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The Role of HvSnRK2.7 and HvSnRK2.9 in Stomatal Regulation of Barley

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The Role of HvSnRK2.7 and HvSnRK2.9 in Stomatal Regulation of Barley

Abstract

This thesis investigated the role of barley SnRK2 kinases HvSnRK2.7 and HvSnRK2.9 in stomatal regulation using two double mutant lines, L15 and L20, and the wild type cultivar Golden Promise. Gas exchange measurements with Licor-6800 showed that mutant plants had higher stomatal conductance and slower stomatal closure in response to reduced relative air humidity compared with wild type plants. Mutants also maintained more stable net CO_2 assimilation under low humidity conditions. No significant differences in stomatal density were detected, indicating that the altered stomatal regulation was caused by changes in stomatal aperture rather than stomatal development. Increased stomatal conductance was also observed in awns and leaf sheaths, suggesting that HvSnRK2.7 and HvSnRK2.9 regulate gas exchange in multiple above-ground organs. Overall, the results demonstrate an important role for these SnRK2 kinases in barley stomatal regulation and responses to atmospheric humidity. Higher stomatal conductance and associated rise in photosynthesis may result in yield gains in well-watered conditions.

Keywords:

Barley, stomatal regulation, stomatal conductance, relative air humidity, barley awns, leaf sheath

CERCS: B310 Physiology of vascular plants; B225 Plant genetics

Institute name: Institute of Technology

Research group: Model and Crop Plant Ecophysiology

HvSnRK2.7 ja HvSnRK2.9 roll odra õhulõhede regulatsioonis

Lühikokkuvõte

Käesolev väitekiri uuris odra SnRK2 kinaaside HvSnRK2.7 ja HvSnRK2.9 rolli õhulõhede regulatsioonis, kasutades kahte topeltmutantliini, L15 ja L20, ning metsiktüüpi, sorti Golden Promise. Gaasivahetuse mõõtmised Licor-6800 gaasivahetusaparatuuriga näitasid, et topeltmutantidel oli metsiktüüpi taimedega võrreldes kõrgem õhulõhede juhtivus ja aeglasem õhulõhede sulgumine madala õhuniiskuse toimel. Kaksik mutantidel säilis madala õhuniiskuse tingimustes ka stabiilsem CO_2 netoassimilatsiooni kiirus. Õhulõhede tiheduses olulisi erinevusi uuritud genotüüpide vahel ei tuvastatud, mis viitab sellele, et muutunud õhulõhede regulatsioon oli põhjustatud pigem muutustest õhulõhe pilu laiuses kui kui õhulõhede arengus. Suurenenud õhulõhede juhtivust täheldati ka lehetuppedes ja ohetes, mis viitab sellele, et HvSnRK2.7 ja HvSnRK2.9 reguleerivad gaasivahetust mitmetes maapealsetes organites. Kokkuvõttes näitavad tulemused SnRK2 kinaaside olulist rolli odra õhulõhede regulatsioonis ja reaktsioonides õhuniiskusele. Kõrgem

õhulõhede juhtivus ning sellega kaasnev fotosünteesi tõus võib viia saagitõusuni hea veevarustusega taimedes.

Võtmesõnad:

Oder, õhulõhede regulatsioon, õhulõhede juhtivus, suhteline õhuniiskus, ohted, lehetupp

CERCS: B310 Soontaimede füsioloogia; B225 Taimegeneetika

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TERMS, ABBREVIATIONS AND NOTATIONS

ABA – Abscisic acid

Anet – Net CO_2 assimilation rate

gs – Stomatal conductance

RH – Relative air humidity

SD – Stomatal density

VPD – Vapor pressure deficit

L15 – independent barley double mutant in SnRK2.7 and SnRK2.9

L20 - independent barley double mutant in SnRK2.7 and SnRK2.9

OST1 – OPEN STOMATA 1 protein kinase

WT – Wild type

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops cultivated in temperate regions, including Northern and Eastern Europe, where it is widely grown for animal feed, food production, and malting industries (FAO, 2023; European Commission, n.d.). Its broad environmental adaptability allows cultivation under diverse climatic conditions, including regions characterized by low temperatures, drought, and relatively poor soil quality (Sattorova, 2024). Due to this adaptability and its economic importance, barley represents an important model crop for studying plant responses to environmental stress.

Climate change increasingly threatens cereal productivity worldwide through rising temperatures, altered precipitation patterns, and more frequent drought events. In Europe, climate trends have contributed to stagnation and reduction of barley yields, although changes in agricultural management and policy also play important roles (Moore & Lobell, 2015). Because drought stress directly affects plant water status and photosynthetic performance, understanding the mechanisms that regulate plant water use has become increasingly important for crop improvement.

Stomata play a central role in balancing CO_2 uptake for photosynthesis with water loss through transpiration. Their opening and closing are tightly regulated by environmental signals such as air and soil humidity, light, and CO_2 concentration, as well as by hormonal signalling pathways involving plant stress hormone abscisic acid (ABA) (Hetherington & Woodward, 2003; Lawson & Blatt, 2014). Protein kinases belonging to the SnRK2 family are key components of ABA-mediated stomatal regulation and drought responses in plants (Umezawa et al., 2010).

Much of the current understanding of stomatal signalling originates from studies in Arabidopsis thaliana, particularly regarding the OST1/SnRK2.6 kinase, which functions as a central regulator of stomatal closure (Mustilli et al., 2002). However, it remains unclear to what extent these regulatory mechanisms are conserved in grasses such as barley, which possess distinct stomatal morphology and physiology. Investigating stomatal regulation in barley therefore contributes both to understanding fundamental plant physiology and to identifying traits relevant for improving crop water-use efficiency under changing environmental conditions.

1 LITERATURE REVIEW

1.1 Stomatal regulation

Stomata are small openings located mainly on the surfaces of leaves, stems and some flower organs that act as important gateways for gas exchange, enabling photosynthesis and transpiration (Hetherington & Woodward, 2003; Lawson & Blatt, 2014). Stomata consist of two specialized guard cells that enclose a central pore, together forming a stomatal complex (Hetherington & Woodward, 2003). These guard cells contain chloroplasts and adjust their size to control the opening and closing of the pore (Shimazaki et al., 2007). Stomata regulate the exchange of gases between the interior of the leaf and the external environment, enabling carbon dioxide (CO_2) to enter and oxygen (O_2) to leave, which are vital processes for photosynthesis and cellular respiration (Lawson & Blatt, 2014). Changes in stomatal opening, influenced by environmental conditions and internal signals, control both CO_2 uptake and water loss through transpiration (Buckley, 2005). By managing these gas exchanges, stomata ensure sufficient CO_2 supply to the mesophyll photosynthetic machinery, help maintain suitable leaf temperature via evaporative cooling and preserve the plant's overall water balance (Hetherington & Woodward, 2003; Buckley, 2005). Furthermore, transpiration-driven water movement through the xylem contributes to the uptake and transport of mineral nutrients from the soil to aerial plant tissues (Sattelmacher, 2001).

Stomatal aperture is controlled by dynamic changes in the turgor pressure of the two guard cells that surround each pore, allowing plants to optimize water-use efficiency while maintaining proper gas exchange (Blatt, 2000; Kim et al., 2010). When guard cells gain turgor, the stomatal pore opens; when they lose turgor, the pore closes (Blatt, 2000). Stomatal conductance represents the rate of water vapour diffusion out of leaves per unit leaf area and is largely determined by stomatal density and stomatal aperture width (Lawson & Blatt, 2014)

Guard cell turgor is continuously regulated by environmental conditions and hormonal signals (Kim et al., 2010). Because mature guard cells lack plasmodesmata, most solute movement occurs through ion channels, transporters, and pumps located in the plasma membrane (Blatt, 2000). The uptake of ions into guard cells, together with water movement through aquaporins, generates the pressure required to keep stomata open, while ion and water efflux reduces turgor and promotes closure (Maurel et al., 2008; Schroeder et al., 2001).

Stomata are also regulated by environmental signals, with light being one of the most influential factors controlling their behavior. Light-induced stomatal opening is driven by two interconnected processes: activation of plasma membrane H^+- ATPases in guard cells by light signals, and a reduction in internal leaf CO_2 concentration caused by photosynthetic activity (Roelfsema & Hedrich, 2005; Marten et al. 2007).

CO_2 inside the leaf acts as a key regulator: elevated intercellular CO_2 enhances guard-cell anion channel activity and promotes closure, whereas low CO_2 tends to favor opening (Mott, 1988; Hu et al., 2010).

Stomatal regulation is strongly influenced by atmospheric humidity through its effect on vapor pressure deficit (VPD), which represents the difference between the moisture inside the leaf and that in the surrounding air.(Zait et al.,2024) VPD can be expressed either as VPDair based on saturation vapor pressure at air temperature, or as VPDleaf, based on saturation vapor pressure at leaf temperature.(Kubota, 2023) Stomata respond not simply to relative humidity itself, but to the evaporative demand created by this gradient of vapour pressures. When humidity is low (high VPD), the driving force for water loss increases, causing more rapid transpiration from the leaf. In response, guard cells adjust their internal solute concentrations, altering turgor pressure and leading to a reduction in stomatal aperture, which serve to limit water loss. (Zait et al., 2024)

Increases in VPD can trigger active stomatal closure through biochemical signaling pathways, including those involving abscisic acid (ABA) and other guard-cell-specific regulators. When VPD rises enough to reduce leaf turgor, ABA levels can increase, promoting solute efflux from guard cells and decreasing stomatal conductance (Zait et al., 2024).

1.2 Abscisic acid signalling

Abscisic acid (ABA) is a key phytohormone involved in plant responses to abiotic stress, particularly drought, where it regulates stomatal aperture and reduces water loss through transpiration (Mustilli et al., 2002). During water-deficit conditions, ABA accumulates in plant tissues and triggers signalling pathways in guard cells that promote stomatal closure, thereby enhancing plant survival (Mustilli et al., 2002).

The ABA signalling pathway is based on a conserved core module consisting of ABA receptors, protein phosphatases, and protein kinases (Umezawa et al., 2010; Fujii et al., 2009). ABA perception leads to the inhibition of type 2C protein phosphatases (PP2Cs), which act as negative regulators of the pathway in the absence of the hormone (Umezawa et al., 2010). This inhibition of PP2Cs allows the activation of SNF1-related protein kinases 2 (SnRK2s), which function as central positive regulators of ABA signalling (Fujii et al., 2009). See Figure 1.

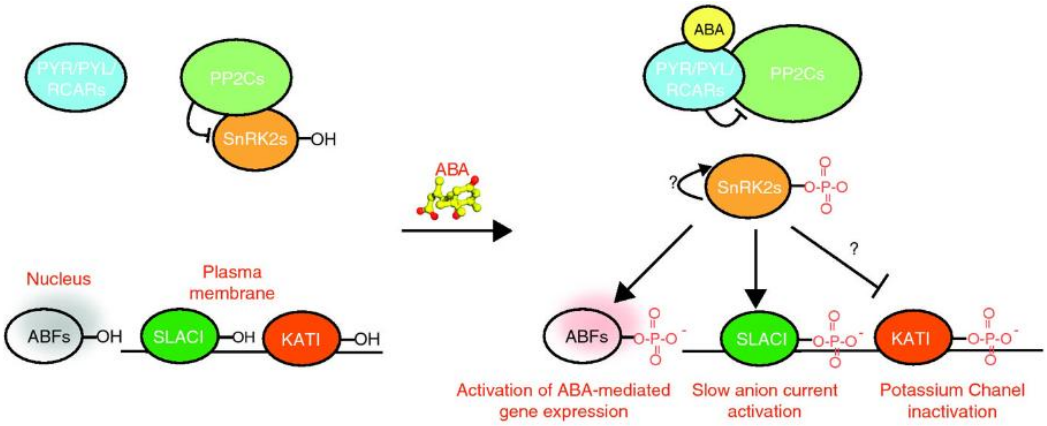

Figure 1. The core ABA signalling pathway in plants. In the absence of ABA, PP2C phosphatases inhibit SnRK2 protein kinases, preventing activation of ABA responses. When ABA is present, it binds to PYR/PYL receptors, which then inhibit PP2Cs. This releases SnRK2s from inhibition and allows them to become active. Activated SnRK2s phosphorylate downstream targets, including ABFs(ABRE-binding factors, transcription factors that recognize and bind to ABA-responsive elements(ABREs) involved in ABA-responsive gene expression, SLAC1 channels that promote anion transport, and KAT1 potassium channels, leading to physiological responses such as stomatal closure and stress adaptation. Figure from Weiner et al, 2010.

Activated SnRK2 kinases phosphorylate downstream targets, including transcription factors and membrane ion channels, resulting in both rapid cellular responses and longer-term changes in gene expression (Umezawa et al., 2010). In guard cells, these signalling events lead to ion efflux, reduction of turgor pressure, and ultimately stomatal closure (Mustilli et al., 2002). Additionally, secondary messengers such as calcium ions and reactive oxygen species (ROS) play an important role in amplifying and integrating ABA signals within guard cells (Mustilli et al., 2002).

A key component of the ABA signalling pathway is the protein kinase OST1 (OPEN STOMATA 1), a member of the SnRK2 family (Mustilli et al., 2002). OST1 is essential for Arabidopsis ABA-induced stomatal closure, as mutations in the OST1 gene result in impaired responses to ABA in guard cells (Mustilli et al., 2002). Functionally, OST1 acts downstream of ABA perception and contributes to the activation of signalling processes leading to ion channel regulation and stomatal movement (Mustilli et al., 2002; Umezawa et al., 2010).

The core components of ABA signalling are evolutionarily conserved across land plants, although their specific functions have diversified (Umezawa et al., 2010; Fujii et al., 2009). The presence of SnRK2 kinases and related regulatory elements in early plant lineages indicates that ABA signalling pathways were established early in plant evolution and later adapted to more complex physiological roles (Umezawa et al., 2010).

1.3 OST1 in *Arabidopsis thaliana*

OST1 (SnRK2.6) is a central component of ABA-induced stomatal closure in *Arabidopsis thaliana*, functioning as a key protein kinase in guard cell signalling. Genetic evidence from OST1 loss-of-function mutants demonstrates that OST1 is essential for stomatal responses not only to ABA, but also to reduced air humidity, light, and elevated CO_2 concentration, loss of OST1 function results in defective stomatal closure and increased water loss under drought conditions. (Mustilli et al., 2002; Merilo et al., 2017; Merilo et al., 2013).

At the molecular level, OST1 directly regulates ion transport processes that control guard cell turgor. One of its primary targets is the S-type anion channel SLAC1, which is phosphorylated and activated by OST1, leading to the efflux of anions such as chloride and nitrate from guard cells (Geiger et al., 2009; Brandt et al., 2012). In addition to SLAC1, OST1 also regulates other anion channels, including the R-type channel QUAC1, further contributing to ion release during stomatal closure (Imes et al., 2013). Studies of double mutants defective in both SLAC1 and QUAC1 further demonstrated that their rapid stomatal responses to environmental stimuli are largely abolished, highlighting the complementary roles of these anion channels in fast guard cell signaling (Jalakas et al., 2021). Concurrently, suppression of the guard cell plasma membrane H^+-ATPase contributes to plasma membrane depolarization, thereby favoring outward ion fluxes associated with stomatal closure (Jezek & Blatt, 2017). At the same time, OST1 inhibits inward potassium channels, thereby preventing potassium uptake that would otherwise promote stomatal opening (Sato et al., 2009). These coordinated changes in ion fluxes reduce guard cell osmotic potential, resulting in water efflux, loss of turgor pressure, and stomatal closure.

OST1 also plays a crucial role in the generation of ROS during ABA signalling in guard cells. It directly phosphorylates NADPH oxidases, leading to ROS production (Sirichandra et al., 2010). These ROS act as secondary messengers that amplify ABA signalling and further regulate ion channel activity, reinforcing stomatal closure.

In addition to ROS signalling, OST1 contributes to calcium-dependent signalling pathways. It promotes increases in cytosolic Ca^{2+} concentration, partly through the activation of calcium-permeable channels, thereby linking ABA signalling to calcium-mediated responses in guard cells (Brandt et al., 2015). This integration of calcium-dependent and independent pathways ensures a robust and finely tuned stomatal response to environmental stress.

Beyond its role in stomatal movement, OST1 functions as a central regulator of plant responses to osmotic stress at the molecular level. OST1 belongs to the SnRK2 kinase family and integrates both ABA-dependent and ABA-independent signalling pathways. Its activity is tightly controlled by type 2C protein phosphatases (PP2Cs), which maintain the kinase in an inactive, dephosphorylated state under non-stress conditions. Upon stress perception, inhibition of PP2Cs allows OST1 to

autophosphorylate and adopt an active conformation, thereby enabling downstream signalling (Yunta et al., 2011).

Structurally, OST1 contains a conserved regulatory motif that stabilizes key elements of the kinase domain, such as the αC helix, which is essential for catalytic activity. In addition, the C terminus of OST1 contains distinct regulatory domains involved in ABA-dependent and ABA-independent activation, with Domain II specifically required for ABA-responsive signaling while other regions contribute to osmotic stress responses independent of ABA. This structural feature provides a mechanistic basis for how OST1 can be activated independently of ABA, highlighting its broader role in osmotic stress signaling beyond hormone-dependent pathways (Yunta et al., 2011; Yoshida et al., 2006).

Importantly, OST1 also participates in broader stress adaptation pathways beyond immediate guard cell responses. It has been shown to regulate cold stress responses by phosphorylating and stabilizing transcription factors such as ICE1, thereby enhancing the expression of cold-responsive genes (Ding et al., 2015). This demonstrates that OST1 links environmental stress perception to transcriptional regulation and long-term adaptive responses.

1.4 Stomatal development

Stomatal development is a highly ordered developmental process characterized by a sequence of asymmetric and symmetric cell divisions that generate specialized epidermal cell types (Pillitteri & Dong, 2013).

The developmental pathway begins when a protodermal cell acquires meristemoid mother cell (MMC) identity. The MMC undergoes an asymmetric division, producing a smaller meristemoid cell and a larger pavement cell. Protodermal cells that do not enter the stomatal lineage can also directly differentiate into pavement cells. This first division establishes the stomatal lineage while contributing to the formation of epidermal pavement cells. (Pillitteri & Dong, 2013). This developmental sequence is illustrated in Figure 2.

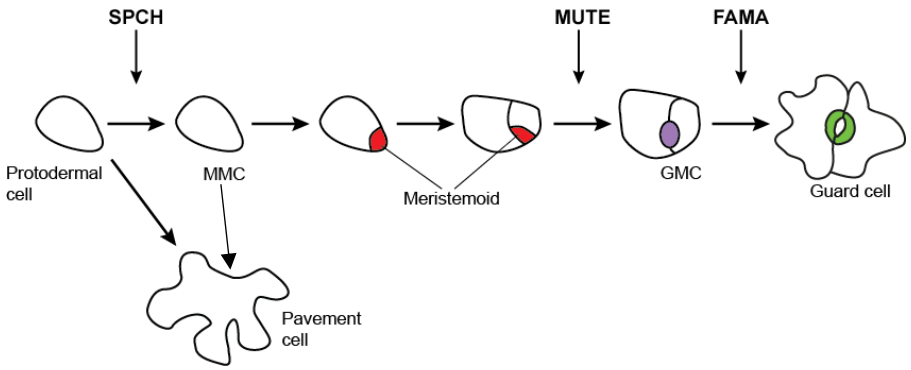

Figure 2. Schematic representation of stomatal development showing the progression from protodermal cell to mature guard cells via MMC, meristemoid, and GMC stages. Figure taken from Jalakas, 2019, modified by Klavina.

The meristemoid functions as a transient, proliferative intermediate cell type. It may undergo additional rounds of asymmetric divisions, expanding the stomatal lineage while also contributing to surrounding pavement cell formation. These repeated asymmetric divisions represent a key amplification phase before terminal commitment (Pillitteri & Dong, 2013).

Eventually, the meristemoid transitions into a guard mother cell (GMC), marking the termination of asymmetric divisions and the onset of a terminal developmental state. The GMC then undergoes a single symmetric division to produce two equivalent daughter cells that differentiate into guard cells (Vatén & Bergmann, 2012).

This progression is regulated by a set of three transcriptional regulators acting in a sequential manner. SPEECHLESS (SPCH) initiates stomatal lineage entry and enables the asymmetric division of MMCs. MUTE promotes the transition from meristemoid to GMC and terminates proliferative activity. FAMA controls the final symmetric division and ensures correct terminal differentiation (Lampard & Bergmann, 2007).

Grass stomatal development differs from that of dicots such as *Arabidopsis thaliana*. In grasses, stomata develop in organized parallel cell files and form characteristic four-celled complexes consisting of two dumbbell-shaped guard cells flanked by subsidiary cells, which contribute to efficient gas exchange and rapid stomatal movements (McKown & Bergmann., 2020; Hepworth et al., 2018)

1.5 Barley, important agronomic crop

Barley is widely cultivated as both a spring and winter crop, with its production systems varying depending on climatic conditions and irrigation availability (Sattorova, 2024). In addition, barley is considered suitable for integration into different farming systems, including organic agriculture. It is often included in crop rotations due to its ability to contribute to system diversity and reduce reliance on high-input production systems (Baker et al., 2020). Its relatively low nitrogen requirement compared with other cereals such as wheat further increases its suitability for low-input and sustainable farming systems (Baker et al., 2020).

Barley also provides important agronomic benefits through its biological characteristics. Rapid establishment and early canopy development in spring barley allow it to compete effectively with weeds, making it useful for weed suppression in agricultural rotations (Baker et al., 2020). Furthermore, barley can improve cropping system flexibility because its earlier maturity allows timely planting of subsequent crops, thereby improving overall land-use efficiency (Baker et al., 2020).

Barley is a highly versatile crop in terms of end use. It is cultivated for food, animal feed, and malting purposes, making it economically important for farmers due to its multiple market opportunities (Baker et al., 2020; Sattorova, 2024). In particular, barley serves as a key raw material for the brewing and distilling industries, while also being used directly as livestock feed and human food (Sattorova, 2024). This

multifunctionality increases its economic value and reduces production risk for farmers by enabling access to different markets.

Nutritionally, barley grains contain significant levels of carbohydrates, proteins, lipids, dietary fiber (notably β-glucans), and bioactive compounds such as phenolics. These components contribute not only to human nutrition but also to the growing use of barley as a functional food ingredient in the food industry . The presence of β-glucans is particularly important due to their recognized health-related properties, while phenolic compounds further enhance its value as a health-promoting crop (Zhang et al., 2023; Mubai et al., 2025).

In addition to its nutritional significance, barley is increasingly recognised as a valuable industrial crop. Its biochemical composition supports its use in food processing and the development of functional food products, especially under conditions of modern food industry demand for health-oriented ingredients (Mubai et al., 2025). This positions barley not only as a staple cereal crop but also as a raw material for value-added food and beverage production.

1.5.1 Trends in barley yields

Climate change has had a measurable negative impact on barley yields in Europe, primarily through long-term trends in temperature and precipitation. These climate trends since 1989 have resulted in a 3.8% reduction in continent-wide barley yields. If Europe had not experienced the warming trends seen since 1989, barley yields would be estimated at 3.8% higher than they currently are. In addition to climatic factors, non-climatic influences such as changes in agricultural policy, fertilizer regulations, and farm management practices may also contribute to variations in barley yield. (Moore & Lobell, 2015)

1.5.2 Barley awns

Barley awns are specialized morphological structures extending from the lemma of florets and probably represent a modified leaf blade that plays multiple physiological and ecological roles in the plant (Huang, 2021). See Figure 3.

Figure 3. Elongated awns from the lemma. Adapted from Borisjuk et al. (2020)

One of the most important functions of barley awns is their strong photosynthetic capacity. Awns contain chlorenchyma tissue and are positioned at the top of the canopy, allowing efficient light interception and CO_2 assimilation, which significantly contributes to plant productivity (Olugbemi et al., 1976; Yuo et al, 2012; Abebe et al., 2009).

Due to this high photosynthetic activity, awns directly contribute to grain filling by supplying assimilates to developing grains (Huang, 2021). In fact, awns can account for a substantial proportion of total spike photosynthesis, up to 90% in barley, and therefore play a central role in determining grain yield (Abebe et al., 2009).

Moreover, barley awns can contribute between 30–50% of the final grain weight, highlighting their quantitative importance in yield formation (Huang, 2021).

In addition to their role in photosynthesis, awns improve plant performance under environmental stress. Their structural adaptations, such as thick epidermis, enhance transpiration efficiency, which can support photosynthesis and grain filling under high temperature and drought conditions (Peleg et al., 2010; Simkin et al., 2020).

Beyond physiological functions, awns also have ecological roles. They can protect grains from animal predation, assist in seed dispersal, and help anchor seeds into the soil during germination (Huang, 2021).

1.5.3 Barley leaf sheaths

Barley leaf sheath is the basal part of the leaf that encloses the stem and connects the leaf blade to the shoot axis. In grasses, including barley, this structure forms a tubular sheath that provides mechanical support and contributes to the overall architecture of the plant. Early anatomical descriptions of grass morphology identify the sheath as a key structural component that stabilizes the stem and supports the erect growth habit typical of cereals (Evert, 2006). This structural function is essential for maintaining canopy architecture and optimizing light interception in dense crop stands.

Beyond its mechanical role, the barley leaf sheath is also physiologically active. It contributes to photosynthetic carbon assimilation, particularly during later developmental stages when leaf blades begin to senesce. Photosynthetically active non-foliar organs such as sheaths and stems can contribute substantially to grain filling and final yield formation in cereals (Araus et al., 2002; Tambussi et al., 2007)

The sheath also plays an important role in plant water relations. It contains functional stomata that enable gas exchange and transpiration. Variation in sheath stomatal traits has been associated with differences in canopy conductance and water use efficiency, highlighting its role in whole-plant physiological regulation, particularly under drought stress conditions (Zhen et al., 2025). Through this mechanism, the sheath contributes to balancing carbon assimilation with water loss, which is crucial for maintaining productivity under environmental stress. In grasses, sheath stomatal conductance and transpiration rates can be comparable to those of leaf blades, and

sheaths may contribute substantially to total plant water use. Furthermore, sheath stomata exhibit distinct environmental responses, including sensitivity to vapor pressure deficit but limited response to elevated CO_2, suggesting that the sheath actively participates in regulating plant water status and adaptation to changing climatic conditions rather than functioning solely as a structural organ (Sadok et al., 2020)

In addition, the barley leaf sheath is involved in nutrient dynamics, especially nitrogen storage and remobilization. It can temporarily accumulate nutrients during vegetative growth and later redistribute them to developing grains during reproductive development. This process supports grain filling and influences final yield formation. Experimental research has shown that sheath biomass and nitrogen content are closely linked to nitrogen uptake and remobilization efficiency in barley (Barmeier & Schmidhalter, 2017).

1.5.4 L15 and L20 lines

It is important to understand whether Arabidopsis based knowledge about stomatal regulation can be applied to grasses.

Barley SnRK genes are involved in plant development, metabolism, and responses to abiotic stresses such as drought and salinity. Several HvSnRK2 genes showed stress-responsive expression patterns, indicating their roles in environmental stress signaling and adaptation.(Chen et al., 2021). The lines in my study, L15 and L20, are double mutants in HvSnRK2.7 and HvSnrk2.9.

L15 and L20 are barley lines characterized by altered stomatal regulation and reduced sensitivity to ABA. Both lines exhibit higher stomatal conductance compared with the wild type, indicating a weaker ability to close stomata in response to environmental stress signals such as increased evaporative demand. This leads to enhanced gas exchange and increased water loss under conditions that normally induce stomatal closure (Samantara et al., 2026). Physiologically, both L15 and L20 show disrupted ABA-mediated control of guard cell behavior, resulting in delayed or incomplete stomatal closure during stress. Despite these changes in stomatal function, photosynthetic rates and biomass accumulation under optimal watering conditions remain largely comparable to wild type plants, indicating that their primary phenotypic difference is in water-use regulation rather than growth capacity. (Samantara et al., 2026)

2 THE AIMS OF THE THESIS

1. Evaluate the effect of reduced relative air humidity on stomatal conductance and net CO_2 assimilation rate in barley double mutants L15 and L20 compared with wild type using Licor-6800 commercial device.
2. Determine whether the mutations in HvSnRK2.7 and HvSnRK2.9 affect stomatal density in barley leaves and sheaths of double mutant line L15 and WT.
3. Assess whether the mutations in HvSnRK2.7 and HvSnRK2.9 influence stomatal conductance and gas exchange in less studied photosynthetic organs, specifically leaf sheaths and inflorescence awns.

3 EXPERIMENTAL PART

3.1 Materials and Methods

3.1.1 Plant material and their growth condition

Three barley (*Hordeum vulgare* L) lines generated in Model and Crop Plant Ecophysiology group were used in this study (Samantara et al., 2026). These plants were double mutants defective in two barley proteins homologous to Arabidopsis OST1, HvSnRK2.7 and HvSnRK2.9 (further abbreviated as L15 and L20) and the corresponding wild type, Golden Promise barley cultivar.

Germinated seeds of three genotypes were sown into 1l pots containing 2:1 (v/v) peat: vermiculite mixture. Plants were cultivated in Percival growth chamber (41AR2cLED; Percival Scientific, Perry, IA, USA) under controlled environmental conditions with a photosynthetically active radiation intensity of 700 µmol m^{-2} s^{-1} measured at plant level. A 12 h light / 12 h dark photoperiod was maintained throughout the growth period. Daytime temperature was set to 24°C, while nighttime temperature was maintained at 20°C. Relative humidity was controlled at 70% during the day and 75% during the night. Plants were kept well-watered.

3.1.2 Measurements

To evaluate the effect of relative air humidity (RH) on leaf gas exchange traits (stomatal conductance (gs) and net assimilation rate (Anet)), a LI-6800 Portable Photosynthesis System together with 3x1 cm leaf chamber was used. Figure 4.

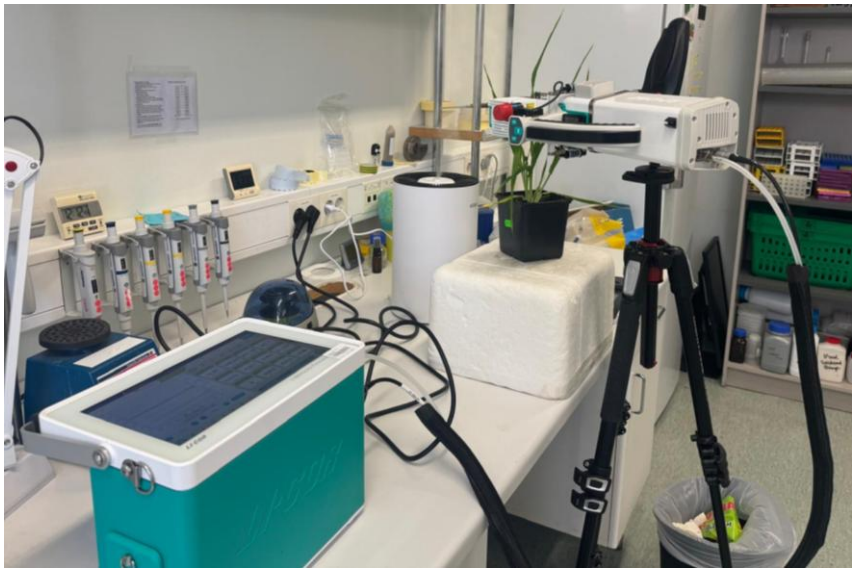

Figure 4. Leaf measurements using Licor-6800. Leaf chamber encloses 3x1 cm leaf section, the rest of the plant is kept in room conditions. Photo: Marta Klavina.

Measurements were performed on juvenile plants (10 - 27 days old). During measurements, the flow rate was set to 600 µmol s^{-1}, reference CO_2 concentration to 440 µmol mol^{-1}, air temperature to 24°C, and light in the leaf chamber to 700 µmol m^{-2} s^{-1}. The fourth leaf was inserted into the measurement chamber for gas exchange

measurements. RH inside the chamber was maintained at 70% during the stabilization period. After one hour, RH was reduced to 20% to assess the physiological response of the leaf to decreased humidity. After one hour of low RH conditions, experiment was terminated and the width of measured leaf was determined using digital caliper in order to correct for leaf area during data analysis. The experimental set up can be seen in Fig. 4.

Stomatal imprints were prepared using a two-part dental silicone kit. Silicone was applied to the central part of both the lower (abaxial) and upper (adaxial) surfaces of the third leaf, as well as to the outer (abaxial) side of the leaf sheath. Adaxial side of the leaf sheath was not imprinted, because it has been shown that this side contains very few stomata (Zhen et al. 2025). After the silicone had set, a layer of transparent nail polish was applied to the imprints. Once dried, the nail polish film was carefully removed using transparent adhesive tape and transferred onto glass microscope slides. Microscopic videos with area of 0.28 mm^2 of the prepared slides were recorded for stomatal analysis using a microscope (Kern OBF 133; KERN & SOHN GmbH; Balingen-Frommern, Germany) at 200× magnification. Images extracted from the videos were analyzed using ImageJ to determine stomatal density. Measurements were conducted on 27-day old plants. Stomatal density measurements were performed only for WT and L15 plants because sufficient L 20 plant material was not available.

After gas exchange measurements, juvenile plants were planted into larger 3L pots and transferred to growth room, where abiotic conditions were 24℃ temperature and 50% RH (day and night), diurnal cycle was 16h day/8h night and photosynthetically active radiation was 250-300 µmol m^{-2} s^{-1} at upper canopy level.

Sheath stomatal conductance and net CO_2 assimilation rate were measured using a LI-6800 Portable Photosynthesis System. Measurements were performed on 4-months old plants. For each measurement, the green leaf sheath of flag leaf was enclosed in the measurement chamber (3x1cm). Chamber conditions were maintained at 70% relative humidity, with a reference CO_2 concentration of 440 µmol mol^{-1}, flow rate of 600 µmol s^{-1}, leaf temperature of 23°C, and light intensity of 500 µmol m^{-2} s^{-1}. Samples were allowed to stabilize in the chamber for 20 minutes before data collection. After measurements, sheath widths were determined using digital caliper to correct for area in the gas exchange analysis file.

Stomatal conductance of awns was measured four months after sowing using a hand-held LI-600N Porometer. LI-600N is meant to measure stomatal conductance of narrow leaves and needles. During each measurement, three awns were placed inside the porometer chamber simultaneously for stomatal conductance analysis. After measurements, awn widths were determined using digital caliper to correct for area in the gas exchange analysis file and the reported stomatal conductance values represent the combined conductance of the three awns measured simultaneously

To investigate systemic responses to localized abscisic acid treatment, only juvenile wt plants from Percival growth chamber were used, mutant lines do not respond to

ABA and were not used in systemic response experiment (Samantara et al., 2026). Measurements were performed on 11-21 days old plants. The systemic leaf (5^{th}) was enclosed in a LI-6800 Portable Photosynthesis System chamber under controlled conditions, with a flow rate of 600 µmol s^{-1}, reference CO_2 concentration of 440 µmol mol^{-1}, air temperature of 24°C, and light intensity of 700 µmol m^{-2} s^{-1}. After the systemic leaf had remained in the chamber for 40 minutes, stomatal conductance of the local leaf was measured using a LI-600 Porometer meant to measure wider leaves. The local leaf was then sprayed with an ABA solution containing 5 mL H_2O, 10 µL of 10 mM ABA, and 1 µL Silwet. Final ABA concentration in the spraying solution was 20 µM. Twenty minutes after ABA application, stomatal conductance of the local leaf was measured again using the LI-600 porometer. Measurements of the systemic leaf continued for 50 minutes after the local leaf had been treated with ABA to assess systemic physiological responses. The aim of this experiment was to study the effect of the rest of the plant on the gas exchange of the leaf section enclosed in gas exchange measurement chamber.

3.1.3 Statistical analysis

To evaluate significant differences between experimental groups, statistical analysis was performed using one-way ANOVA followed by Tukey's HSD post hoc test. For stomatal density, two-way ANOVA followed by Tukey's HSD post hoc test was performed. All statistical analyses were conducted using Statistica (version 14.0, StatSoft Inc., Tulsa, OK, USA).

3.2 Results

3.2.1 Effect of decreased relative air humidity on barley leaves

In all plants, an initial increase in stomatal conductance (wrong-way response) was detected immediately after RH transition to 20%, which was followed by a rapid decrease in stomatal conductance in WT plants (Fig. 5A). In contrast, L15 and L20 plants showed a slower, more gradual decline in stomatal conductance after wrong-way response, which lasted throughout the experiment. Unlike in double mutants, a partial recovery of stomatal conductance over time was evident in wild type.

Net CO_2 assimilation rate decreased markedly in WT after the humidity shift, whereas L15 and L20 displayed smaller reductions and greater stability in Anet throughout the measurement period. (Fig 5B.)

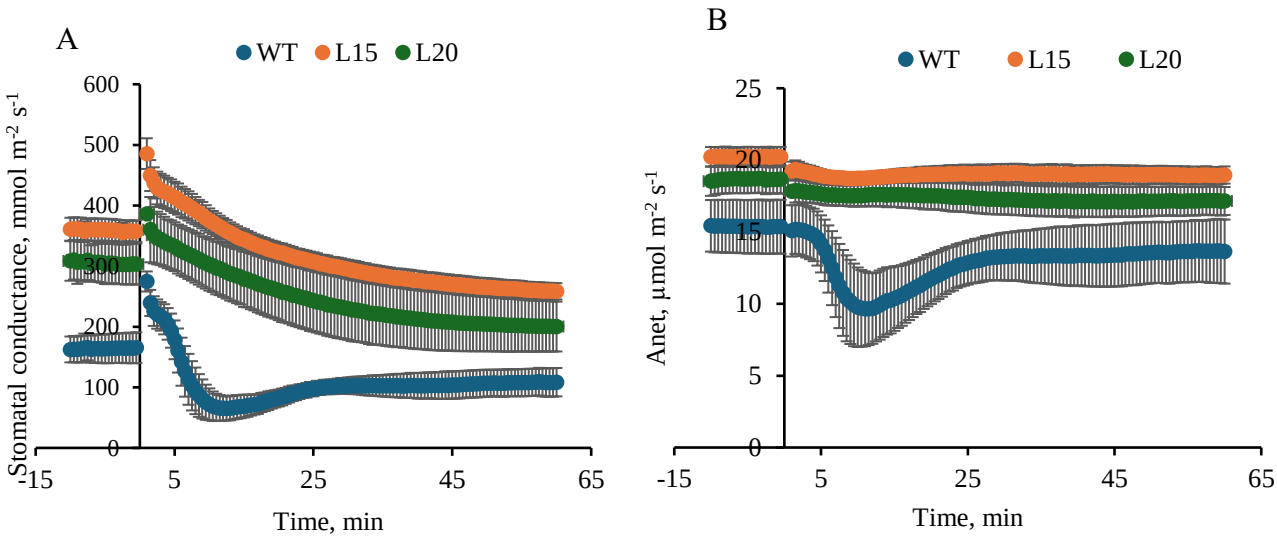

Figure 5. Changes in (A) stomatal conductance and (B) net CO_2 assimilation rate in WT, L15, and L20 leaves. Time 0 marks the transition from 70% to 20% air RH. Values represent mean ± SE, n=3.

Figure 6 shows statistical analyses of average values of stomatal conductance and net assimilation rate for specific timepoints of Figure 5. There was a significant difference in leaf stomatal conductance between WT and both mutant lines under 70% RH: double mutants had higher leaf stomatal conductance. Following the reduction in relative humidity to 20%, WT exhibited a significant decrease in stomatal conductance after 30 min, whereas neither mutant line showed a significant response at this time point. After 60 min under low RH conditions, WT stomatal conductance partially recovered and was not significantly different from its pretreatment value, while stomatal conductance of both mutant lines continued to decline and differed significantly from their initial conductance values. (Fig 6 A.)

Even though the values of net assimilation rate were higher in double mutants, this difference was not statistically significant (Figure 6 B). Anet was significantly less under low air RH in WT, whereas in L20, only Anet at the end of the experiment significantly differed from pre-treatment Anet. In contrast, in L15, a significant difference was observed between pre-treatment Anet and Anet measured 30 min after the change in air RH, while no significant difference was detected between pre-treatment Anet and Anet at the end of the experiment.

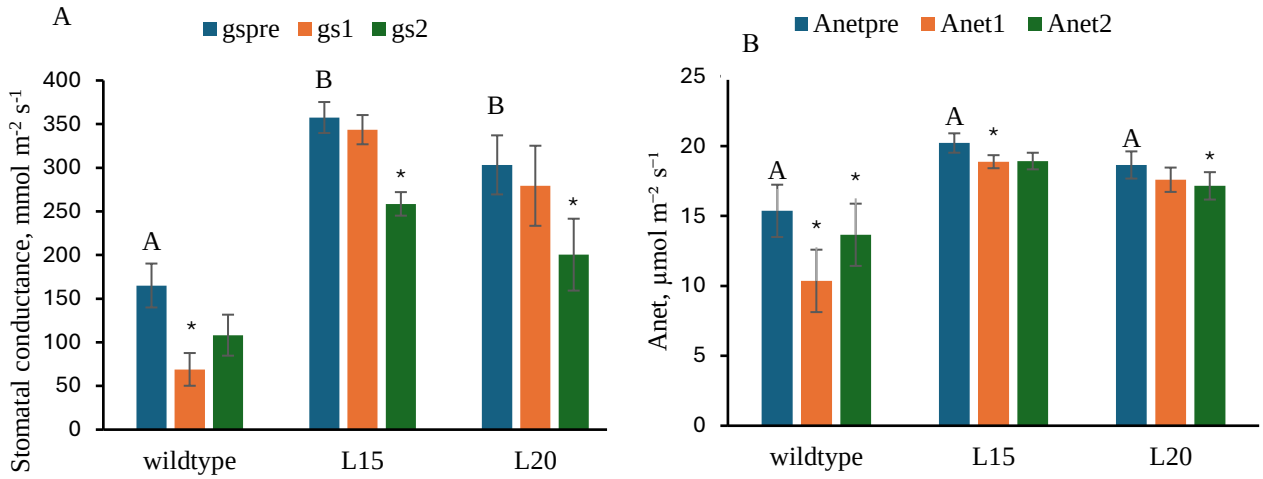

Figure 6. Average values of (A) stomatal conductance and (B) net CO_2 assimilation rate of WT, L15, and L20 leaves. Stabilized values under 70% RH (gspre, Anetpre) and 30 and 60 minutes after RH was decreased to 20% (gs1, Anet1 and gs2, Anet2 respectively). Values represent mean ± SE, n=3. Different capital letters above gspre and Anetpre values indicate statistically significant differences between different lines (one-way ANOVA followed by Tukey HSD test), whereas star points at a significant difference from gspre or Anetpre of that line (Repeated measures ANOVA followed by HSD test.)

Higher values of gs resulted in increased Anet under lower gs values, but at higher gs values, Anet did not increase any more, indicating that photosynthesis was not stomatally limited any more. (Fig.7)

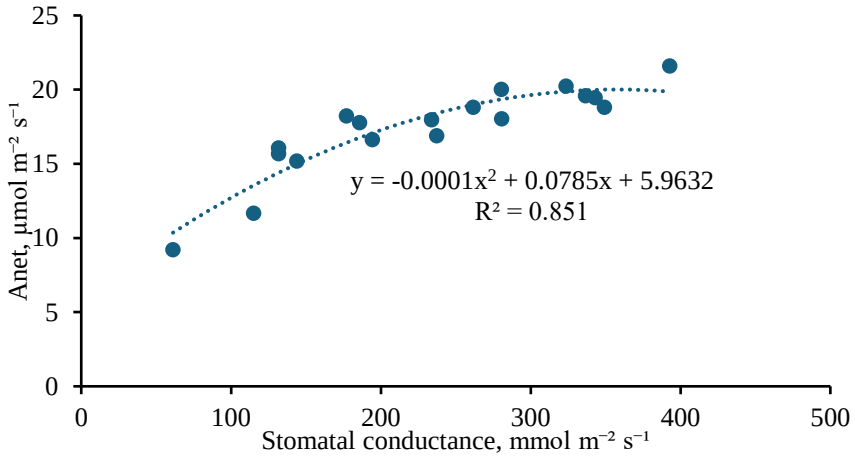

Figure 7. Correlation between average values of stomatal conductance and net CO_2 assimilation rate of all genotypes, pre (at 70% RH) and final timepoints (60 minutes at RH 20%) of Figure 6 were used. P=0.008 for gs^2 and P=0.0003 for gs (polynomial regression of Statistica)

3.2.2 Stomatal density

Stomatal density analysis included only WT and L15 plants, as L20 plants were unavailable for this experiment. No significant difference was detected between leaf stomatal density values of WT and mutant plants for both the top and bottom sides of the leaf. No significant leaf side effect on SD was evident either. The stomatal density of leaf sheath was significantly lower than that of leaves, but not different between the lines (Fig.8). The absence of significant differences in stomatal density indicates that altered gas exchange in mutant plants is not explained by stomatal number.

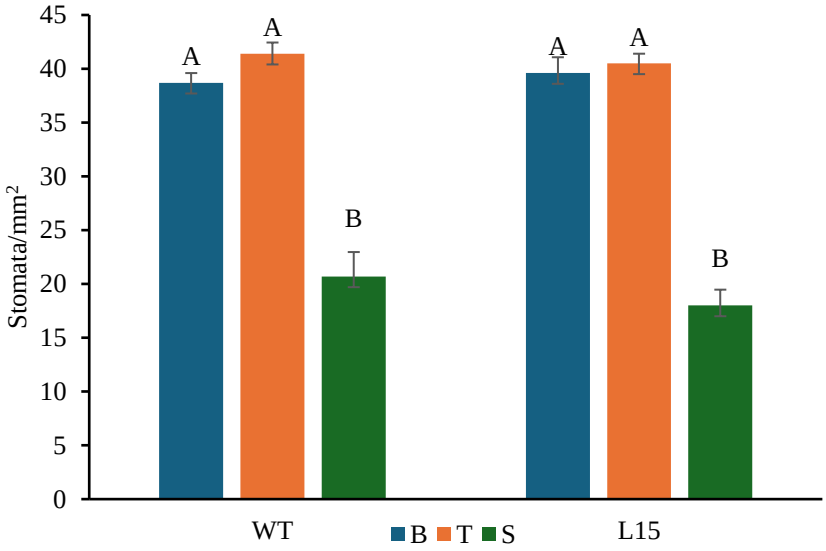

Figure 8. Stomatal density leaf bottom (abaxial) side, top (adaxial) side and of sheath (B, T and S, respectively) of studied lines. Values represent mean ± SE, n= 4. Different letters denote significant differences between SD values of genotypes and organs. (two-way ANOVA and Tukey HSD test)

3.2.3 Barley leaf sheath stomatal conductance and net CO_2 assimilation

There was a significant difference in the values of sheath stomatal conductance between WT and L15. L20 showed higher sheath stomatal conductance than WT, but this difference was not statistically significant relative to either WT or L15. In contrast, no significant differences were observed among the three lines in net CO_2 assimilation rate of the leaf sheaths. (Fig.9).

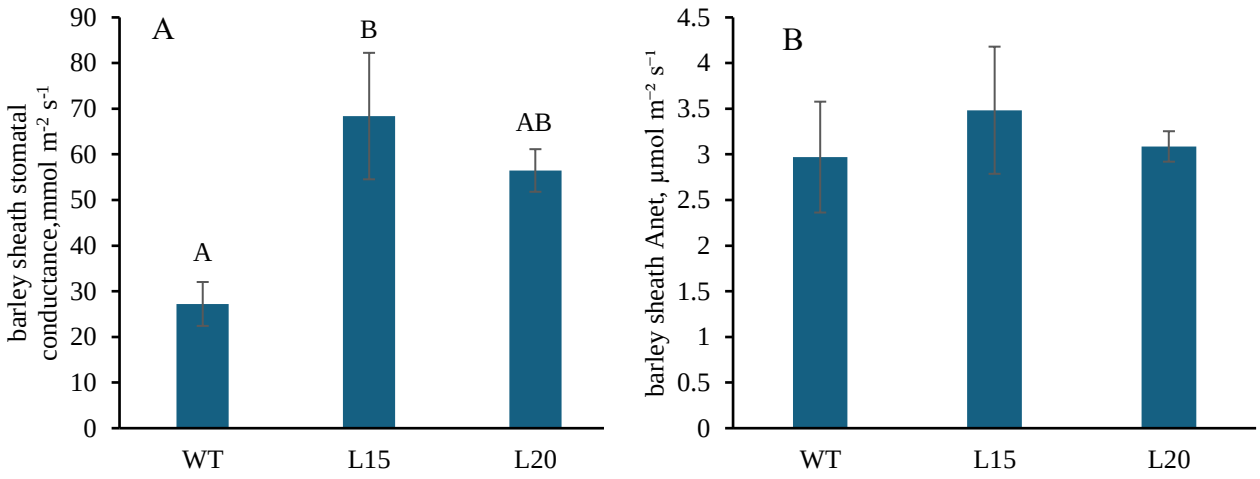

Figure 9. (A) Stomatal conductance and (B) net CO_2 assimilation rate of barley leaf sheaths for WT, L15, and L20 plants. Stabilized values under 70% RH (gspre, Anetpre). Values represent mean ± SE, n=3. Different letters denote statistically significant differences between lines (one-way ANOVA and Tukey HSD test).

3.2.4 Barley awn stomatal conductance

Significant differences in awn stomatal conductance were observed between WT and both mutant lines (L15 and L20), whereas L15 and L20 did not differ significantly from each other. (Fig.10)

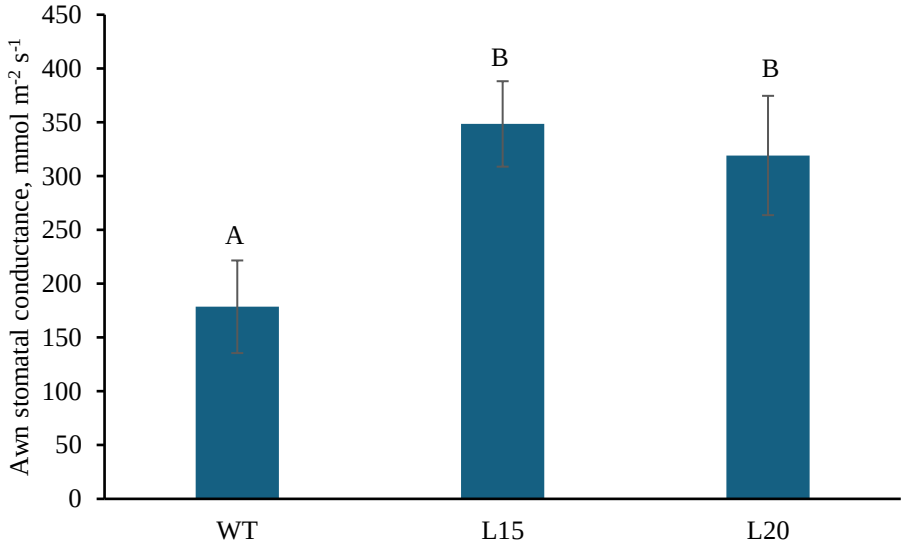

Figure 10. Stomatal conductance of barley awns for WT, L15, and L20. Each measurement represents the combined stomatal conductance of three awns measured simultaneously. Values represent mean ± SE, n= 3, 4 and 5 for WT, L20 and L15, respectively. Statistically significant differences are indicated by different letters. (one-way ANOVA and Tukey HSD test).

3.2.5 Systemic responses to local ABA treatment

Measurements using Licor-6800 enclosed leaf section into 3x1 cm leaf chamber, whereas the rest of the plant was kept under room conditions with uncontrolled environment. Systemic ABA spraying experiment was performed to understand whether the gas exchange of the leaf section enclosed into Licor chamber is affected by changes taking place in the other plant parts. Systemic and local leaf had very different stomatal conductances, since room conditions were not supportive of high gs (lower light level, decreased RH). There was no change in stomatal conductance of the systemic leaf measured before and after ABA application to the local leaf of the same plants. Although no statistically significant change was detected (probably due to small sample size), stomatal conductance of the local, ABA-treated leaf showed a decrease following ABA treatment. These results indicate that the leaf measured in the chamber is not significantly affected by ABA application to another leaf, suggesting that gas exchange responses in the chamber-enclosed leaf operate independently of what happens to other leaves. (Fig.11)

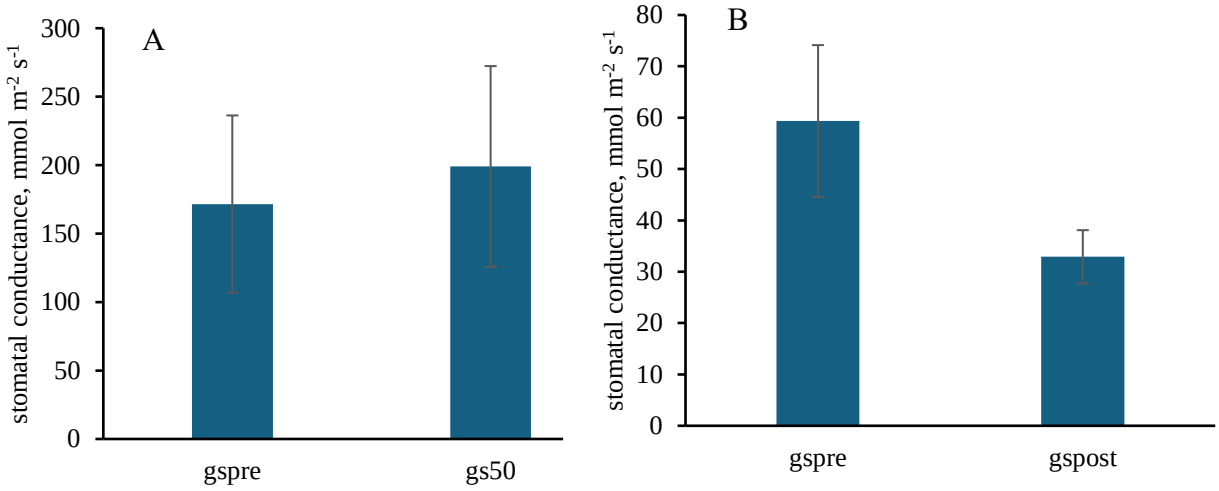

Figure 11. (A) Stomatal conductance of barley 5th leaf (systemic leaf) under 70% RH before (gspre) and 50 minutes after 4th leaf (local leaf) was sprayed with abscisic acid (gs50). (B) Stomatal conductance of barley 4th leaf (local leaf) measured before spraying with abscisic acid (gspre), and 20 minutes after spraying with abscisic acid (gspost).

3.3 Discussion

In this study the involvement of barley SnRK2.7 and SnRK2.9 in leaf blade, leaf sheath and awn stomatal regulation was demonstrated. Barley double mutant lines L15 and L20, defective in SnRK2.7 and SnRK2.9 exhibited higher steady-state stomatal conductance of leaf blades, leaf sheaths and awns and altered stomatal responses of leaf blades compared with wild type plants under changing humidity conditions. Stomatal density did not differ between genotypes.

Following a reduction in relative air humidity, wild type plants showed a rapid decrease in stomatal conductance and net CO_2 assimilation rate, whereas both mutant

lines responded more gradually and maintained more stable photosynthetic activity. These differences were not associated with changes in stomatal density, indicating that altered gas exchange responses are more likely explained by differences in stomatal aperture regulation than by stomatal development.

This is in agreement with (Samantara et al. 2026), who reported that SnRK2.7/SnRK2.9 double mutants exhibited higher steady-state stomatal conductance and impaired stomatal responses to reduced relative humidity and ABA under whole-plant growth chamber conditions. Although the relative humidity regimes were not exactly comparable (RH change was stronger in the current experiment) and measurement systems differed between the two studies, similar physiological trends were observed, particularly the higher steady-state stomatal conductance and weaker humidity-induced stomatal closure in mutant plants compared with wild type. A similar tendency was observed for net CO_2 assimilation rate, which was generally higher or more stable in mutant plants, although these differences were not statistically significant in my study. The consistency between whole-plant and leaf chamber experiments suggests that the observed mutant phenotype is reproducible across experimental setups and further supports a role for SnRK2.7 and SnRK2.9 in regulating stomatal responses to atmospheric humidity changes. This contrasts with the conclusions of Zait et al. (2024), who proposed that the response of stomatal regulatory mutants can be conditional on the experimental chamber configuration. Also, the results of my study showed that local ABA application did not influence stomatal conductance of the systemic leaf enclosed in the measurement chamber. This suggests that stomatal behavior of the measured leaf mainly reflects local physiological regulation and operates largely independently from responses occurring in other leaves under these experimental conditions.

Similarly to (Wong et al. 1979), there was a correlation between stomatal conductance and net CO_2 assimilation, further showing that mutants had higher net assimilation rates, even though statistically, the differences were not significant. At higher gs values, net assimilation rate showed no further increases, pointing at non-stomatal limitation of Anet. The saturating response observed between stomatal conductance and net assimilation is consistent with the framework described by Farquhar and Sharkey (1982), where photosynthesis becomes progressively less responsive to increases in stomatal conductance once biochemical limitations dominate over diffusional limitations.

The results also show that HvSnRK2.7 and HvSnRK2.9 are involved in regulating stomatal conductance of barley leaf sheaths and awns. In both tissues, the mutant lines exhibited higher stomatal conductance compared with wild type plants. This indicates that the functional role of these kinases in stomatal regulation may extend beyond leaf blades to other above-ground organs. To my knowledge, this is the first report showing the involvement of SnRK2 kinases in leaf sheath and awn stomatal regulation.

(Biscoe et al. 1973) showed that barley awns possess active stomatal regulation and contribute substantially to ear transpiration and photosynthesis. This is consistent with my result that higher stomatal conductance was observed in mutant awns compared with wild type plants, confirming active stomatal regulation in awns. In addition, (Abebe et al., 2009) established the awn as the major photosynthetic organ of the barley, with preferential expression of genes involved in photosynthesis, chlorophyll biosynthesis, reactive oxygen species scavenging, and nearly all enzymes of the Calvin cycle. This supports the idea that awns function similarly to leaves in terms of photosynthetic activity and therefore may also share similar mechanisms of stomatal regulation. Hein et al., (2016) used LI-6400 gas exchange system and reported relatively high stomatal conductance values in barley awns. In my study, awn stomatal conductance values were lower, even in mutant lines. In my study, measurements were performed on green awns that began turning yellow shortly afterwards, suggesting the onset of senescence, whereas differences in tissue age and condition may explain differences stomatal conductance between studies.

Similarly, the leaf sheath measurements in my study confirm that barley sheaths are physiologically active tissues capable of gas exchange, supporting earlier work by (Sadok et al. 2020) In my study, stomatal conductance and net CO_2 assimilation were lower in leaf sheaths than in blades. Sheath stomatal conductance observed in my study was considerably lower than that reported by (Sadok et al. 2020), who found that sheath stomatal conductance could be comparable to leaf blade conductance under certain environmental conditions. Differences in tissue age and experimental conditions may partly explain this variation. The sheaths measured in my study were approximately four months old, whereas the plants studied by (Sadok et al. 2020) were only 22–38 days old. Older sheath tissues may exhibit reduced physiological activity, partial senescence, or lower stomatal responsiveness compared with younger tissues, which could contribute to decreased stomatal conductance and photosynthetic activity. Similar age-dependent declines in photosynthesis and stomatal conductance have been reported in ageing cereal leaves (Jahan et al.2023). However, Sadok et al. (2020) also showed that sheath stomata were generally less responsive to environmental drivers. Consistent with Sadok et al. (2020), stomatal density of the sheath was lower than in the leaf blade. The sheath abaxial surface contained approximately 20 stomata mm^{-2}, comparable to values observed in my study. Net CO_2 assimilation rates in barley leaf sheaths were also substantially lower than those of leaf blades, in agreement with (Sadok et al. 2020), although the assimilation rates measured here were even lower, again potentially reflecting differences in plant age.

Sadok et al. (2020) further reported that sheath stomatal conductance showed limited responsiveness to environmental conditions compared with leaf blade stomata. Environmental responses of sheath stomata were not directly investigated in my study, and therefore no conclusions can be drawn regarding sheath stomatal sensitivity to changing environmental factors. Nevertheless, the stomatal conductance values

measured in barley sheaths in my experiment support the idea that sheath stomata are actively regulated and contribute to whole-plant gas exchange.

In conclusion, this study demonstrates that HvSnRK2.7 and HvSnRK2.9 play an important role in regulating stomatal conductance and humidity-induced stomatal responses in barley. The double mutant lines showed higher steady-state stomatal conductance and slower stomatal closure of leaf blades after reductions in relative humidity, indicating impaired stomatal regulation compared with wild type plants. These effects were observed not only in leaf blades but also in awns and leaf sheaths, suggesting that the function of these kinases extends to multiple above-ground organs involved in gas exchange. The results further support previous findings that altered stomatal regulation in the mutants is associated with changes in stomatal aperture rather than stomatal density or development.

Further research is needed to explore whether double mutants also show altered stomatal responses to CO_2 and light, and how awn and sheath stomata adapt to changing environmental conditions.

SUMMARY

This study demonstrated that barley SnRK2 kinases HvSnRK2.7 and HvSnRK2.9 play an important role in stomatal regulation and plant responses to atmospheric humidity. Using the double mutant barley lines L15 and L20 defective in SnRK2.7 and SnRK2.9 and the wild type cultivar Golden Promise, showed that mutations in HvSnRK2.7 and HvSnRK2.9 altered stomatal behavior in leaves, leaf sheaths, and awns. Gas exchange measurements using different Licor devices revealed that both mutant lines exhibited higher steady-state stomatal conductance of leaf blades than wild type plants and displayed slower stomatal closure following a reduction in relative air humidity. In contrast to the rapid decrease in stomatal conductance observed in wild type plants, the mutant lines responded more gradually and maintained higher stomatal conductance of leaf blades throughout the low humidity treatment. Mutant plants also showed more stable net CO_2 assimilation rates under reduced air humidity conditions, whereas wild type plants experienced a stronger decline in photosynthetic activity, which matched with the lowest drop in stomatal conductance under low RH. These results indicate that HvSnRK2.7 and HvSnRK2.9 contribute to efficient leaf blade stomatal responses to changing atmospheric conditions and regulation of plant water loss.

Analysis of stomatal density showed no significant differences between mutant and wild type plants on either the abaxial or adaxial leaf surfaces. Although stomatal density in leaf sheaths was lower than in leaves, there were no significant effects of genotype. The absence of differences in stomatal density demonstrates that the altered stomatal conductance observed in the mutant lines resulted from changes in stomatal aperture regulation rather than from developmental changes in stomatal number.

The study further showed that the role of HvSnRK2.7 and HvSnRK2.9 extends beyond leaf blades to other photosynthetically active organs. Both mutant lines exhibited increased stomatal conductance in awns, while leaf sheath conductance was significantly higher in the L15 line compared with wild type plants. Net CO_2 assimilation of leaf sheaths did not differ significantly between genotypes, suggesting that the primary effect of the mutations was on stomatal behavior rather than photosynthetic capacity. These findings confirm that HvSnRK2.7 and HvSnRK2.9 regulate gas exchange across multiple above-ground organs involved in transpiration and photosynthesis.

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