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The role of SPEN3 in stomatal development and plant growth in *Arabidopsis thaliana*

Bachelor's Thesis (12 ECTS)

Curriculum Science & Technology

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Tartu 2024

The role of SPEN3 in stomatal development and plant growth in *Arabidopsis thaliana*

Abstract

Stomata are small openings on the surface of the leaves, which are crucial for plants' gas exchange. This study focused on analyzing stomatal density and conductance in plants deficient in the *SPEN3* gene. SPEN3, as an RNA-binding protein, assists in transcriptional regulation, posttranscriptional processing, and nuclear export of mRNA. By growing plants under controlled conditions, taking stomatal imprints and measuring stomatal conductance we were able to assess stomatal traits in wild-type and *spen3* mutants. Those assessments showed a lower stomatal density on the bottom leaf side of the *spen3* mutants compared to wild type plants, whereas no statistically significant differences in stomatal conductance or plant growth were observed. Further studies are needed to elucidate the role of SPEN3 in plant water use efficiency.

Keywords

Stomata, stomatal development, stomatal density, stomatal conductance, SPEN3, *Arabidopsis thaliana*

CERCS: B225 plant genetics

SPEN3 roll hariliku müürlooga õhulõhede arengus ja taimekasvus

Lühikokkuvõte

Õhulõhed, väikesed avad lehtede pinnal, on olulised taimede gaasivahetuse jaoks. SPEN3 on RNA-d siduv valk, mis osaleb traskriptsiooni reguleerimises, post-transkriptsioonilises RNA töötlemises ja mRNA ekspordis tuumast. Käesolevas töös uuriti, kas vigane *SPEN3* avaldab mõju õhulõhede tihedusele ja juhtivusele. Leiti, et kontrollitud tingimustes kasvanud *spen3* mutantide õhulõhede tihedus lehe alaküljel on oluliselt väiksem kui metsiktüüpi taimedel, kuid nende õhulõhede juhtivus ega kasv ei erinenud metsiktüüpi taimedest. On vaja täiendavaid uuringuid, et selgitada SPEN3 rolli taime veekasutuse reguleerimisel.

Võtmesõnad:

Õhulõhe, õhulõhede areng, õhulõhede tihedus, õhulõhede juhtivus, SPEN3, *Arabidopsis thaliana*

CERCS: B225 Taimegeneetika

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ABBREVIATIONS

ABA – abscisic acid

ANOVA – analysis of variance

EPF1 – EPIDERMAL PATTERNING FACTOR 1

EPF2 – EPIDERMAL PATTERNING FACTOR 2

GC – guard cell

GMC – guard mother cell

HSD – honestly significant difference

MMC - meristemoid mother cell

SE – standard error

SLGC - stomatal lineage ground cell

SPEN3 - SPEN FAMILY TRANSCRIPTIONAL REPRESSOR 3

INTRODUCTION

Stomata – pores on the surface of leaves – have been a center of attention among scientists for a long time. Not only do they serve one of the most significant functions in plants, i.e., photosynthesis, but also help plants to quickly respond and adapt to their environment. Recent scientific advances have shed light to the understanding of the physiology and evolution of stomata (Chater *et al.*, 2017; Lawson and Matthews, 2020a).

Nowadays, water scarcity brings up new challenges for agricultural productivity. This indicates the need for a better understanding of plants' water use as well as their response to different water deficit circumstances. Central to this complex topic is the concept of water-use efficiency which is a key determinant of a plant's ability to balance carbon assimilation with transpiration. Plants themselves have developed an ability to respond to water deficit. Such ability lies in the stomatal opening and closure. If water deficit occurs, stomata close to reduce the loss of water through transpiration. However, under prolonged water deficit conditions, additional adaptations may be seen. These adaptations include the production of leaves with altered stomatal density and size, which results in reduced maximum stomatal conductance. How such a developmental response to drought occurs in leaves remains unclear but it affects plant water use efficiency. Understanding stomatal development regulation is crucial for improvement of plants resilience to water scarcity as well as optimization of their survival in a changing environment.

There are still a lot of aspects of stomata, important parts of plant vegetative structure, which remain undiscovered. It is pivotal to bring up and answer new questions regarding the role and function of the stomata in plants as not only will it contribute to the expansion of apprehension of plant physiology but also assist in the understanding of plants' behavior and their adjustment to specific growth environments and climate conditions.

1 LITERATURE REVIEW

1.1 STOMATA AND THEIR FUNCTION

Stomata are microscopic pores surrounded by pairs of guard cells found in the epidermis of plant leaves. Guard cells are kidney- or dumbbell-shaped cells situated on the aerial parts of plants (Figure 1). Kidney-shaped guard cells are mainly found in dicots as well as in mosses, gymnosperms, and ferns, while dumbbell-shaped guard cells are present in monocots, including grasses (Bertolino, Caine and Gray, 2019). Stomata play a pivotal role in gas exchange between the environment and the plant. Gas exchange helps to carry out photosynthesis which is essential for plant's life cycle, and evaporative cooling of the plant leaf by water loss. Namely, oxygen, which is the main component of plant respiration, and carbon dioxide (CO_2) pass through the stomata when guard cells are turgid and stomata open. At the same time, stomata can prevent unnecessary water loss in plants by closing under water-limited conditions. The positioning of stomata differs in plant leaves. The majority of plants have stomata only on the lower leaf surface (hypostomy), however, some species including the model plant Arabidopsis (*Arabidopsis thaliana*) have stomata on both the upper and lower leaf surfaces (amphistomaty) (Muir, 2015; Drake *et al.*, 2019).

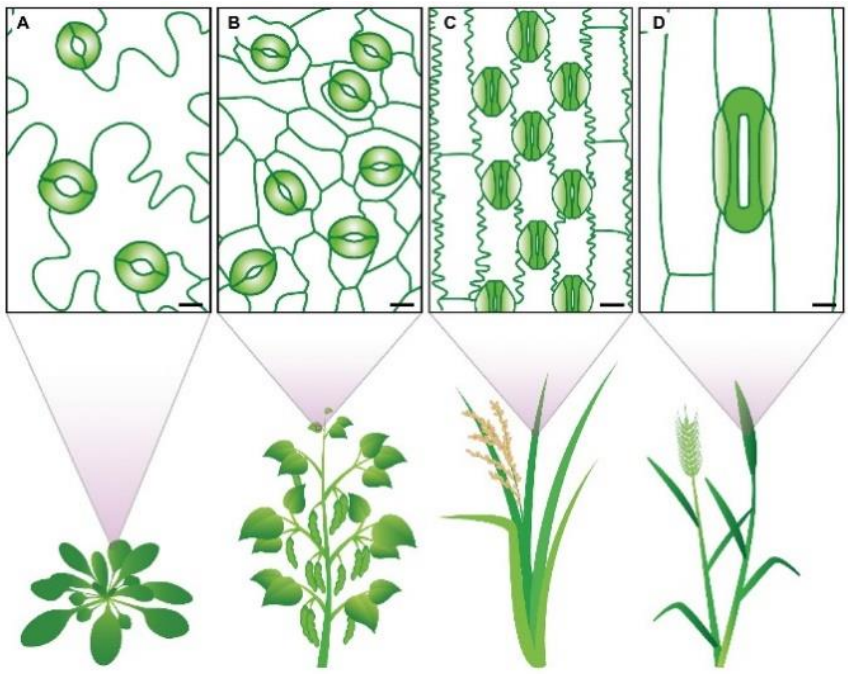

Figure 1. Guard cell shapes in different plant species. Kidney-shaped guard cells in the eudicots (A) *Arabidopsis thaliana* and (B) *Phaseolus vulgaris*. Dumbbell-shaped guard cells (solid green) and specialized subsidiary cells (light green) in the grasses (C) *Oryza sativa* and (D) *Triticum aestivum* (Bertolino, Caine and Gray, 2019).

The outer surface of the epidermis is covered by a layer named cuticle. The cuticle helps in the reduction of water loss through transpiration as well as protects the leaf interior from external environmental factors (Guzmán-Delgado, Laca and Zwieniecki, 2021).

Guard cells are the main opening and closing regulators of the stomatal pore. The stomatal pore together with the guard cells, and sometimes subsidiary cells, makes up the stomatal complex. The cells of the stomatal complex immediately surrounding the guard cells provide structural support together with assisting in stomatal opening and closing (Clark *et al.*, 2022; Nguyen and Blatt, 2024). Guard cell walls consist of polysaccharide-based wall polymers (Doblin, Pettolino and Bacic, 2010). They are the main providers of the elasticity and strength of cell walls, which assist in stomatal opening and closure. The organization of cellulose microfibrils in the cell wall results in proper stomatal opening mechanisms (Yi *et al.*, 2018; Pautov *et al.*, 2019). The inner side of guard cells has a thicker cellulose cell wall compared to the outer side (Shtein *et al.*, 2017). This asymmetry in thickness is the reason for the shape of the guard cells.

The opening and closing of stomata are regulated by the turgor pressure within the guard cells. When environmental conditions become less favorable the guard cells lose turgor pressure, which facilitates the closure of stomatal pores, reducing transpiration. Stomatal opening takes place when K^+, Cl^-, malate^{2-}, and sucrose build up inside the cells (Lawson and Matthews, 2020a). This enables water entry into the guard cells by opening up the stomatal pore. Stomatal closure occurs when the efflux of previously mentioned ions takes place, resulting in a reduction in cell turgor and prevention of water loss by the closure of pores. Such process of stomatal closure reduces plant transpiration. Stomatal transpiration is a dynamic process that is influenced by various factors. Those factors include intensity of light, temperature, and relative air humidity. Increased light intensity stimulates stomatal opening since it is associated with higher rates of photosynthesis (Lawson and Matthews, 2020b). Warmer temperatures may enhance the transpiration rate by increasing the water-carrying capacity of the air, while lower relative air humidity creates a steeper water potential gradient moving the water molecules out of the leaf.

1.2 STOMATAL CONDUCTANCE

Stomatal conductance refers to the ease with which gases, particularly water vapor and carbon dioxide move through the stomatal pores in plant leaves. Therefore, it mainly depends on the size, density, and degree of opening of stomata, where widely open stomata allow for greater

conductance as well as higher photosynthesis and transpiration rates. Stomatal conductance is measured in mmol m^{-2} s^{-1} and is affected by different factors.

One of the factors that control stomatal conductance are the photoreceptors which play an important role in stomatal opening (Kinoshita *et al.*, 2001; Wang *et al.*, 2010). Sufficient intensity of light leads to the opening of stomata in leaves and as a result of this, carbon dioxide would be taken up efficiently during photosynthesis, which leads to increased productivity. On the opposite, in the dark, stomata close and stomatal conductance decreases. Additionally, stomata open up in response to temperature. Mostly, the stomatal opening occurs in the same direction as that of the temperature until it gets too high or low (Driesen *et al.*, 2020). Another factor affecting stomatal opening mechanism is relative air humidity. Low relative air humidity leads to stomatal closure (Jalakas *et al.*, 2021). Under high CO_2 concentrations stomatal conductance decreases.

1.2.1 Regulation of stomatal conductance by hormones

Stomatal conductance is mostly regulated by a network of hormonal signals that act as molecular messengers, integrating various internal and external cues. Abscisic acid (ABA) is a key stress hormone that has a direct influence on stomatal conductance (Pantin *et al.*, 2013; Negin and Moshelion, 2016). ABA synthesis is usually activated by various environmental stressors, such as drought or high salinity, expressing the need for water conservation (Damour *et al.*, 2010). Consequently, ABA induces stomatal closure, minimizing water loss by transpiration (Hsu *et al.*, 2021). Contrasting with ABA, hormones like auxins and cytokinins contribute to stomatal opening and growth under favorable conditions (Damour *et al.*, 2010).

As stress conditions directly influence plants' hormonal response, ABA levels increase accordingly. ABA binds to its receptors and initiates a signaling cascade that activates protein kinases such as OPEN STOMATA 1 (OST1) and GUARD CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1) (Lee *et al.*, 2009; Hua *et al.*, 2012). They phosphorylate target proteins involved in stomatal regulation. Such phosphorylation results in the opening of anion channels, causing an efflux of anions. This results in stomatal closure (Merilo *et al.*, 2015; Hsu *et al.*, 2021).

1.3 STOMATAL DEVELOPMENT

Stomata are produced in a specific cell lineage (Figure 2). The lineage consists of several cell divisions, which can be categorized into asymmetric and symmetric divisions (Bergmann and Sack, 2007). Stomatal development starts with an undifferentiated epidermal cell, known as a

protodermal cell, that enters the stomatal lineage by adopting a meristemoid mother cell (MMC) identity. The first division of an MMC is considered asymmetric since it produces one meristemoid cell and a larger sister cell, also known as a stomatal lineage ground cell (SLGC) (Shpak *et al.*, 2005). Meristemoids divide only a few times before differentiating into guard mother cells (GMCs), which undergo a single symmetric division and form a pair of guard cells, the terminal cells in the lineage (Lau and Bergmann, 2012). SLGCs can divide asymmetrically again or become pavement cells. These divisions play a pivotal role in increasing the total number of epidermal cells. The spacing divisions occur after amplifying divisions and help in positioning of stomata, so they will not be in direct contact with one another.

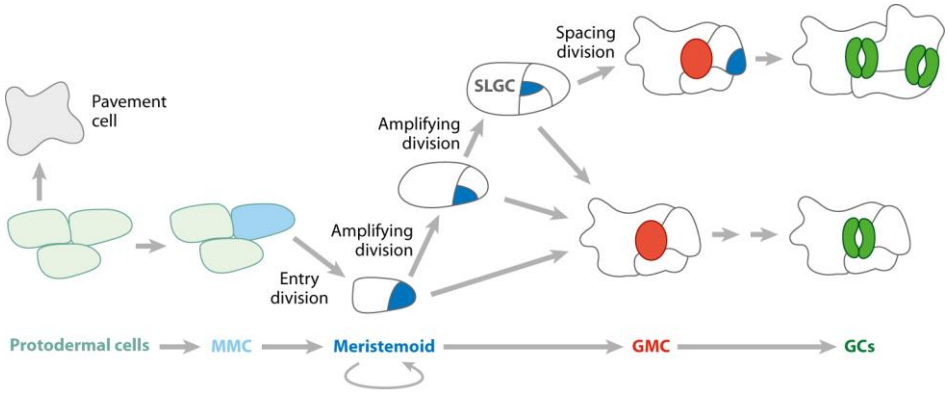

Figure 2. Major stages of stomatal development in Arabidopsis, where MMC stands for meristemoid mother cell, SLGC – stomatal lineage ground cell, GMC – guard mother cell, and GC – guard cell (Pillitteri and Torii, 2012).

There are several main regulators which are essential for stomatal lineage transitions. Those are SPEECHLESS (SPCH), MUTE, FAMA, and epidermal patterning factors EPF1 and EPF2. The *SPCH* gene, which encodes a basic helix–loop–helix (bHLH) transcription factor, is crucial for the occurrence of asymmetric divisions that initiates stomatal linage (MacAlister, Ohashi-Ito and Bergmann, 2007). The bHLH transcription factor MUTE promotes the change of meristemoid cell to guard mother cell (GMC) (Pillitteri *et al.*, 2007). Another important bHLH transcription factor is FAMA. It is mainly responsible for the final differentiation of the stomata (Ohashi-Ito and Bergmann, 2006).

Stomatal density, the number of stomata per unit leaf area on the plants' aerial surfaces, varies; however, there is a consistent feature, which is the one-cell spacing rule between stomata (Larkin *et al.*, 1997). This rule ensures that all stomata are separated by at least one pavement cell. The one-cell spacing pattern occurs during asymmetric cell division next to an already existing stoma. This aids in the right positioning of the stoma, SLGC, and meristemoid. The

stoma is positioned next to the SLGC, which separates it from the meristemoid. This process occurs in every asymmetric division, where the potential formation of the new stoma takes place. Such orientation of the cells happens due to the cell-to-cell signaling process driven by the peptides EPF1 and EPF2. EPF1 is necessary for the maintenance of the spacings between stomata, whilst EPF2 determines the number of cells that enter the stomatal lineage (Hara *et al.*, 2007; Hunt and Gray, 2009). It has been found that the lack of aforementioned patterning factors increased stomatal density, while overexpression of them has been shown to reduce stomatal numbers (Hunt and Gray, 2009; Hughes *et al.*, 2017; Caine *et al.*, 2019; Dunn *et al.*, 2019a).

Stomatal spacing is most likely an adaptive mechanism of the plants, which serves several functions. Firstly, this spacing might generate solute reservoirs between stomata, which help in the fair distribution of nutrients to the plant (Bergmann and Sack, 2007). Secondly, it minimizes the mechanical interference that may occur between adjacent cells, and this in turn optimizes the distribution of gas diffusion (Bergmann and Sack, 2007).

1.4 ROLE OF SPEN3 IN ARABIDOPSIS

In Arabidopsis, the *SPEN FAMILY TRANSCRIPTIONAL REPRESSOR 3* (*SPEN3*) gene encodes a 117.47-kDa protein that has a conserved RNA recognition motif (RRM) and a SPOC (Spen Paralog and Ortholog C-terminal) protein binding domain (Woloszynska *et al.*, 2019). Spen (Split Ends) proteins function in transcriptional regulation, posttranscriptional processing, and nuclear export of mRNA (Ariyoshi and Schwabe, 2003). Wolonszynska et al. (2019) showed that SPEN3 along with another RNA-binding protein KHD1 (KH-DOMAIN PROTEIN 1) interact with the HUB1/HUB2 complex to influence pre-mRNA processing of the circadian clock and flowering time regulatory genes. Single *spen3-1* mutant displays a delayed flowering time and reduced primary root length, while double mutant of *spen3-1 hub1-4* displayed an early flowering phenotype similar to *hub1* single mutant, suggesting that HUB1 is epistatic to SPEN3 (Woloszynska *et al.*, 2019). At present, the role of SPEN3 in stomatal regulation is unknown.

2 THE AIMS OF THE THESIS

The aims of this study are:

- Characterization of stomatal development in plants deficient in the *SPEN3* gene
- Characterization of stomatal physiology and growth in plants deficient in the *SPEN3* gene

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Plant lines and growth conditions

Arabidopsis wild type (Col-0) and *spen3* mutant lines in the same background were used for experiments. The information on the mutant lines is highlighted in Table 1. The T-DNA insertion mutants of *SPEN3* were ordered from Nottingham Arabidopsis Stock Centre (NASC, (Scholl, May and Ware, 2000)) and checked for homozygosity prior to experiments in the host lab.

Table 1. Plant lines used in this study.

***A. thaliana* lines**	**T-DNA insertion line name**	**Position of the insertion**
Col-0	-	-
spen3-1	GABI_626H01	2nd exon
spen3-2	SAIL_363_H11	2nd intron
spen3-3	GABI_461F01	6th intron

Plants were grown in growth cabinets (Microclima Arabidopsis MCA1600-3LP6-E, Snijders Scientific, Tilburg, Netherlands), under the photoperiod of light for 10 hours at a light intensity of 250 μmol m^{-2} s^{-1} and darkness for 14 hours. Relative air humidity was 60% during the day and 80% at night and temperature was 23°C during the day and 19°C at night.

3.1.2 Stomatal patterning

In order to observe the stomatal densities of the leaves of different mutants, leaves were numbered according to the spiral order of *Arabidopsis thaliana* growth pattern (Figure 3) (Farmer *et al.*, 2013) and the 10th leaf was determined on the 4th and 5th week of the plant's growth.

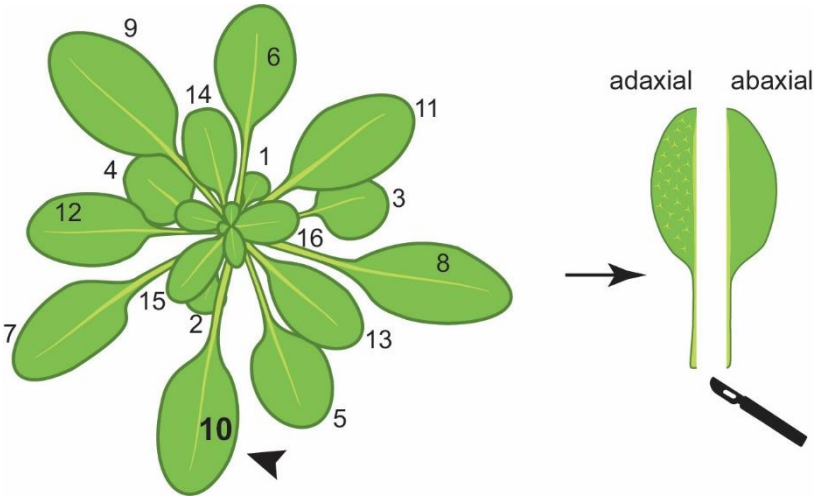

Figure 3. Arabidopsis growth spiral (Modified from (Jalakas *et al.*, 2024)). The 10th leaf was sampled for stomatal anatomical trait analyses.

Leaf number 10 was sampled from plants at 7 weeks of age. The extracted 10th leaf was cut in half to make impressions of the stomata situated on both the top (adaxial) and the bottom (abaxial) sides of the leaf. The impressions were collected by application of the following steps. Dental silicone (Speedex light body, Coltene/Whaledent AG for top leaf side; Alstätte, Switzerland and oranwash L, Zhermack; www.zhermack.com for bottom leaf side) was applied to each side of the leaf. After the silicone hardening, nail varnish was applied to the silicone, and the imprints were taken via transparent tape and transferred to a microscope slide. An area of 0.254 mm^{-2} from each imprint was recorded via microscope (Kern OBF 133; Kern & Sohn GmbH; www.kern-sohn.com) with a 200 x magnification. Stomatal density (number of stomata mm^{-2}), total stomatal density (sum of top and bottom stomatal densities, stomata mm^{-2}), and stomatal ratio (stomatal density on the upper side of the leaf divided by stomatal density on the bottom side of the leaf) were determined by ImageJ software (National Institutes of Health, USA; Schneider et al. 2012).

3.1.3 Projected rosette area

In the second replicate of the experiment, whole plants were photographed at 4 weeks of age. The projected rosette area was determined with ImageJ software (Schneider, Rasband and Eliceiri, 2012).

3.1.4 Measurement of stomatal conductance

Stomatal conductance (mmol m^{-2} s^{-1}) was measured from both the top and bottom sides of one leaf per plant using porometer LI-600 (LI-COR Biosciences, Lincoln, NE, USA).

3.1.5 Statistical analysis of the acquired data

Pooled data from two independent experiments are shown. The statistical test conducted was one-way analysis of variance (ANOVA) with Tukey *post hoc* test or linear regression, which is indicated in the corresponding figure legends. The results with a p-value less than 0.05 were considered significant. Statistical analyses were done using the STATISTICA 7.0 program.

3.1.6 Usage of AI tools

I used the assistance of ChatGPT (OpenAI, communication was held on 24th April 2024) in the literature review part of my thesis as a paraphrasing tool for several sentences in order to convey my thoughts in a clearer and reader-friendly structure. ChatGPT is an AI-driven text generator developed by OpenAI (2023).

3.2 RESULTS

3.2.1 Stomatal patterning traits in the *spen3* mutants

To test whether SPEN3 is involved in stomatal development, we measured stomatal density from both the top and bottom sides of the leaf. Stomatal density was significantly lower on the bottom side of the leaf in all the studied *spen3* mutant lines compared with wild type Col-0 plants (Figure 4). However, there were no differences in stomatal densities on the top side of the leaf (Figure 4; $p = 0.42$, one-way ANOVA).

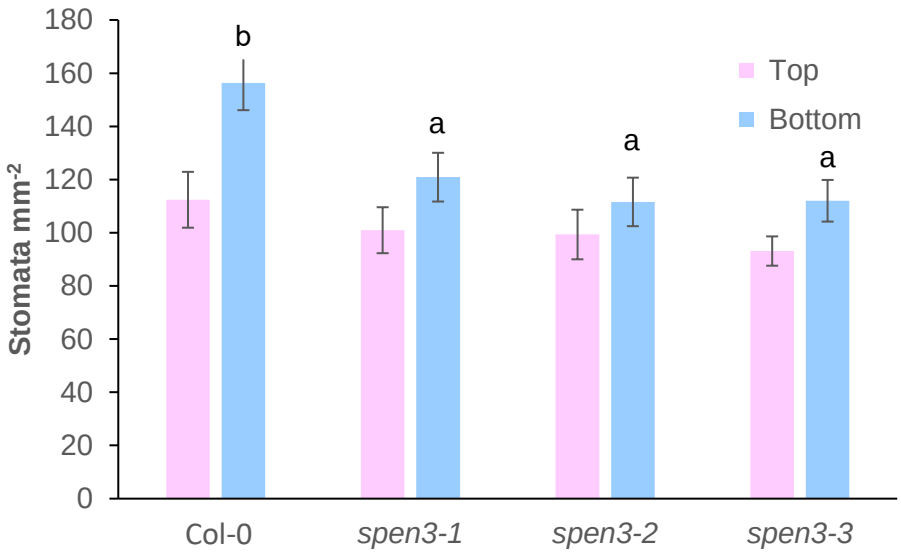

Figure 4. Stomatal density of seven to eight-week-old plants on the top and bottom side of the leaf. Letters denote statistically significant differences between stomatal density on the bottom side of the leaf (ANOVA with Tukey Unequal N HSD *post hoc* test, $p < 0.05$; n=21-22 plants). The data is represented as average ± SE.

Total stomatal densities were also measured. Although all the *spen3* mutants showed a decrease in total stomatal density, it was significantly decreased only in the *spen3-3* mutant compared to Col-0 (Figure 5).

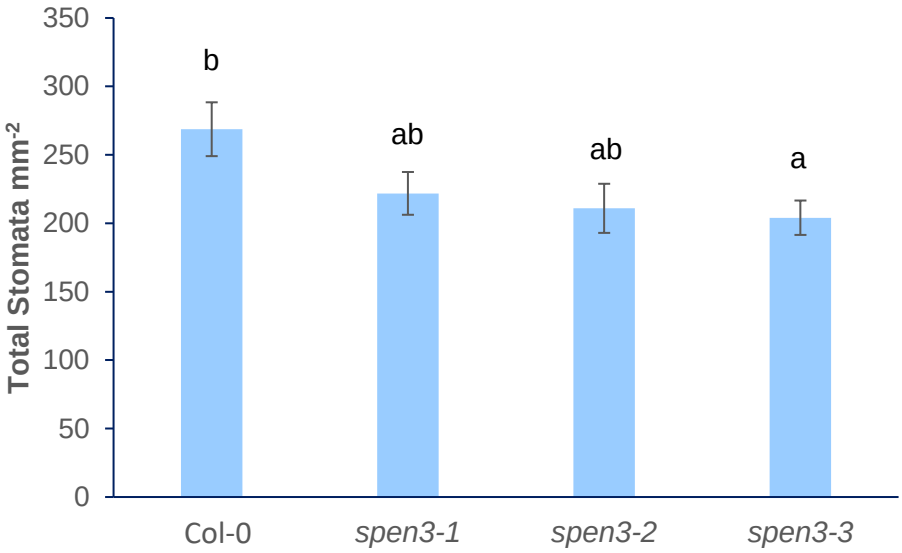

Figure 5. Total stomatal density of seven to eight-week-old plants. Letters denote statistically significant differences between the total stomatal density (ANOVA with Tukey Unequal N HSD post hoc test, $p < 0.05$; n=21-22). The data is represented as average ± SE.

We measured stomatal ratio to see how evenly stomata were distributed across both leaf surfaces. By dividing the stomatal density of the top side of the leaf to the bottom side we found that there was a significant difference between Col-0 and *spen3-2* plants, whereas Col-0 had the lowest stomatal ratio (Figure 6).

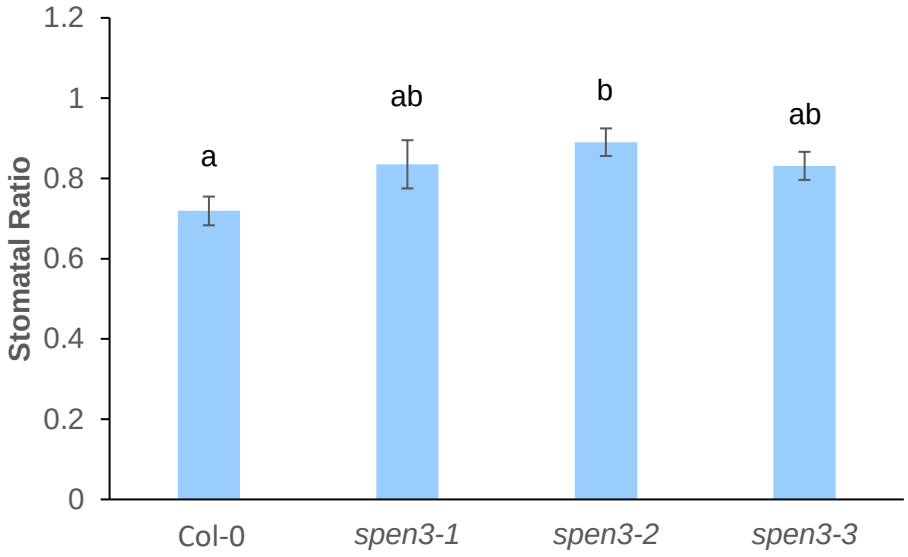

Figure 6. Stomatal ratio of seven to eight-week-old plants. Letters denote statistically significant differences between the stomatal ratio (ANOVA with Tukey Unequal N HSD post hoc test, $p < 0.05$; n=21-22). The data is represented as average ± SE.

3.2.2 Stomatal conductance in the *spen3* mutants

By measuring stomatal conductance using porometer we found no significant difference in stomatal conductance in the bottom and top sides of the leaf between different plant lines. However, stomatal conductance differed between bottom and upper sides of the same plants, possibly due to the reduced number of stomata on the upper side of the leaf (Figure 4, Figure 7).

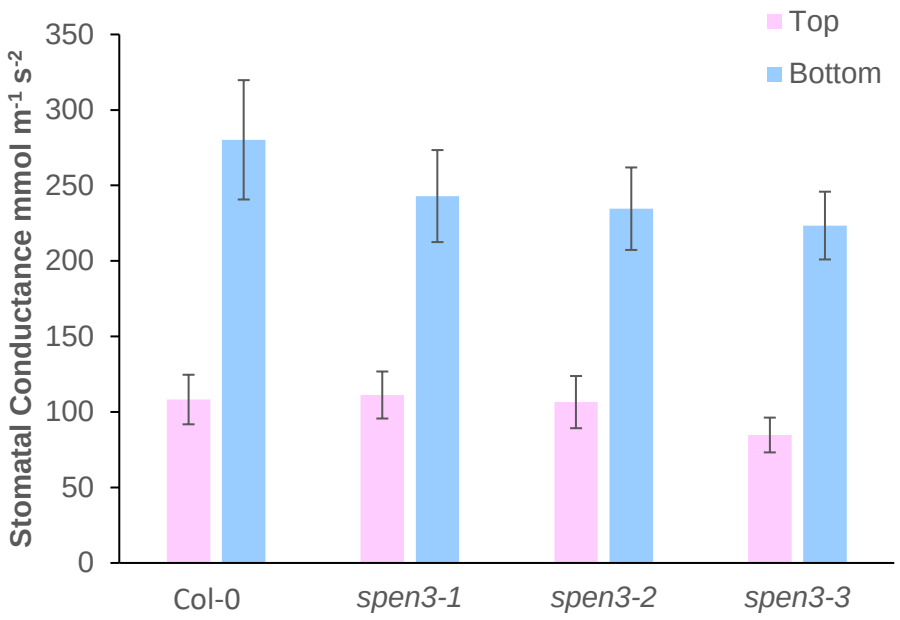

Figure 7. Stomatal conductance of five to six-week-old plants on top and bottom side of the leaf. The data is represented as average ± SE; p (bottom conductance) = 0.6; p (top conductance) = 0.6, one-way ANOVA.

Stomatal conductance ratio, measured by division of the conductance on the top side to the bottom side of the leaf, showed us that there were no significant differences in stomatal conductance ratio among genotypes. Nevertheless, *spen3-2* appeared to have the highest stomatal conductance ratio among given plants (Figure 8).

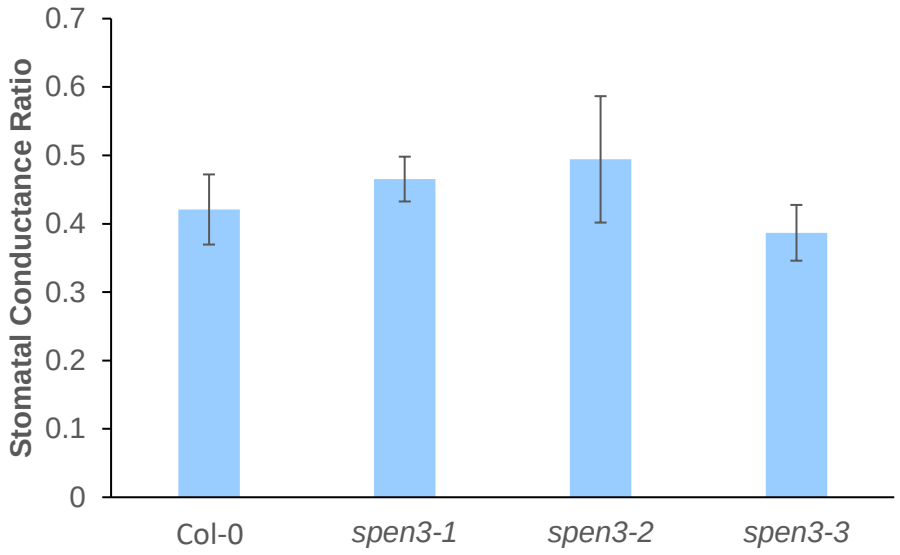

Figure 8. Stomatal conductance ratio of five to six-week-old plants. The data is represented as average ± SE; p=0.58, one-way ANOVA.

3.2.3 Growth and its relationship with stomatal density in the *spen3* mutants

Additionally, we measured the rosette area of four-week-old plants. Although *spen3-2* had the largest rosette area, there were no significant differences in the area found between the studied plant lines (Figure 9; p=0.5, one-way ANOVA).

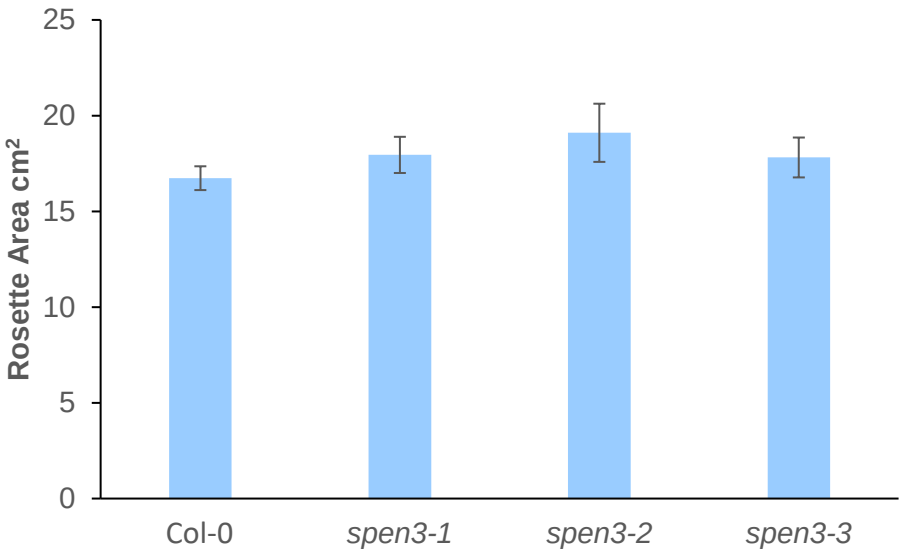

Figure 9. Projected rosette area (cm^2) measured in the second experiment of four-week-old plants. The data is represented as average ± SE.

We also analyzed the relationship between total stomatal density and projected rosette area by linear regression analysis. All of the plants were in a similar rosette area and stomatal density range and there was no significant relationship between stomatal density and projected rosette area (Figure 10; p=0.5, linear regression).

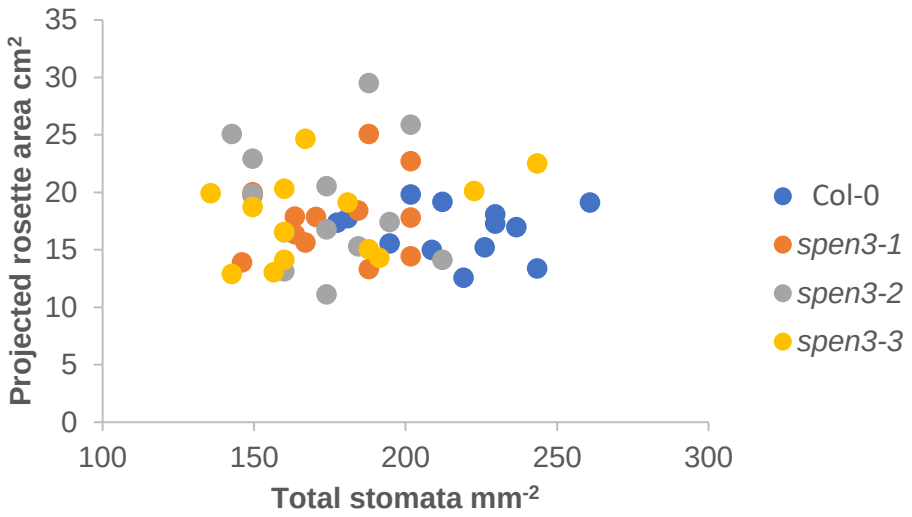

Figure 10. Projected rosette area (cm^2) corresponding to total stomatal density. Each dot represents one plant.

3.3 DISCUSSION

The outcomes of this study provide insights into the difference in stomatal density and stomatal conductance between the Col-0 wild-type and *spen3-1*, *spen3-2,* and *spen3-3* plants.

According to the results obtained within the conducted experiments (Figure 4), we were able to observe that stomatal density differs between the bottom and top sides of the leaf of every plant line, with relatively fewer stomata present on the upper leaf side. Such common distribution of stomata has also been seen in recent studies, which support our experimental outcomes (Jalakas *et al.*, 2024). We were able to observe that even though no significant differences were found within stomatal density on the top side of the leaves of the plants, a noticeable change was found within the bottom sides of the leaves: *SPEN3* deficient plants appeared to have lower stomatal density in comparison to Col-0 wild type plants. This suggests that the *SPEN3* gene is important for the development of stomata on the lower side of the leaf, whilst stomatal development on the upper side possibly has different development mechanism.

Research on the topic of stomatal density has shown that stomatal density is correlated with the expression of EPF1 and EPF2 epidermal patterning factors (Franks *et al.*, 2015). The lack of expression of both EPF1 and EPF2 resulted in overall higher stomatal density of the leaves of *Arabidopsis thaliana*, whereas stomatal density was increased relatively more on the lower leaf side (Jalakas *et al.*, 2024). This brings up the possibility that the *SPEN3* gene may affect the

expression of those patterning factors and therefore influence the stomatal density on the bottom side of the leaf. In order to test such an assumption, analysis of EPF1 and EPF2 gene expression in the *spen3* mutants would give insight into this hypothesis. Nevertheless, since *SPEN3*-deficient plants have shown a significant reduction of stomatal density only on the bottom side of the leaf, the *SPEN3* gene may only have an impact on the stomatal development on the bottom side, whilst the top side of the leaf can have different developmental mechanisms. Similarly, STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1) and TOO MANY MOUTHS (TMM) seem to be more important for regulating stomatal development on the bottom side of the leaf (Jalakas *et al.*, 2024). This supports the suggestion of stomatal development on the lower and upper sides of the leaves being regulated by different developmental mechanisms.

Stomatal conductance is strongly related to stomatal density. Commonly, higher stomatal density corresponds to higher stomatal conductance in the same plants (Franks and Beerling, 2009). However, the results of our experiment showed that this does not take place in *SPEN3*-deficient plants and their stomatal conductance is not significantly affected by lowered stomatal density on the bottom sides of the leaves (Figure 7). This may be due to *SPEN3*-deficient plants potentially having more open stomata than Col-0 wild type plants. Such an assumption may be tested by measurements of stomatal apertures in the *spen3* mutants. The uptake of CO_2 and overall gas exchange is higher in the stomata with a wider opening (Franks and Farquhar, 2007).

The outcomes of the experiment also provided insight into the possible correlation of plant growth with the state of the *SPEN3* gene (Figure 9). The results show that there was no significant difference in rosette area between *SPEN3*-deficient and Col-0 wild type plants. There was also no significant relationship between stomatal density and plant growth in the studied plants (Figure 10). Such results at first sight oppose past studies, which have shown that stomatal density is negatively related with plant growth (Tulva, Koolmeister and Hõrak, 2023; Jalakas *et al.*, 2024). The obtained data from our experiment may indicate that despite lower stomatal density on the bottom side of the leaves, *SPEN3*-deficient plants overall stomatal density is in a suitable range for unaltered plant growth. Additionally, *SPEN3*-deficient plants may create an opportunity for the development of plants with optimal growth and better water-use efficiency ability, as lower stomatal densities without a penalty on growth reduce plant water loss. Such traits may be useful for agricultural applications. A similar impact has been found before for overexpression of epidermal patterning factors in wheat (Dunn *et al.*, 2019b).

It has been shown that lower stomatal density has its effects on the overall disease resistance of the plants (Dutton *et al.*, 2019). Lower stomatal density means fewer entry points for various

plant pathogens. Such a correlation between stomatal density and disease resistance brings up the possibility of *SPEN3*-deficient plants to be more resistant to different pathogens. By infecting *spen3* mutants with a certain stomata-entering pathogen and analyzing disease progression it may be possible to test the possibility of *spen3* mutants being resistant to pathogens.

In conclusion, despite the reduced stomatal density on the lower leaf side, stomatal conductance remained unchanged in the *spen3* mutants, indicating the presence of possible compensatory mechanisms. Additionally, there was no significant difference in plant growth between the *spen3* mutants and wild-type plants. Such findings suggest that the *spen3* mutants could offer sufficient growth together with better water-use efficiency due to lower stomatal density. This indicates the possibility of *spen3* mutants possessing better survival rate in drought conditions. Nevertheless, *SPEN3*-deficient plants response to different environmental conditions has not been measured during the conducted experiment, and therefore responses of *spen3* mutants to such altered conditions as changes in air or soil humidity should be measured in order to test possible application of *spen3* mutants in drought conditions. Overall, further research into the role of SPEN3 in stomatal development and its impact on plant growth would provide valuable data for the improvement of crops in agriculture.

SUMMARY

Stomatal opening and closure are irreplaceable processes, which facilitate and directly affect plant's ability for photosynthesis, balance carbon assimilation with transpiration, and impact plant water-use efficiency. One of the important aspects of the development of such mechanisms is the *SPEN3* gene, which affects transcriptional regulation, posttranscriptional processing, and nuclear export of mRNA. The main purpose of this study was to characterize the role of the *SPEN3* gene in both stomatal development and growth patterns in *Arabidopsis thaliana*. The study included stomatal and growth analyses of *spen3-1*, *spen3-2*, and *spen3-3* mutants, as well as Col-0 wild type plants.

Our findings showed that *SPEN3*-deficient plants had significantly lower stomatal density on the bottom sides of the leaves in comparison to Col-0 wild type plants. However, no significant changes in stomatal density on the upper side of the leaves between *spen3* mutants and wild type plants was seen. This suggests that there are different stomatal development mechanisms on the upper and lower sides of the leaves. Despite past studies, which have shown direct correlation between stomatal density and stomatal conductance, stomatal conductance was not affected in the *SPEN3*-deficient plants. Lower stomatal density may also affect plant growth; however, no significant growth alterations were detected in *SPEN3*-deficient plants. Although past studies showed a negative relationship between stomatal density and plant growth, such relationship was not seen here.

This study also suggests possible future implementation of *SPEN3*-deficient plants in agriculture, as these plants may have better water-use efficiency together with optimal growth patterns. *SPEN3*-deficient plants may also have better pathogen resistance due to lower stomatal density.

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