaf, often without specifying its position. However, in Arabidopsis, leaves can vary considerably in size, shape, and developmental stage, which may confound comparisons of stomatal traits. To reduce this source of variability, fully expanded leaf 10 was sampled in all plants. In experiment 1, the mutants *el19y-2*, *prx34* and *el21x-1* (Fig. 4A) had significantly higher SI on the abaxial leaf surface, whereas on the adaxial leaf surface (Fig. 4B) only *el19y-2* had significantly higher SI than wild-type plants. Stomatal density (Appendix, Fig. 1A, 1B) and stomatal ratio (Fig. 5) were not significantly different compared with wild type in any mutant.

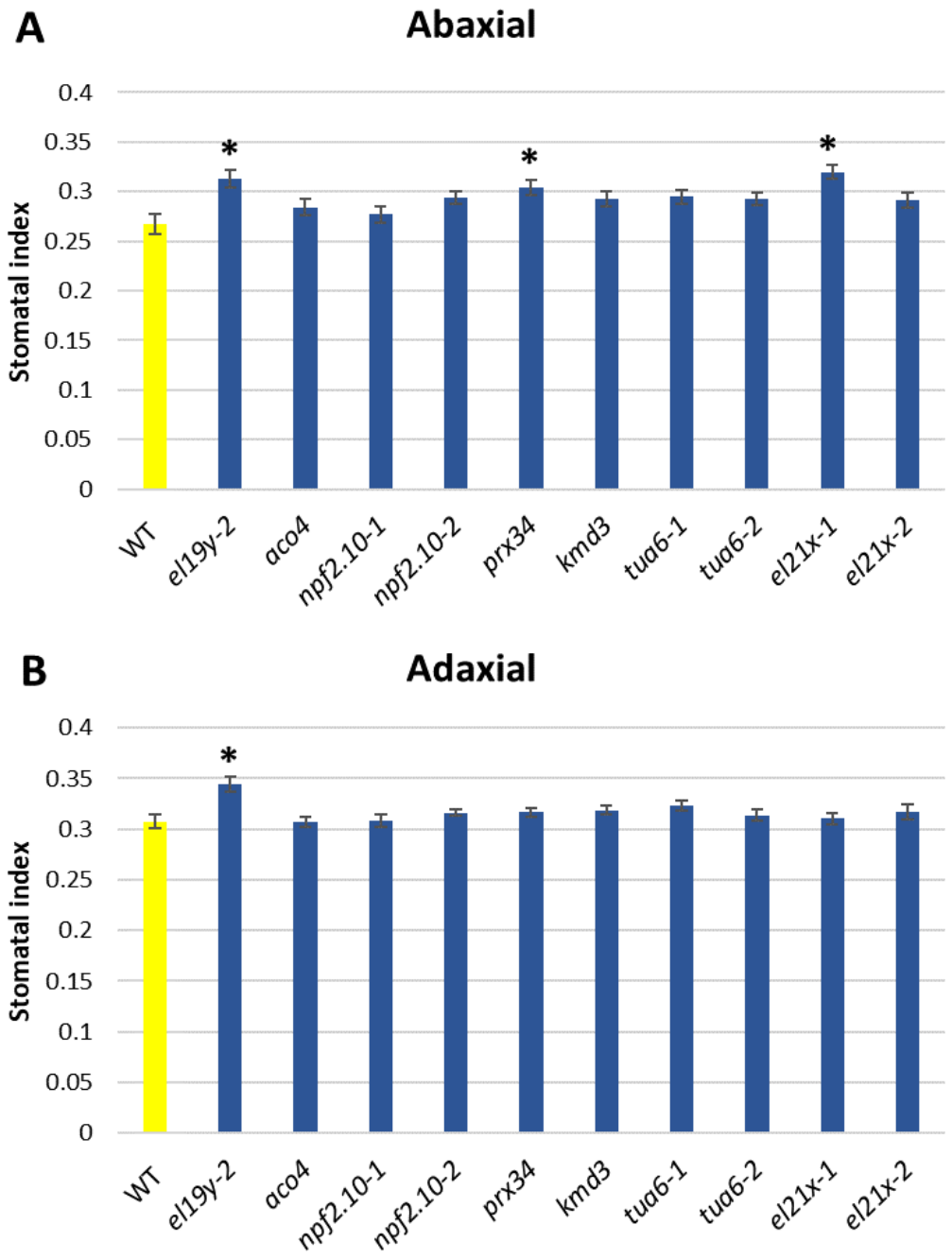

Fig. 4. Stomatal index in loss-of-function mutants and wild type control. Bars show average stomatal index in abaxial (A) and adaxial (B) leaf surfaces; n=17-18 plants for each genotype, error bars mark SEM. Significant differences were determined by one-way ANOVA with Dunnet's T3 post-hoc test. Asterisks above bars represent statistically significant differences compared with wild type (WT) at $P<0.05$.

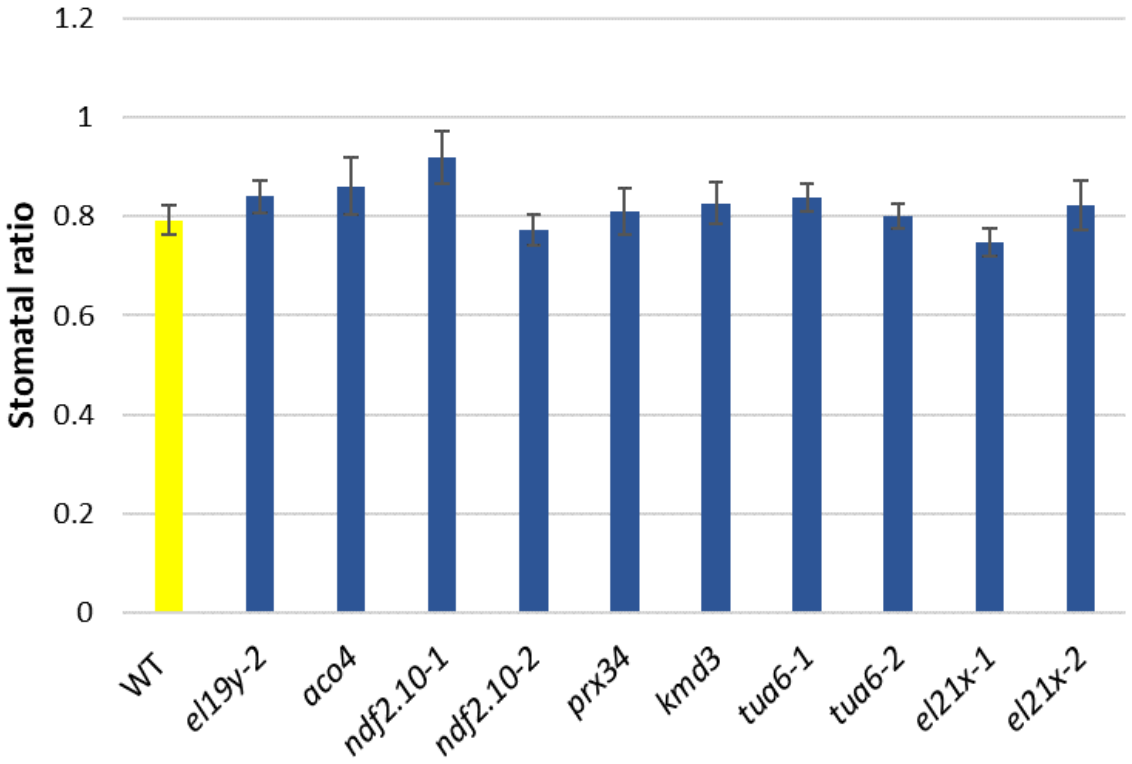

Fig. 5. Stomatal ratio in loss-of-function mutants and wild type control. Bars show average stomatal ratio; n=17-18 plants for each genotype, error bars mark SEM. Significant differences were determined by one-way ANOVA. No statistically significant differences were found compared with wild type (WT) at $P<0.05$.

3.2.2. Physiological characterization and re-examination of stomatal patterning in a mutant subset

While no mutants showed significant differences in stomatal density (Appendix, Fig. 1), mutants *el19y-2*, *aco4*, *npf2.10-1*, and *npf2.10-2* seemed to show a trend of increased stomatal density adaxially (Appendix, Fig. 1B). To further investigate this potential trend, a subset of mutants were re-analysed in experiment 2 for stomatal density, and stomatal conductance was also analysed. Mutants *el19y-2*, *aco4*, *npf2.10-1*, and *npf2.10-2* were examined, and the mutant *scrm* was added for adaxial stomatal characterization. Congruent with stomatal index results in experiment 1 (Fig. 4A), mutant *el19y-2,* showed significantly increased stomatal density abaxially (Fig. 6A). Additionally, the mutant *npf2.10-2* also showed significant increase in abaxial stomatal density (Fig. 6A). In contrast, the mutant *scrm* showed significantly decreased stomatal density both abaxially and adaxially (Fig. 6A, 6B). The mutant *npf2.10-1* had significantly higher stomatal conductance on the abaxial leaf

surface (Fig. 6C), while on the adaxial surface (Fig. 6D) only *scrm* had significantly higher stomatal conductance than WT.

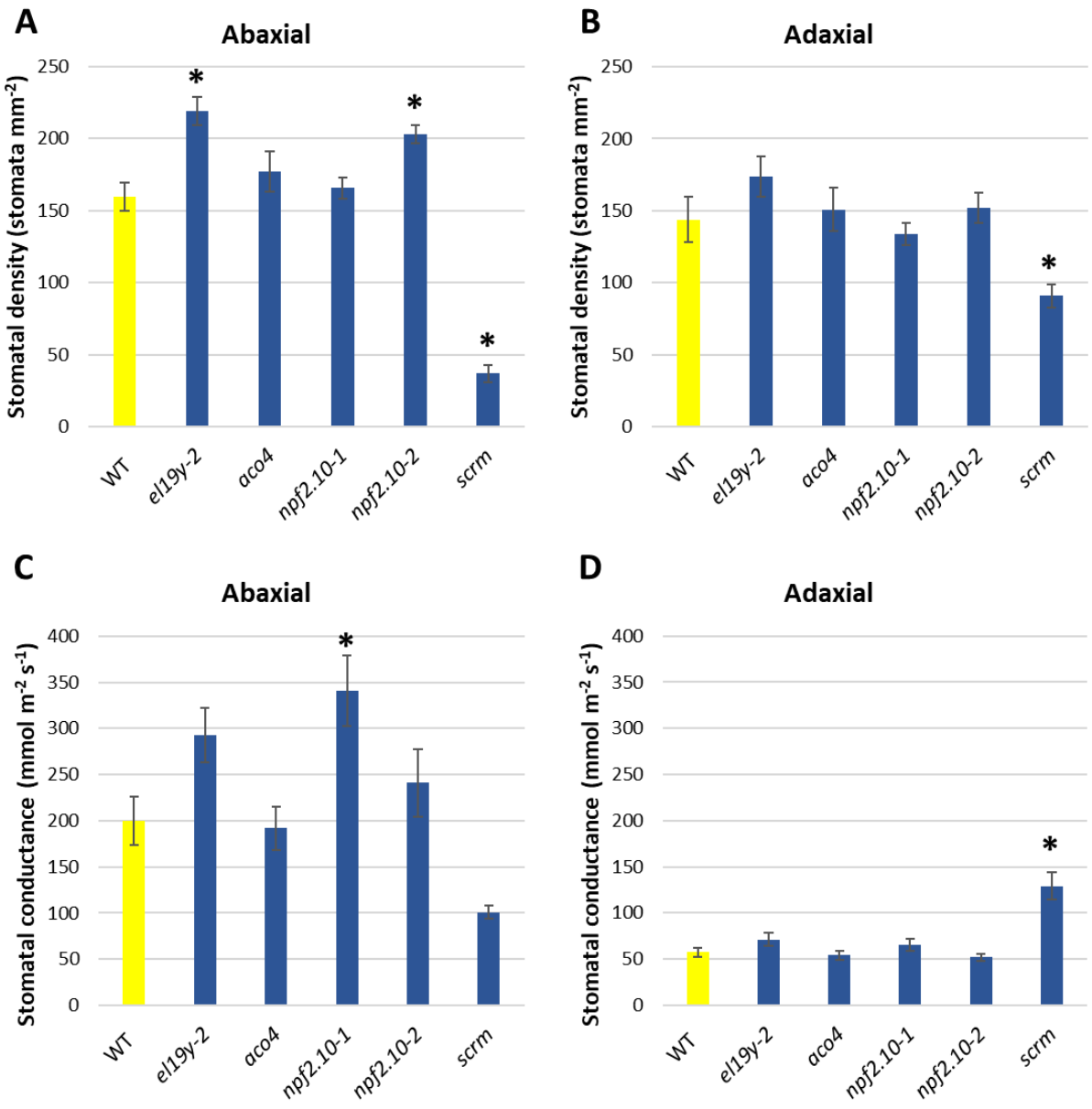

Fig. 6. Variation in stomatal density and stomatal conductance. Bars show average stomatal density in abaxial (A) and adaxial (B) leaf surfaces, and average stomatal conductance in abaxial (C) and adaxial (D) leaf surfaces; n=10 plants for each genotype, error bars mark SEM. Significant differences were determined by one-way ANOVA with Dunnet's T3 post-hoc test. Asterisks above bars represent statistically significant differences from wild type (WT) at $P<0.05$.

3.2.3. Stomatal ratio and stomatal conductance ratio in the mutant subset

The reexamination of the mutant subset showed the mutant *scrm* had significantly increased stomatal ratio with a relatively higher number of stomata confined to the adaxial leaf surface (Fig. 7A), while the other mutants had similar stomatal ratios with wild type, and had more equal stomatal distribution between leaf surfaces (Fig. 7A). The mutant *scrm* also had significantly higher stomatal conductance ratio compared with wild type (Fig. 7B), with relatively higher stomatal conductance on the adaxial leaf surface. The other mutants showed similar conductance ratio with wild type, while had relatively higher stomatal conductance on the abaxial leaf surface (Fig. 7B).

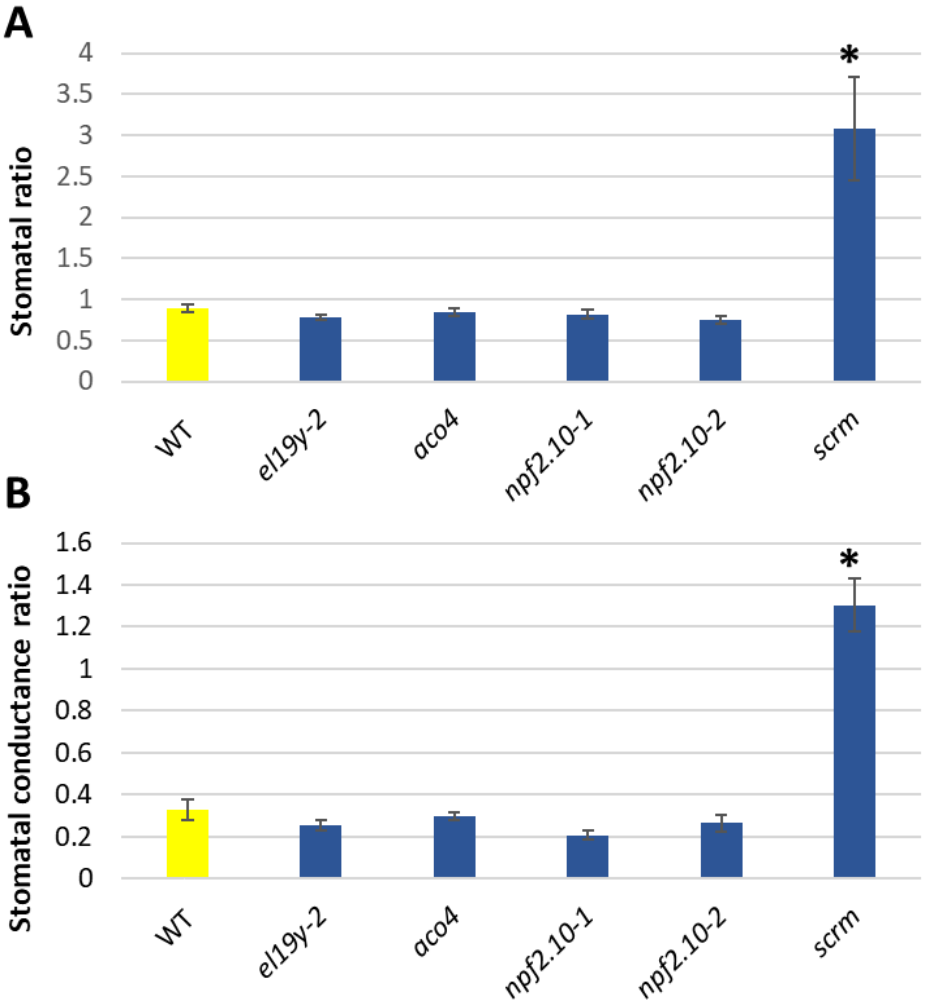

Fig. 7. Stomatal ratio and stomatal conductance ratio. Bars show average stomatal ratio (A) and average stomatal conductance ratio (B); n=10 plants for each genotype, error bars mark SEM. Significant differences were determined by one-way ANOVA with Dunnet's T3

post-hoc test. Asterisks above bars represent statistically significant differences from wild type (WT) at $P<0.05$.

3.2.4. Conductance of individual stomata

To further investigate stomatal conductance at the single stoma level, conductance per stoma was also calculated. Stomatal conductance per stoma was higher in *scrm* mutant in both leaf surfaces (Fig. 8A, 8B). Notably, stomatal conductance per stoma for all genotypes was nearly two times higher on the abaxial surface (Fig. 8A), compared with the adaxial surface (Fig. 8B).

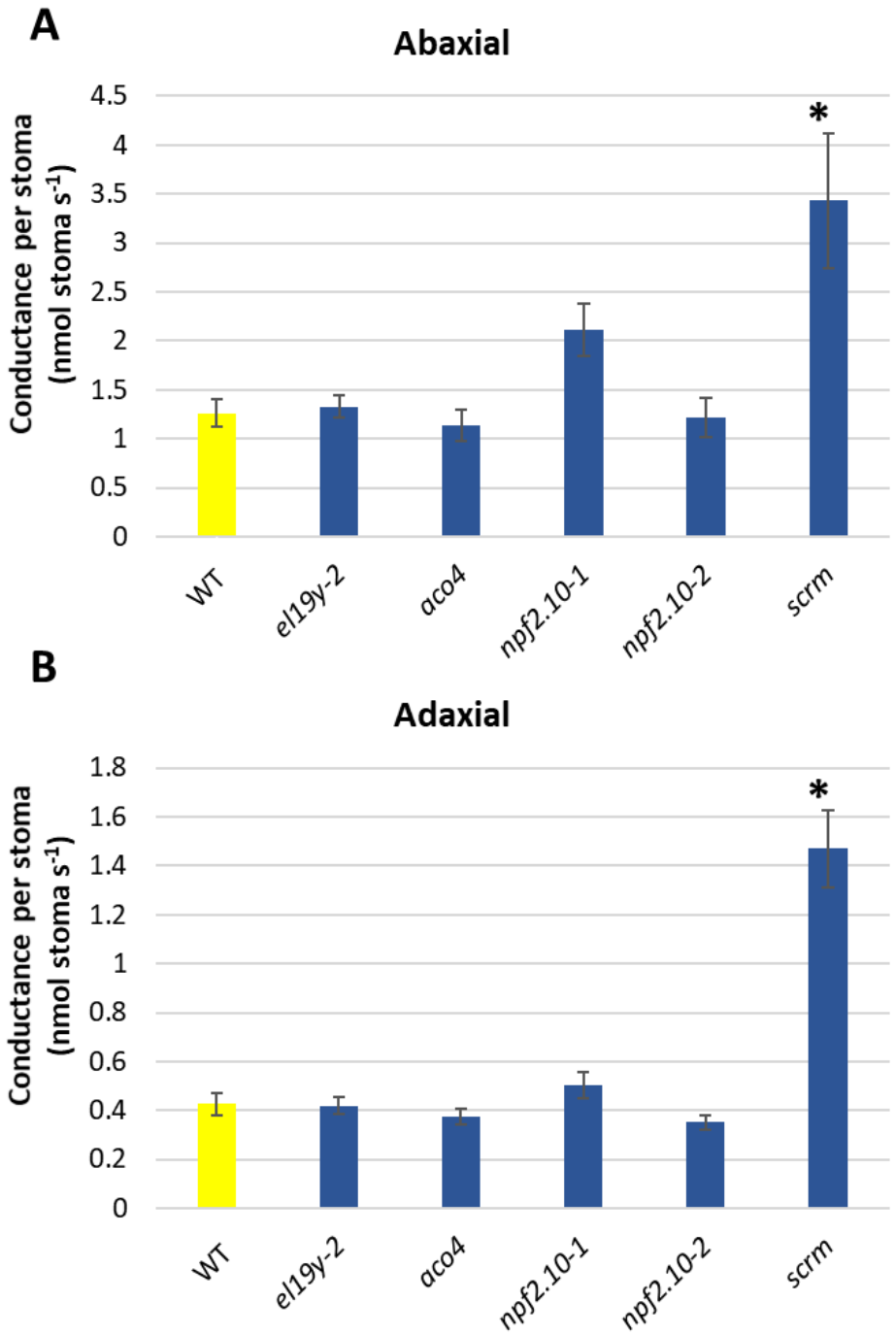

Fig. 8. Variation in conductance per stoma. Bars show average stomatal conductance per stoma in abaxial (A) and adaxial (B) leaf surfaces; n=10 plants for each genotype, error bars mark SEM. Significant differences were determined by one-way ANOVA with Dunnet's T3 post-hoc test. Asterisks above bars represent statistically significant differences from wild type (WT) at $P<0.05$.

3.2.5. Stomatal index and stomatal lineage cell index in wild type and *scrm*

Stomatal index alone does not reflect how many stomatal lineage cells were initiated, given that it accounts only for mature stomata. To further elucidate the stomatal development in the *scrm* mutant, stomatal lineage cell index was calculated, as this accounts also for stomatal precursor cells, shown in Fig. 10C, 10D with red triangles, which failed to become mature stomata. The *scrm* mutant had significantly lower stomatal index on both leaf surfaces (Fig. 9A, 9B) compared with WT, however the stomatal index on the adaxial surface (Fig. 9B) was over 2 times higher than on the abaxial surface (Fig. 9A). Similarly, *scrm* stomatal lineage cell index was also significantly lower compared with wild type on both leaf surfaces (Fig. 9C, 9D). Notably, the stomatal lineage cell index on both leaf surfaces for *scrm* was closer to that of WT (Fig. 9C, 9D) than the *srcm* stomatal index with WT (Fig. 9A, 9B).

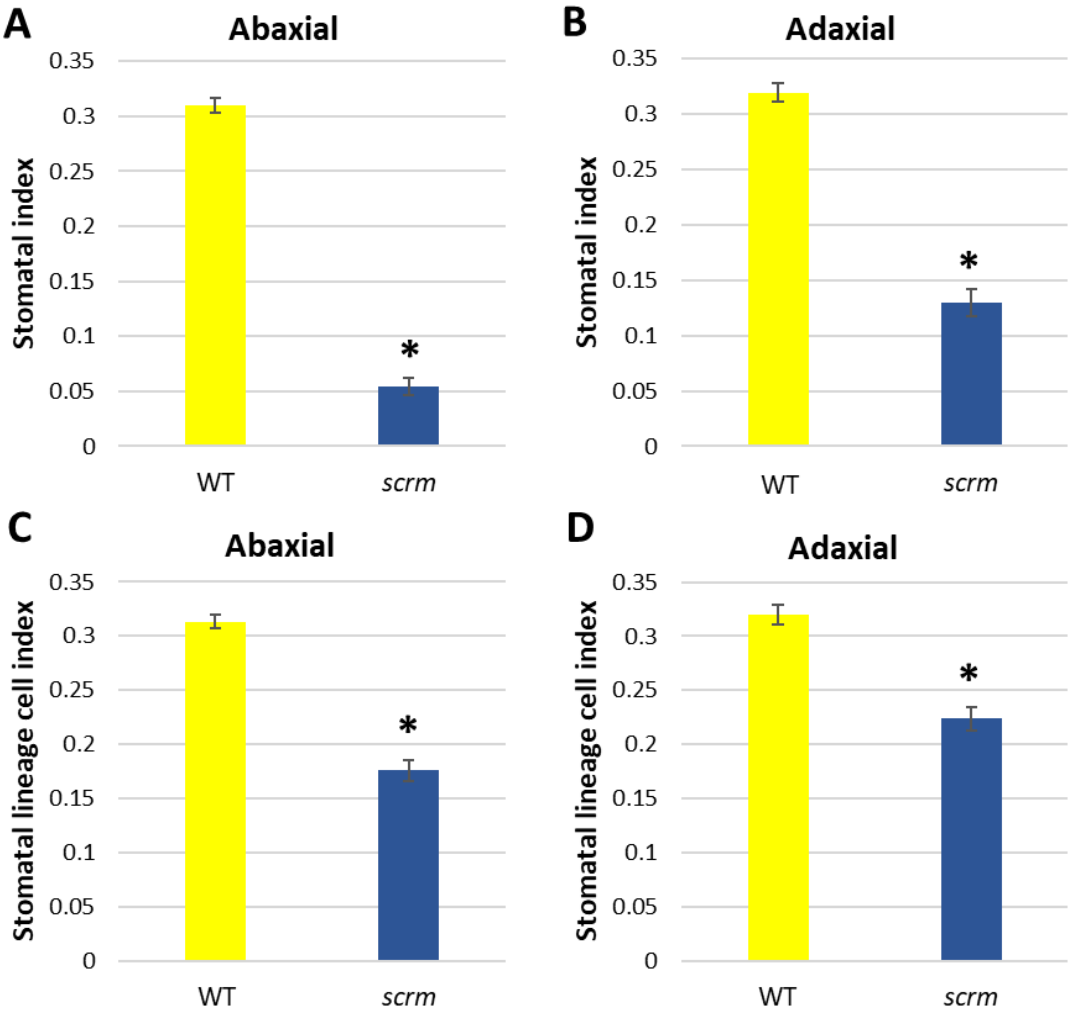

Fig. 9. Stomatal index and stomatal lineage cell index in wild type and *scrm* plants. Bars show average stomatal index in abaxial (A) and adaxial (B) leaf surfaces, and average

stomatal lineage cell index in abaxial (C) and adaxial (D) leaf surfaces; n=10 plants for each genotype, error bars mark SEM. Significant differences were determined by unpaired student's t-test. Asterisks above bars represent statistically significant differences from wild type (WT) at $P<0.05$.

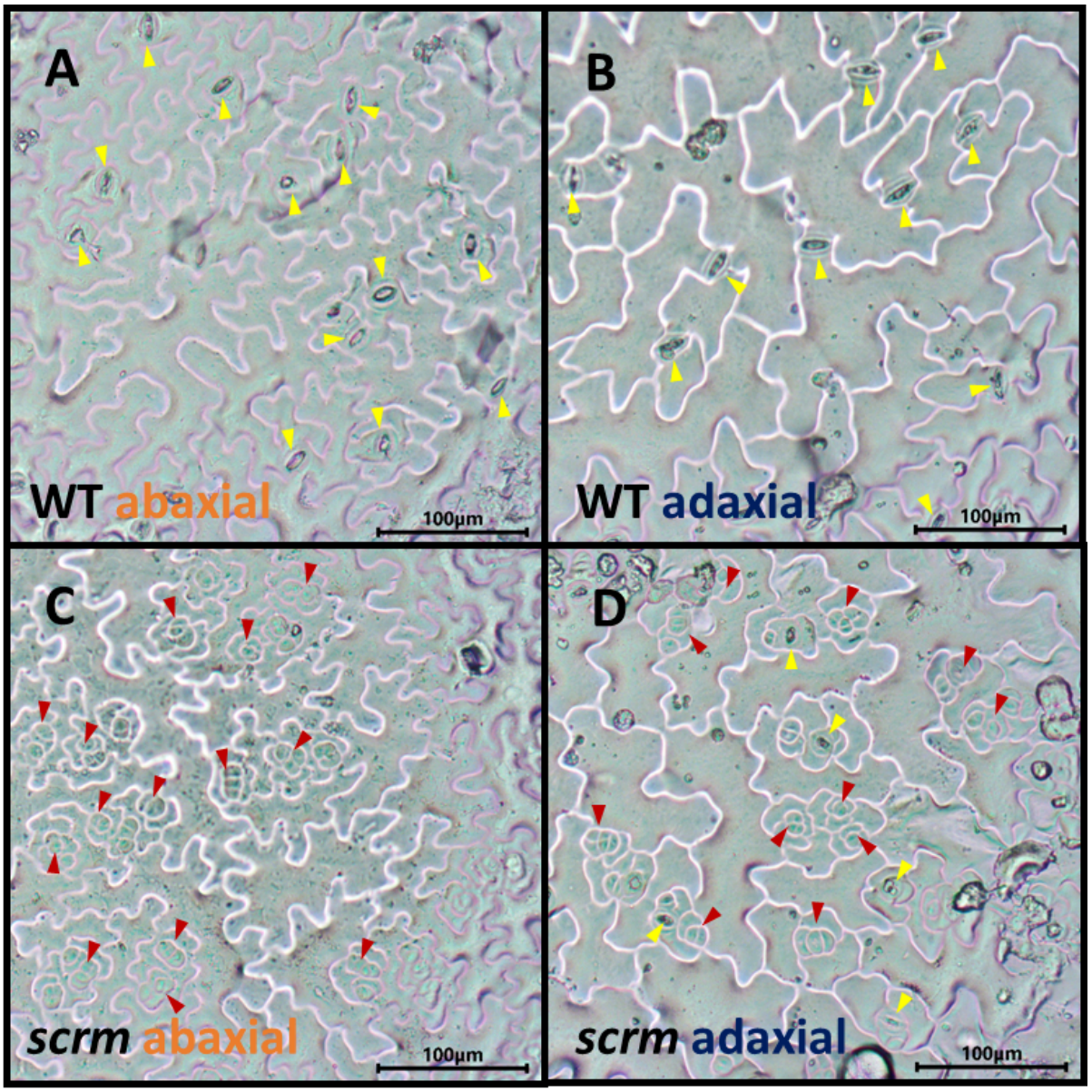

Fig. 10. Microscopy images of epidermal impressions of wild type Arabidopsis and *scrm* abaxial and adaxial leaf surfaces at 200x magnification. Yellow triangles show stomata, red triangles show cell clusters containing stomatal precursor cells. Not all visible stomata or stomatal precursors are marked by triangles in the images. The scale bar is 100 µm.

3.3. DISCUSSION

3.3.1. Effects of studied genes on stomatal distribution

In this study I examined the stomatal patterning and development of a set of loss-of-function mutants (Table 2) of genes, which have preferential expression in one leaf surface, and are expressed in the stomatal lineage (Lopez-Anido et al., 2021; Tenorio Berrío et al., 2022). Increasing evidence shows that both patterning and the development of stomata may function differently and partly independently on the two leaf surfaces (Hõrak, 2026). Altered and unequal distribution of stomata can arise from mutations in key stomatal development regulators, such as the stomatal initiation transcription factor SPCH in Arabidopsis. Silencing of *SPCH* results in the loss of abaxial stomata, yet adaxial stomata persisted (Dow et al., 2014). Similarly, loss-of-function mutants of *TMM*, *EPF1* and *EPF2* genes have consistently exhibited stronger increase in abaxial than adaxial stomatal density, resulting in more stomata being confined to the lower leaf surface (Jalakas et al., 2024). Environmental conditions too unequally affect the distribution of stomata between the two surfaces, for instance, low relative air humidity leads to accumulation of relatively more stomata on the adaxial leaf surface, increasing stomatal ratio in Arabidopsis (Tulva et al., 2024). This is further seen in increased adaxial SI, and decreased abaxial SI in dry air conditions (Tulva et al., 2025). Increases in adaxial SI occurred also upon increased light intensity, while abaxial SI remained mostly unchanged (Hronková et al., 2015).

We had selected genes with preferential leaf surface-specific activity, and I examined stomatal density, ratio and stomatal index of respective mutants in experiment 1. We expected to see differences of stomatal distribution between the leaf surfaces, however no differences were observed for the most part. In contrast to strong stomatal patterning phenotypes observed in core regulatory mutants (Jalakas et al., 2024), the mutants analysed here exhibited only minor effects. No significant differences in stomatal density were observed compared with WT (Appendix, Fig. 1), and no significant differences in stomatal ratio either (Fig. 5). This suggests that, unlike core stomatal regulators, the analysed candidate genes have a limited functional contribution to surface-specific stomatal patterning. I did observe a small, but significant increase in stomatal index in some mutants, namely *el19y-2*, *prx34* and *el21x-1*, which had significantly increased SI on the abaxial leaf surface (Fig. 4A), whereas on the adaxial leaf surface (Fig. 4B) only *el19y-2* had significantly higher SI. This may suggest that *PRX34* and *EL21X* have some, albeit minor, leaf surface-specific effect, as the increased SI appears only abaxially. The second experiment re-analysing select few mutants

confounds this with significantly increased stomatal density occurring only abaxially in *el19y-2* and *npf2.10-2*, whereas the mutant *scrm* had significantly decreased stomatal density of both surfaces. Notably, the reduction in stomatal density was much stronger on the abaxial surface for *scrm* (Fig. 6A), leading to a higher stomatal ratio with 3x more stomata produced adaxially than abaxially (Fig. 7A). These findings are in line with previous studies with stronger phenotypic effects observed on the abaxial surface in stomatal developmental regulator mutants (Dow et al., 2014; He et al., 2025; Jalakas et al., 2024). As most studies have focused exclusively on the abaxial surface, there is a possibility that adaxial-specific stomatal regulators remain to be discovered. The results here indicate that the analysed genes are unlikely to act as primary regulators of stomatal patterning, as evidenced by minor phenotypic effects, and may instead play a modulatory role. The observed effects are mostly confined to the abaxial surface in the studied mutants, indicating a leaf surface-specific role for respective genes.

3.3.2. Effects of studied genes on stomatal development

Stomatal patterning is dependent on stomatal development, which in turn is governed by a range of regulators and expansive cell signalling. This study focused on genes, of which the majority have no known stomatal development-specific functions, yet have enriched expression in a single leaf surface and are expressed in the stomatal lineage (Table 2), with the exception of SCRM, which is a central transcription factor involved in stomatal development (Kanaoka et al., 2008), though currently lacks adaxial characterization. Among the studied genes in the first experiment, three showed significantly increased stomatal index abaxially (Fig. 4A), and one adaxially (Fig. 4B). Two of these genes - *EL19Y* and *EL21X* - are ribosomal protein encoding genes (Cheng et al., 2017). These data may suggest a role for ribosomal processes in stomatal development. Disruption of these genes led to increased SI abaxially (Fig. 4A). Interestingly, in the second experiment examining some mutants again, the *el19y-2* mutant also showed effects confined only to the abaxial surface (Fig. 6A), echoing other studies demonstrating stronger modulation of abaxial stomatal development by mutations in developmental regulators (Jalakas et al., 2024). Still, given that the mutant *el19y-2* had significantly higher stomatal index on both leaf surfaces (Fig. 4A, 4B), *EL19Y* may act as a negative regulator of stomatal differentiation on both leaf surfaces. The *el19y-1* mutant line failed to germinate entirely, suggesting some line-specific detrimental effect, and thus was not included in the experiment. An additional *el19y* line should be studied to

understand if *EL19Y* affects stomatal development. Only *el21x-1* but not *el21x-2* had significant increase in abaxial stomatal index (Fig. 4A), suggesting a line-specific phenotype. The effect on stomatal development in the abaxial epidermis only in *el21x-1* may be due to the position of T-DNA insertion at the beginning of the coding sequence in the first exon, whereas in *el21x-2*, the T-DNA is located towards the end of the gene (Table 1) and thus may not hinder gene function completely. To test the potential role of EL21X in stomatal development, another mutant line with a T-DNA insert in a different location of the first exon may be examined in future studies. Roles of *EL19Y* and *EL21X* may be further explored by generating double loss-of-function mutants to test if these genes may act in the same pathway to control stomatal development.

The third mutant in experiment 1 to show significant effect was *prx34*, which had increased SI and this effect was confined only to the abaxial surface (Fig. 4A). Similarly, in the second experiment, the mutant *npf2.10-2* showed significantly increased stomatal density, also confined to the abaxial surface (Fig. 6A). PRX34 is a class III plant peroxidase (POX) involved in generating reactive oxygen species (ROS). POX have been suggested to be involved in various cellular processes such as cross-linking cell wall structural proteins, pathogen defense, senescence, auxin catabolism, lignification and other processes (Hiraga et al., 2001). *PRX34* is involved in Arabidopsis disease resistance (Zhao et al., 2019). Given the increased stomatal index, *PRX34* may have a role in stomatal development too, perhaps via ROS signalling. Indeed, the studied *prx34* mutant does not produce any transcripts and may have an effect on ROS generation (Zhao et al., 2019), which in turn may have an effect on stomatal development. Additional POX knockout lines could be tested, to implicate ROS in stomatal development. Similarly, the *NPF2.10* gene, belonging to one of the largest transporter families in the plant kingdom - nitrate and peptide transporter family (NPF) - is responsible for encoding a major transporter for glucosinolates, compounds involved in plant defense, as well as the vast majority of specialized plant metabolite classes are transported by members of the NPF (Kanstrup & Nour-Eldin, 2022). *NPF2.10* may also have a role in plant development, as a previous study (Saito et al., 2015) has implicated *NPF2.10* in Arabidopsis stamen development. It may be reasonable to think that *NPF2.10* may also play a part in stomatal development, although it is important to note that only the *npf2.10-2* line showed any significant effects, as opposed to *npf2.10-1* (Fig. 6A). This may be explained by T-DNA insertion of *npf2.10-2* being located in the exon, while *npf2.10-1* having T-DNA insertion located in the intron region (Table 1).

3.3.3. Stomatal physiology in the studied mutants

To further examine stomatal function, experiment 2 included physiological characterization alongside patterning. This was done by taking stomatal conductance measurements and performing impression analysis for the same leaves for a subset of mutants to determine whether changes in stomatal patterning translate into functional differences in gas exchange (Fig. 6C, 6D). Abaxially, only *npf2.10-1* had significantly increased stomatal conductance, while *npf2.10-2* had no significant effect (Fig. 6C), as opposed to stomatal density (Fig. 6A), which was increased only in *npf2.10-2*. This reveals a discrepancy between the observed significant effects of the mutants *npf2.10-1* and *npf2.10-2* in stomatal density and stomatal conductance, and the observed phenotypes may be due to random variation or background mutations in studied plant lines, as stomatal density and stomatal conductance often positively correlate (Tulva et al., 2024, 2025; Xu & Zhou, 2008; Yin et al., 2020). Adaxially, only the mutant *scrm* had significantly increased stomatal conductance (Fig. 6D), providing another example of the mismatch between anatomy and physiology.

Comparing stomatal density ratio (Fig. 7A) and stomatal conductance ratio (Fig. 7B) from experiment 2, a slight difference between the two surfaces can be seen. Stomatal density ratio for all mutants, except *scrm*, is similar with wild type and shows a roughly equal distribution of stomata on the upper and lower leaf surface (Fig. 7A), however most of the stomatal conductance is taking place abaxially for all mutants, except *scrm*, and is similar to wild type (Fig. 7B). This suggests independent control of stomatal apertures on different leaf surfaces, allowing plants to adjust stomata to local microclimates and potentially improve the balance of water loss and CO_2 uptake. Upper leaf surface stomata are exposed to higher light and higher evaporative demand, thus may be relatively more closed, as opposed to lower surface stomata, which may be fully open for maximized CO_2 uptake, while being exposed to lower light via shading and decreased evaporative demand (Hõrak, 2026). However, it has also been shown that abaxial conductance is affected more strongly by changes in environment. One example is where stomatal conductance was shown to increase significantly more abaxially, than adaxially under increasing light conditions (Wei et al., 2025).

To further examine the effects in stomatal conductance, the conductance at an individual stoma level was calculated for both leaf surfaces (Fig. 8A, 8B), in order to shed light on alterations to stomatal activity, by indirectly inferring aperture, as it is a large determinant of stomatal conductance. All mutants, except *scrm*, exhibited similar conductance per stoma compared with wild type, however higher conductance and thus potentially more open

stomata were observed abaxially, with over two times higher conductance per stoma, in line with previous differences in stomatal aperture being attributed to local microclimate (Hõrak, 2026). Overall, these results suggest that all mutant lines keep adaxial stomata more closed (Fig. 7B).

3.3.4. The mutant *scrm* has altered stomatal distribution and more open stomata

I show that stomatal development patterning is severely impaired in *scrm* mutants on the adaxial surface in a manner comparable to that reported for the abaxial surface (Kanaoka et al., 2008). This mutant consistently produced significantly lower stomatal density (Fig. 6A, 6B) and stomatal index (Fig. 9A, 9B) on both leaf surfaces, and produced a great deal of stomatal precursors on both surfaces (Fig. 10A, 10B). Stomatal ratio reveals a relatively higher production of stomata on the adaxial surface, leading to a significantly higher stomatal ratio with three times the amount of stomata allocated to the adaxial surface compared with abaxial surface (Fig. 7A). This phenotype resembles that of increased stomatal density and sensitivity mutants of Arabidopsis tested by (Tulva et al., 2024) in low relative air humidity, where more stomata were produced on the adaxial surface, however these effects observed in response to a change in growth environment were much milder. Previous studies have reported decreased stomatal ratio in mutants (Berger & Altmann, 2000; Dow et al., 2014; Hronková et al., 2015; Jalakas et al., 2024). Exceptions to this are mutant lines related with SPCH transcription factor: the *SPCH* silenced mutants (Dow et al., 2014) had a complete loss of stomata on the abaxial surface, while adaxial stomata remained unaffected, and the overexpression of a *SPCH* repressor resulted in higher reduction of stomatal density and index occurring on the abaxial surface, compared with adaxial surface (Qi et al., 2019). The increase of stomatal ratio in the *scrm* mutant lacking a core partner to SPCH matches these results, as the lack of *SPCH* function seems to similarly preferentially suppress abaxial stomata as does the lack of *SCRM*.

Stomatal conductance (Fig. 7B) ratio in *scrm* follows stomatal ratio (Fig. 7A), which can be explained by more stomata confined to the adaxial surface. Despite the reduced stomatal density on the abaxial surface for *scrm* (Fig. 6A), stomatal conductance was not significantly reduced abaxially (Fig. 6C). Conversely, adaxial stomatal conductance (Fig. 6D) in the *scrm* mutant was significantly higher than wild type, despite the reduced stomatal density seen on this surface (Fig. 6B). This posed a question whether stomatal aperture may be differentially

affected in the *scrm* mutant and may be responsible for the discrepancy in stomatal density and stomatal conductance.

In order to elucidate this discrepancy, stomatal conductance for individual stoma was calculated for both surfaces (Fig. 8A, 8B). On both leaf surfaces, *scrm* showed significantly higher per stoma conductance, about 3-fold higher compared with wild type, suggesting that on both surfaces of the *scrm* mutant, increased stomatal apertures may compensate for lower density. These findings support previous work showing that stomatal conductance is not solely determined by stomatal density but also by stomatal aperture and regulation (Faralli et al., 2019; Ochoa et al., 2024; Tulva et al., 2024). Future studies could be done to examine the responsiveness of stomata in the *SCRM* loss-of-function mutants in order to test whether this mutant has had its ability to close stomata compromised or is its higher conductance per stoma simply to compensate for the low stomatal density in order to allow for sufficient gas exchange.

To further characterise stomatal development in the *scrm* mutant, stomatal index and stomatal lineage cell index were calculated to gain an understanding how many stomata are initiated versus how many differentiate fully (Fig. 9). As expected from previous work (Kanaoka et al., 2008), stomatal index on both leaf surfaces was significantly lower compared with wild type (Fig. 9A, 9B). Stomatal lineage cell index followed this trend and *scrm* exhibited significantly lower indices on both leaf surfaces (Fig. 9C, 9D), however the stomatal lineage cell index was closer to that of wild type compared with stomatal index of *scrm* and wild type (Fig. 9A, 9B). This indicates that loss of *SCRM* does not fully prevent stomatal lineage initiation, and the mutant produces a considerable amount of stomatal precursor cells, many of which are unable to differentiate into mature stomata (Fig. 10). This is consistent with the established role of *SCRM* as a core partner of SPCH, MUTE, and FAMA in regulating stomatal lineage progression (Kanaoka et al., 2008), but perhaps suggests that major role of SCRM occurs in the stomatal differentiation step, whereas its role in stomatal initiation is less pronounced.

SUMMARY

Stomata are small pores found in the epidermis of plants that facilitate the balance of water loss with uptake of CO_2. Stomata are most often found on a single leaf surface, typically the lower or abaxial leaf surface, however some plants, including the model plant *Arabidopsis thaliana,* have stomata also on the upper or adaxial leaf surface. Mechanisms responsible for regulating the development and patterning of stomata between the two surfaces remain incompletely understood, as most previous studies have focused exclusively on the lower leaf surface, and mostly on core stomatal development regulators. This has left a knowledge gap both about other potential surface-enriched stomatal regulators, and the regulation of stomatal development on the adaxial surface. The aim of this thesis therefore was to investigate the role of select abaxially or adaxially enriched genes in Arabidopsis stomatal development and physiology.

Overall, the investigated genes had limited impact on stomatal ratio, suggesting they are not key regulators of stomatal distribution between leaf surfaces. However, several mutants showed significant changes in stomatal index. In particular, the *el19y-2* mutant showed increased stomatal index on both leaf surfaces, and mutants *prx34* and *el21x-1* showed increased stomatal index only on the lower leaf surface. The *scrm* mutant exhibited strongly reduced stomatal density and index. Despite these reductions, *scrm* displayed increased adaxial stomatal conductance and increased conductance per individual stoma on both surfaces, indicating that stomatal density and stomatal function can become uncoupled. Furthermore, *scrm* exhibited altered stomatal distribution between leaf surfaces via increase in stomatal ratio, supporting partially independent regulation of adaxial and abaxial stomatal traits. Together, these findings suggest that the analysed surface-enriched genes generally act more like minor modulators, rather than major regulators, except for *SCRM*, in stomatal development and patterning. This work further demonstrates that stomatal development and stomatal physiology are not always directly linked and highlights the importance of considering both developmental and functional traits when studying stomatal biology. In summary, this study shows that stomatal development and function can be uncoupled between the leaf surfaces, and that leaf surfaces differ in their regulation of these processes. Understanding how stomatal development and function are regulated can help improve crop water use efficiency and photosynthetic performance under changing environmental conditions. Insights into the independent regulation of stomatal characteristics between leaf

surfaces may also support future strategies for developing crops with improved drought tolerance and more stable productivity, driving agricultural sustainability and food security.

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APPENDIX

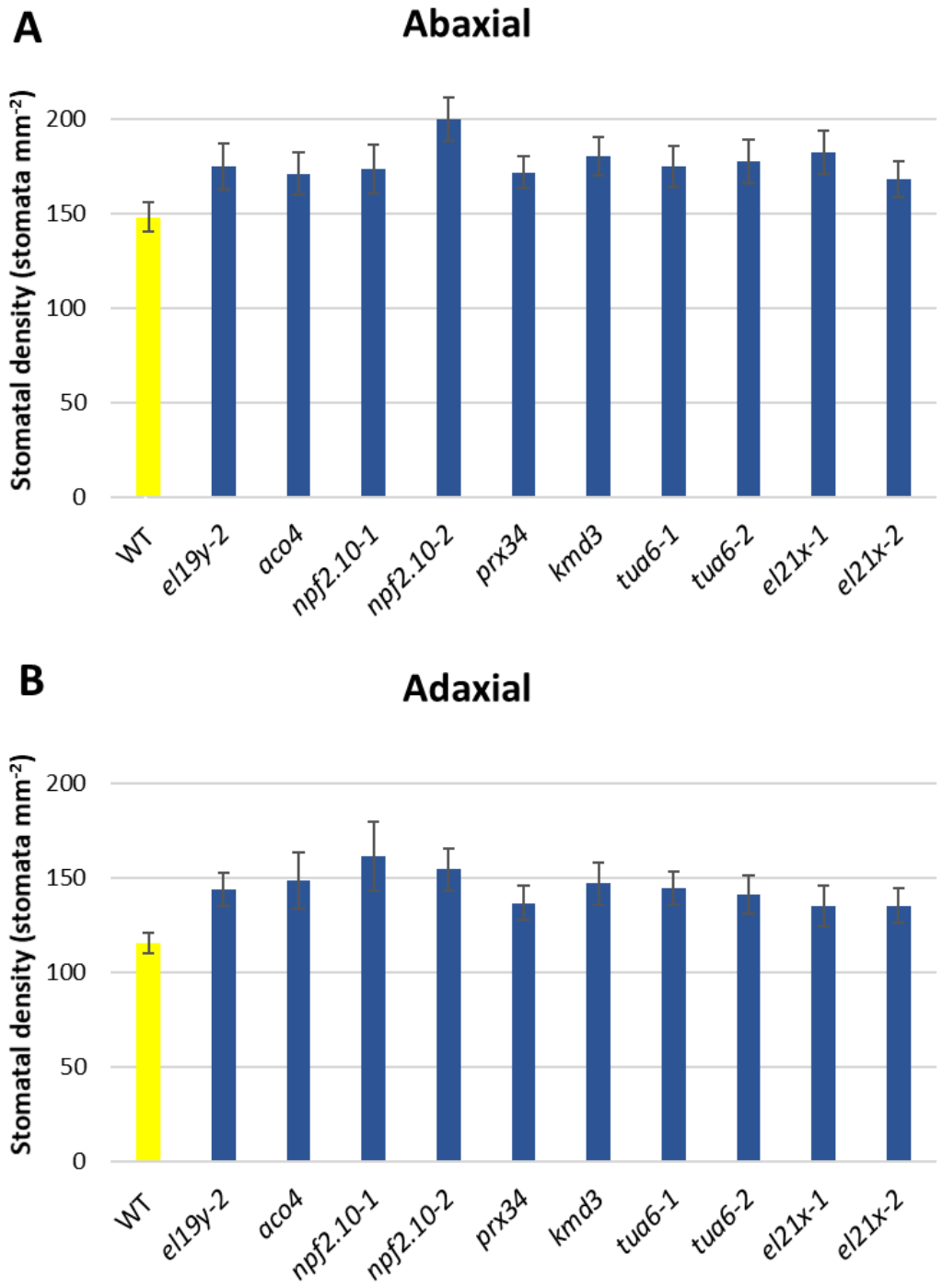

Fig. 1. Bars show average stomatal density in abaxial (A) and adaxial (B) leaf surfaces, n=17-18 plants for each genotype. Significant differences were determined by one-way ANOVA. No statistically significant differences were found compared with wild type (WT) at $P<0.05$.

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