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EGON MEIGAS

Evolution of gas exchange traits and their application in crop breeding

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Evolution of gas exchange traits and their application in crop breeding

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*These authors contributed equally

Author's contributions

I. Participated in experimental planning. Performed half of the gas exchange experiments and supervised the rest. Analysed and visualised all of the gas exchange data and wrote first version of the manuscript.

II. Generated the *Arabidopsis* complementation lines. Performed, analysed and visualised RT-qPCR. Performed and visualised gene alignments. Contributed to the writing of the manuscript.

III. Aided in visualising some of the data, commented on the manuscript.

IV. Contributed to the device methodology. Designed the gas exchange experiments in large parts. Performed or supervised all of the gas exchange experiments. Analysed and visualised all of the gas exchange data. Wrote parts of the manuscript.

ABBREVIATIONS

3-PGA	–	3-PHOSPHOGLYCERATE
ABA	–	ABSCISIC ACID
ABI	–	ABA INSENSITIVE
ATP	–	ADENOSINE TRIPHOSPHATE
bHLH	–	BASIC HELIX-LOOP-HELIX
CA	–	CARBONIC ANHYDRASE
CBC	–	CONVERGENCE OF BLUE LIGHT AND CO2
CNGC	–	CYCLIC NUCLEOTIDE-GATED CHANNEL
CPK	–	CALCIUM-DEPENDENT PROTEIN KINASE
EPF	–	EPIDERMAL PATTERNING FACTOR
GHR1	–	GUARD CELL HYDROGEN PEROXIDE-RESISTANT1
GORK1	–	GUARD CELL OUTWARD-RECTIFYING K^+ CHANNEL
HAB1	–	HYPERSENSITIVE TO ABA 1
HT1	–	HIGH LEAF TEMPERATURE 1
MPK	–	MITOGEN ACTIVATED PROTEIN KINASE
NADPH	–	NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE
OST1/SnRK2.6	–	OPEN STOMATA 1/SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2.6
PAR	–	PHOTOSYNTHETICALLY ACTIVE RADIATION
PIP2;1	–	PLASMA MEMBRANE INTRINSIC PROTEIN 2-1
PP2C	–	TYPE 2C PROTEIN PHOSPHATASE
PPFD	–	PHOTOSYNTHETIC PHOTON FLUX DENSITY
PS	–	PHOTOSYSTEM
PYR/PYL/RCAR	–	PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR
QUAC1	–	QUICK-ACTIVATING ANION CHANNEL 1
RBOHF	–	RESPIRATORY BURST OXIDASE HOMOLOG F
RUBISCO	–	RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE
RuBP	–	RIBULOSE 1,5-BISPHOSPHATE
SLAC1	–	SLOW ANION CHANNEL 1
SLAH3	–	SLAC HOMOLOGUE 3
TP	–	TRIOSE PHOSPHATES
WAASB	–	WEIGHTED AVERAGE OF ABSOLUTE SCORES FROM BLUPS
VPD	–	VAPOR PRESSURE DEFICIT

1. INTRODUCTION

Stomata on plant leaves are the primary gateways that have regulated the terrestrial productivity for hundreds of millions of years. These pores are formed by guard cells and allow CO_2 uptake for photosynthesis. At the same time, plants transpire water they draw from the soil through stomata. To colonise the land and evade drying out, plants have evolved to actively regulate the aperture of the stomatal pores. If plants keep their stomata closed, CO_2 can no longer diffuse in, leading to starvation. Thus plants perpetually balance the ratio of water loss to carbon gain, depending on the environmental signals like light, CO_2 concentration, air humidity and soil water availability. In the face of climate change, plants are increasingly subjected to extreme weather events like heatwaves and droughts. However, human population growth necessitates increase in food production and thus, breeding of higher and stable-yielding and/or more climate-ready varieties is required to sustain food security. Historically, breeding programs were slow, thus new ways to improve the throughput and outcomes are actively incorporated. Statistical evaluation and multi-environment trials have already allowed selection of stable varieties that can produce grain yield in different conditions. Gas exchange is seen as a potential avenue for breeding for drought resistance and increased yields, by creating varieties with increased net CO_2 assimilation of different organs or improved water use efficiency. Understanding stomatal regulation at a molecular level is required for modulating plants' responses to the environment, and for this, knowledge based on model plants needs to be translated into economically important crops. Modifications at the genetic level allow precise tuning of the gas exchange traits of crops. Furthermore, tracing the origins and evolution of the regulatory pathways of stomata yields insights into developing water use efficient plants and helps to understand the role and scope of individual components of stomatal regulation. Research has demonstrated that economically important crops already possess a significant amount of genotypic variation in their gas exchange traits, which could simply be selected for in breeding programs. However, directly measuring CO_2 uptake at a scale in the field necessary for breeding programs is not best done with the current methodology.

In this thesis, the stomatal regulation responsible for air humidity responses was studied in barley, as well as in one of the oldest surviving vascular plants, horsetails. Spring wheat yield components and their stability was assessed in field trials, alongside a new methodology for increased through-put of gas exchange measurements and its potential in breeding application.

2. REVIEW OF LITERATURE

2.1 Evolution of stomata

Stomata are tiny openings on plant leaves that enable uptake of photosynthetic carbon dioxide (CO_2) from the outside air and control transpiration of water vapour into the surrounding air. Transpiration regulates leaf temperature and draws nutrients from the soil. Stomata occur in vascular plants, including horsetails and ferns, and also in some more primitive plants like mosses (Brownlee, 2018). Phylogenomic studies indicate that stomata likely evolved only once in the last common ancestor of all land plants before the divergence of bryophytes (mosses, hornworts, and liverworts) and tracheophytes (vascular plants; Vatén and Bergmann, 2012; Clark *et al*., 2022). The core mechanism for stomatal development, including basic helix-loop-helix (bHLH) transcription factors and epidermal patterning factor (EPF) signaling peptides most likely were already present in this ancestral embryophyte (Vatén and Bergmann, 2012; Chater *et al*., 2017).

As major plant lineages diverged, they followed distinct trajectories that shaped stomatal evolution. While stomata were ancestral to all land plants, they were lost entirely in liverworts and underwent over 60 independent losses across various moss lineages (Clark *et al*., 2022). In most extant bryophytes, stomata are restricted to the sporangium and may function mostly to facilitate drying and spore dispersal rather than vegetative gas exchange (Sussmilch *et al*., 2019; Clark *et al*., 2022). Terrestial brypohytes that lack stomata absorb CO_2 through surface diffusion or as in the case of liverworts, through epidermal air pores that are distinct from stomata (Vatén and Bergmann, 2012; Carriquí *et al*., 2019). Some aquatic plants, including the marine seagrass *Zostera marina*, eventually lost their stomata and necessary genes when adapting to underwater environments (Clark *et al*., 2022; Chen *et al*., 2024). Most stomata, including the ones in ancient lineages, are kidney-shaped, however within the last 150 million years, the most recent and significant advancement was the appearance of dumbbell-shaped guard cells in monocots (Vatén and Bergmann, 2012; Chen *et al*., 2024). These stomata are accompanied by subsidiary cells that provide mechanical assistance and ion storage, allowing for faster and more efficient stomatal responses to abiotic cues (Franks and Farquhar, 2007; Kirkham, 2014; Merilo *et al*., 2014; Woning *et al*., 2025). The mechanical improvement of stomatal complexes possibly enabled grasses to expand into drier areas during periods of global aridification (Clark *et al*., 2022).

Active regulation of stomatal aperture has evolved in plants to respond to changes in the surrounding environment and to ensure an optimal balance between CO_2 uptake and transpiration. Accordingly, stomata open when CO_2 concentration decreases, air humidity increases and in response to light, while they close under low air humidity, water deficit, darkness, and when CO_2 increases (Shimazaki *et al*., 2007; Merilo *et al*., 2014; Kollist *et al*., 2014; Binstock *et al*,. 2024). Stomatal closure in drought is caused by a general decrease in turgor, and via induced stress hormones, primarily abscisic acid (ABA; Munemasa *et al*., 2015).

ABA predates stomatal pores on plant surfaces, and occurs already in algae (Chater *et al*., 2017; Sun *et al*., 2019). Genes encoding ABA-activated receptors required for initiation of stomatal closure in modern plants are predominantly absent in algal genomes, however their analogues as well as orthologues of downstream components have been identified in various algal species (de Vries and Archibald, 2018; Sun *et al*., 2019). Algal ABA receptor analogues interact with the downstream components in an ABA-independent manner and only upon colonisation of land ABA became a regulator of stress responses in plants (Sun *et al*., 2019).

ABA- and CO_2-driven stomatal regulation and activation of the main guard cell anion channel SLAC1 (SLOW ANION CHANNEL 1) required for stomatal closure, has been found in some mosses (Lind *et al*., 2015). Data for early vascular plants above mosses (ferns and lycophytes) are conflicting. In some studies, no effect of ABA on stomata was detected in any fern or lycophyte species and their stomatal regulation was considered to be hydropassive (McAdam and Brodribb, 2011; McAdam and Brodribb, 2012; Cândido-Sobrinho *et al*., 2022). However, the opposite has also been shown: ABA induced stomatal closure in several fern species, although the extent of closure is smaller compared with angiosperms (Cai *et al*., 2017; Hõrak *et al*., 2017; Plackett *et al*., 2021). Genetic studies suggest, that ABA signaling elements pre-date appearance of stomata and that land plants acquired active stomatal control early in their evolution (Ruszala *et al* 2011; Cai *et al* 2017; Sun *et al* 2019; Sun *et al* 2020; Chen *et al*, 2024). Broadly, this has led to the conclusions that either the signalling components in early plants have not yet evolved to regulate stomata, have undergone species-specific secondary losses or require sensitisation that happens only when certain conditions are met (McAdam *et al* 2017; Hõrak *et al* 2017; Gong *et al*., 2021; Plackett *et al*., 2021, Wuyun *et al*., 2023; Sussmilch *et al*., 2024).

2.2 Stomatal responses to ABA

ABA is an important plant hormone, regulating germination, early growth, stress responses, root growth, seed maturation, stomatal responses, pathogen responses, and senescence among many other functions (Finkelstein, 2013; Li *et al*., 2017). It is synthesised in almost all plant cells that contain chloroplasts or amyloplasts, and the main components of the ABA biosynthesis pathway are conserved in all land plants (Li *et al*., 2017). ABA is also present in other living organisms, from bacteria to humans, but does not have a systematically important role in these organisms (Lievens *et al*., 2017; Li *et al*., 2017). It has been shown that in stomatal closure, ABA can originate from multiple sources, including phloem companion cells, mesophyll cells as well as guard cells themselves (Bauer *et al*., 2013; Kuromori *et al*., 2014; McAdam and Brodribb, 2018; Merilo *et al*., 2018; Cotelle and Leonhardt, 2019).

ABA-induced stomatal closure in angiosperms is accomplished via a multi-step signalling pathway. First, ABA is perceived by PYR/PYL/RCAR receptor

proteins (PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR; Figure 1; Ma *et al*., 2009; Park *et al*., 2009). In *A. thaliana*, 14 PYR/PYL/RCAR proteins have been identified (Raghavendra *et al*., 2010). Biochemically they can be divided into two groups—those which form dimers (PYR1, PYL1 and PYL2) and have lower affinity for ABA, and others acting as monomers (e.g. PYL5, PYL6 and PYL8; Joshi-Saha *et al*., 2011; Gonzalez-Guzman *et al*., 2012). The functional division of roles among the receptors is not fully understood, however it has been shown that ABA-dependent stomatal closure requires primarily PYL2 and, to a lesser extent, PYR1 (Dittrich *et al*., 2019). To initiate stomatal closure, ABA and its receptors bind and form a ternary complex with phosphatases of the TYPE 2C PROTEIN PHOSPHATASE (PP2C) family, thereby inactivating PP2Cs (Gonzalez-Guzman *et al*., 2012). There are more than 50 PP2C phosphatases in A. *thaliana*, but the most important in stomatal regulation are ABI1 (ABA INSENSITIVE 1), ABI2 (ABA INSENSITIVE 2) and HAB1 (HYPERSENSITIVE TO ABA 1; Ma *et al*., 2009). Outside of the ternary complex, active PP2C phosphatases dephosphorylate and inactivate downstream components of the signalling pathway, including the protein kinase OST1/SnRK2.6 (OPEN STOMATA 1/SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2.6; Mustilli *et al*., 2002; Kollist *et al*., 2014). OST1 kinase phosphorylates and activates S-type SLAC1 and R-type QUAC1 (QUICK-ACTIVATING ANION CHANNEL 1) anion channels that drive stomatal closure (Kollist *et al*., 2014; Jalakas *et al*., 2021a). In addition, ABI2 inhibits GHR1 (GUARD CELL HYDROGEN PEROXIDE-RESISTANT1), that controls the activation of the SLAC1 anion channel (Hua *et al*., 2012).

In guard cells, CPKs (CALCIUM-DEPENDENT PROTEIN KINASE) are also controlled by PP2C-s (Geiger *et al*., 2011). Most, but not all, CPKs require the presence of free Ca^{2+} ions for their activation, which is only achieved once the closure cascade has been initiated (Geiger *et al*., 2011). CPK-s are able to activate SLAC1 as well as other S-type anion channels such as SLAH3 (SLAC HOMOLOGUE 3; Geiger *et al*., 2011).

Upon formation of the ternary ABA–PYR/PYL/RCAR–PP2C complex, PP2C phosphatases are inactivated and OST1 becomes activated, either through autophosphorylation or through Raf kinases (Figure 1; Fàbregas *et al*., 2020). Activation of OST1 in turn activates the anion channels. SLAC1 activation results in efflux of Cl^-, NO_3^- anions from the guard cell, while QUAC1 transports $malate^{2-}$ (Kollist *et al*., 2014). At the same time, OST1 phosphorylates plasma membrane Ca^{2+} permeable CYCLIC NUCLEOTIDE-GATED CHANNELs like CNGC5, 6, 9 and 12, causing a rise in cytosolic Ca^{2+} (Tan *et al*., 2023). Increased cytosolic Ca^{2+} allows for the activation of CPKs and in *A. thaliana*, CPK15 has been found to be the most important: when it is knocked out, impairment in stomatal closure is comparable with the impairment in *cpk3/4/5/6/11* quintuple mutant (Shen *et al*., 2025). CPK15 is also directly phosphorylated and activated by OST1 (Shen *et al*., 2025). In addition, OST1 also activates NADPH (nicotinamide adenine dinucleotide phosphate) oxidases, such as RBOHF (RESPIRATORY BURST OXIDASE HOMOLOG F), leading to accumulation of reactive oxygen species,

which in turn may add an entourage effect on cytosolic Ca^{2+} rise (Sirichandra *et al*., 2009). The rise of free Ca^{2+} ions in the cytoplasm together with the suppression of PP2C phosphatases in the presence of ABA activates other CPK kinases, which in turn support activation of anion channels and depolarisation of the plasma membrane (Geiger *et al*., 2011; Kollist *et al*., 2014). Depolarisation drives K^+ ions out of the guard cell through the GORK1 (GUARD CELL OUTWARD-RECTIFYING K^+ CHANNEL) channel, resulting in substantial ion efflux, a decrease in the osmotic potential of the guard cell, water exiting the cell and stomatal pore closure (Munemasa *et al*., 2015). ABA is also shown to inhibit stomatal opening through inactivation of H^+-ATPases, which would normally hyperpolarise the plasma membrane and drive ions into the guard cell, increasing its turgor (Zhang *et al.,* 2004; Kollist *et al*., 2014).

2.3 Stomatal responses to VPD

Vapor pressure deficit (VPD) is the difference between the saturating water vapor pressure and the actual vapor pressure of the air. It is the primary driving force for evaporation, where dry air (high atmospheric VPD) increases transpiration, while humid air (low atmospheric VPD) reduces evaporative demand. As global temperatures are rising at a rate of 0.2 °C per decade, combined with declining relative humidity, it is important to understand the mechanisms of stomatal responses to VPD (IPCC, 2022). The rise in atmospheric VPD has been shown to decrease stomatal conductance, photosynthesis and crop yields, thus threatening global food security (Yuan *et al*., 2019; López *et al*., 2021).

High VPD induces stomatal closure through hydropassive and hydroactive mechanisms (Franks *et al*., 1998). At the onset of high VPD plant transpiration through stomata initially increases, which leads to turgor loss in the neighbouring cells of the stomata in the epidermis: this loss of epidermal backpressure triggers a transient "wrong-way response", where the stomatal pores briefly expand (Buckley 2005). This is followed by the hydroactive signalling, where high VPD can induce ABA production, with ABA again forming a complex with PYR/PYL/RCARs and PP2Cs, thus activating the protein kinase OST1 and triggering stomatal closure (Figure 1; Merilo *et al* 2013; McAdam and Brodribb, 2016; Merilo *et al*., 2018). Interestingly, in evolutionarily old species the wrong way response is generally absent, with the exception of *Marsileaceae* (Franks and Farquhar, 2007; Martins *et al*., 2015; Westbrook and McAdam, 2020).

If the active metabolic signaling pathway for stomatal closure (the ABA-OST1-SLAC1) is broken, the plants can still close their stomata hydropassively via guard cell turgor loss, however this response often relies on high initial stomatal conductance and depends on the plant's hydraulic status (Merilo *et al*., 2018; Jalakas *et al*., 2021b; Tulva *et al*., 2023). In addition, it has been shown, that when the ABA-activated Domain II is removed from OST1, but ABA-independent activation (Domain I) domain is intact, the mutants show stronger

response to high VPD compared with *ost1-3* (Tulva *et al.*, 2023). This indicates, that OST1 also regulates stomatal response to high VPD ABA-independently.

Beyond stomatal regulation, OST1 is a regulator of whole-plant water status and stress adaptation (Wang *et al.*, 2020). In *Arabidopsis,* it is involved in regulating cold tolerance and root growth under drought stress, while in tomato SlOST1 is involved in promoting flowering under drought stress (Chong *et al.,* 2022a; Chong *et al.,* 2022b). In barley, there are 10 genes belonging to the SnRK2 subclass and from these, 8 have ABA-responsive elements in their promoters (Chen *et al.*, 2021).

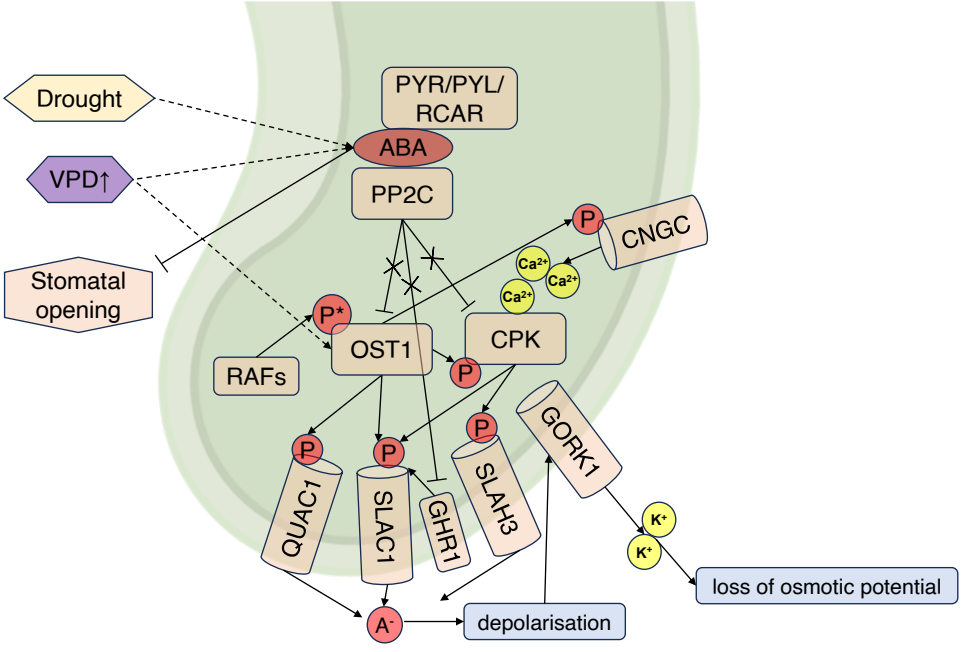

Figure 1. A simplified scheme of absisic acid and vapour pressure deficite triggered stomatal closure signalling pathway. Drought and increase in ambient vapour pressure deficit (VPD) both trigger absisic acid (ABA) synthesis (McAdam and Brodribb, 2016). ABA is sensed by its PYR/PYL/RCAR receptor proteins to form a ternary complex with PP2C-s (Gonzalez-Guzman *et al.*, 2012). Once PP2C-s are bound, they no longer exert inhibiton on OST1, CPKs and GHR1 (Mustilli *et al.*, 2002; Geiger *et al.*, 2011; Hua *et al.*, 2012). Once OST1 is phosphorylated by RAF kinases or autophosphorylated, it triggers stomatal closure cascade by activating CNGC Ca^{2+} channels, CPK-s, GHR1 as well as SLAC1 and QUAC1 anion channels directly (Geiger *et al.*, 2011; Hua *et al.*, 2012; Kollist *et al.*, 2014; Fàbregas *et al.*, 2020; Tan *et al.*, 2022). CPKs further contribute to SLAC1 activation, as well as SLAH3 anion channel activation, while GHR1 also activates SLAC1 (Geiger *et al* 2011; Hua *et al.*, 2012). The three anion channels drive anions out of the guard cell, which depolarises the guard cell activating K^+ efflux channel GORK1 (Munemasa *et al.*, 2015). With the simultaneous loss of anions and K^+ cations, guard cell osmotic potential decreases resulting in water efflux, turgor loss and ultimately stomatal closure (Munemasa *et al.*, 2015). In the presence of ABA, stomatal opening is also inhibited through its effect on H^+-ATPase (Zhang *et al.*, 2004; Kollist *et al.*, 2014).

2.4 Stomatal responses to atmospheric CO_2 change

Global atmospheric CO_2 concentration has been rising at a rate of 2.6 ppm per year between 2020 and 2025 (Lan *et al*., 2026). This has large effects on plant physiology, with meta-analyses of free-air CO_2 enrichment studies showing that light-saturated carbon uptake, diurnal carbon assimilation and above-ground production increase in elevated CO_2, whereas crop nitrogen and nutrient concentrations decrease (Ainsworth and Long, 2004; Ainsworth and Long, 2020). Atmospheric CO_2 concentration, which determines the intercellular CO_2 level, also participates in stomatal regulation. CO_2 taken up through the stomata enters guard cells via the PIP2;1 (PLASMA MEMBRANE INTRINSIC PROTEIN 2-1) aquaporins (Izumi *et al*., 2014). Although inside the cell CO_2 can then passively convert into bicarbonate (HCO_3^-), carbonic anhydrases CA1 and CA4 significantly increase the conversion rate (Hu *et al*., 2010). As bicarbonate levels rise in the cell, the physical interaction between the Raf-like kinase HT1 (HIGH LEAF TEMPERATURE 1) and the MAP kinases MPK4 (MITOGEN ACTIVATED PROTEIN KINASE 4) and MPK12 (MITOGEN ACTIVATED PROTEIN KINASE 12) is enhanced (Yeh *et al*., 2023). The binding between MPK12/MPK4 and HT1 inhibits the HT1 kinase activity, resulting in downregulation of CBC1 and CBC2 (CONVERGENCE OF BLUE LIGHT AND CO_2 1 and 2) activity (Takahashi *et al*., 2022). CBC1 and CBC2 in their active state suppress SLAC1 and SLAH3 anion channels preventing stomatal closure, while under elevated CO_2 conditions this suppression is lifted via HT1 inactivation and stomatal closure is initiated (Hiyama *et al*., 2017).

2.5 Photosynthetic carbon assimilation

Carbon assimilation through photosynthesis is the most important biochemical mechanism for sustaining life on Earth, being the primary source for carbon in almost all organic matter and oxygen in the atmosphere (Blankenship, 2010; Stirbet *et al*., 2020). Its importance extends from the basic energy and carbon needs of individual cells to the regulation of the Earth's atmosphere and biosphere (Hohmann-Marriott and Blankenship, 2011). The process starts at the stomata, where CO_2 diffuses from the atmosphere into the substomatal cavity (Farquhar and von Caemmerer, 1981). Plants' consumption of CO_2 reduces the partial pressure of CO_2 inside the leaf intercellular airspaces compared with atmospheric pressure, thus generating a concentration gradient, which determines the rate at which CO_2 diffuses inward (Farquhar and Sharkey, 1982). From the intercellular spaces, CO_2 must pass through the mesophyll cell wall, plasma membrane, cytosol and chloroplast membranes before it can reach the chloroplast (Moore *et al*., 2021; Huang *et al*., 2022). It has been found that the mesophyll cell wall thickness is the primary driver of the CO_2 mesophyll conductance (g_m), also depending on the chloroplast surface area exposed to intercellular airspace (Huang *et al*., 2022). Mesophyll conductance generally decreases with stress and

leaf senescence and in non-stressed leaves, g_m is negatively correlated with leaf dry mass per unit area, which is a measure of leaf structural toughness (Niinemets *et al*., 2009).

Inside the chloroplast stroma, CO_2 enters the Calvin-Benson cycle, where the enzyme Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) fixes one molecule of CO_2 to a five-carbon sugar acceptor called ribulose 1,5-bisphosphate (RuBP), which turns into two molecules of 3-phosphoglycerate (3-PGA; Farquhar *et al*., 1980; Moore *et al*., 2021). For converting the 3-PGA into triose phosphates (TP) and subsequently into starch and sugars, additional energy is required in the form of ATP (adenosine triphosphate) and NADPH (Eberhard *et al*., 2008).

Inside the chloroplast thylakoid membranes, light is absorbed by pigments in the light-harvesting complexes of Photosystem II (PSII) and Photosystem I (PSI; Stirbet *et al*., 2020). In PSII, electrons are extracted from water as the molecule is split, producing oxygen and protons into the lumen: the latter are used to drive ATP synthase via the generated proton concentration gradient (Hill and Bendall, 1960; Mitchell, 1961; Eberhard *et al*., 2008; Stirbet *et al*., 2020). The electrons move through electron transport chain into PSI, where they are re-energised by light and used to reduce $NADP^+$ into NADPH by the ferredoxin-$NADP^+$-reductase (FNR; Hill and Bendall, 1960; Mitchell, 1961; Eberhard *et al*., 2008; Stirbet *et al*., 2020). In the Calvin-Benson cycle, 3-PGA is first phosphorylated using ATP to form 1,3-bisphosphoglycerate, which then is reduced by NADPH to produce TPs, mainly glyceraldehyde 3-phosphate (Farquhar *et al*., 1980; Stirbet *et al*., 2020). Some of the TPs are combined and rearranged into hexose phosphates inside the chloroplast, where they are synthesised into starch to provide sugars for respiration, maintenance and growth during the night (Pearcy, 1990; Lawlor, 1995; Stirbet *et al*., 2020). The rest of TPs are transported into cytosol where they are also converted into hexose phosphates, and then synthesised into sucrose through sucrose phosphate synthase and sucrose-phosphate phosphatase (Pearcy, 1990; Lawlor, 1995; Albi *et al*., 2016; Stirbet *et al*., 2020). Sucrose is then loaded into the phloem and transported into sink organs such as seeds, fruits, roots and tubers (Lawlor and Mitchell, 1991; Lawlor, 1995; Moore *et al*., 2021).

For the continuous operation of Calvin-Benson cycle, the RuBP regeneration from TPs is needed. RuBP regeneration can become a rate limiting step for overall photosynthesis at high light or CO_2 levels (von Caemmerer and Farquhar, 1981; Farquhar and Sharkey, 1982; Moore *et al*., 2021). Furthermore the Rubisco enzyme itself must be maintained in an active state, requiring ATP-dependent action of Rubisco activase to remove the sugar phosphates that block Rubisco active site (Moore *et al*., 2021).

Rubisco can also fix oxygen because it cannot perfectly distinguish between CO_2 and O_2. It results in the production of 2-phosphoglycolate that cannot be used in the Calvin-Benson cycle and must be thus recycled, resulting in energy losses (Farquhar *et al*., 1980; Stirbet *et al*., 2020). This process, called photorespiration is estimated to reduce the net rate of CO_2 assimilation in C3 plants by 20% to 40% depending on environmental conditions (Lawlor and Mitchell, 1991; Moore *et al*., 2021). However, when CO_2 supply is restricted i.e during drought when

stomata are closed, photorespiration through consuming ATP and NADPH can act as an alternative sink for the electrons from the light reactions, protecting the photosystems from oxidative damage (Eberhard *et al*., 2008; Tcherkez and Limami, 2019). Photorespiration is projected to increase with climate change as rising temperatures decrease CO_2 specificity of Rubisco and reduce the solubility of CO_2 relative to O_2 in the chloroplast, although the increase in atmospheric CO_2 can mitigate this effect (Cavanagh *et al*., 2022).

C4 plants (for example, maize and sugarcane) have evolved a special CO_2-concentrating mechanism called C4 photosynthesis. In these plants, CO_2 is first fixed into organic acids such as malate in mesophyll cells, followed by transportation of organic acids into bundle sheath cells that contain Rubisco (Lawlor and Mitchell, 1991; Moore *et al*., 2021). Next, organic acids are decarboxylated, thus concentrating CO_2 near the Rubisco (Lawlor and Mitchell, 1991; Moore *et al*., 2021). This creates a nearly saturated CO_2 environment in bundle sheath cells (typically 10 times higher than ambient levels) that outcompetes oxygen for Rubisco's active sites (Lawlor and Mitchell, 1991; Lawlor, 1995).

2.6 Wheat and barley as food crops

Wheat is the third most produced cereal, after maize and rice at 800 million metric tons, but is grown on the largest area of any other crop (FAOSTAT, 2025). Its domestication began over 10,000 years ago in the Fertile Crescent of the Middle East (Charmet, 2011). This process involved gradually transitioning from gathering wild grains to deliberate cultivation (Weiss *et al*., 2006; Feldman and Levy, 2023). This resulted in anatomical and morphological changes such as the development of a non-brittle rachis and free-threshing grains (Hammer, 1984; Feldman and Levy, 2023).

Genetically, wheat is separated into diploid einkorn, tetraploid durum, and hexaploid bread wheat (Sax, 1918; Feldman and Levy, 2023). Bread wheat has a massive genome of 16Gb, that comprises of three subgenomes (AABBDD; Arumuganathan and Earle, 1991; IWGSC, 2018). The hexaploid bread wheat came from a hybridisation event between domesticated tetraploid emmer wheat and the wild diploid grass *Aegilops tauschii*, which extended the crops range of adaption to more continental climates and northern latitudes (McFadden and Sears, 1946; Tsunewaki, 1968; Zohary, 1969; Charmet, 2011).

Wheat contributes about a fifth of total human calory intake; on average around 19% of wheat production is used as animal feed, however this percentage is higher in Eastern Europe at approx. 40% (Feldman *et al*., 1995; Baloch and Meija, 1999; Shewry and Hey, 2015; OECD/FAO, 2024). In addition, production of wheat gluten accounts for a significant portion of wheat processing, used in the baking industry, as a meat replacement in vegetarian foods and pet foods (Veraverbeke and Delcour, 2002; Boland *et al*., 2005; Day *et al*., 2006).

Wheat breeding has evolved from traditional selection to highly technical pipelines for improved speed and efficiency. In conventional breeding which is a

10–15 year process, selected parents are crossed, selfed in segregating generations before they are evaluated in mutliple locations based on their harvest index (grain weight/straw weight), number of fertile florets, ear size, quality traits and stress tolerance (Breseghello and Coelho, 2013; Gudi *et al*., 2022). Finally, seed is multiplied and the variety is released (Breseghello and Coelho, 2013; Gudi *et al*., 2022). During the Green Revolution, a strategy called shuttle breeding was popularised where cultivar development time was halved by evaluating the segregating generations already at different geographic locations after each generation, allowing twice per year harvest and ensuring that genotypes are exposed to different diseases, soil types and environmental stresses (Ortiz *et al*., 2007; Borlaug, 2007; Gudi *et al*., 2022). Speed breeding, using long photoperiods (22 hours of light) in controlled environments reduces breeding time by triggering early flowering, allowing for up to six generations per year (Watson *et al*., 2018; Hickey *et al*., 2019; Gudi *et al*., 2022). Newer approaches like double-haploid technology, by which breeders achieve complete homozygosity in a single round of recombination, and genomics-assisted breeding using genomic markers to predict the performance of lines without extensive field testing, have further increased the throughput of breeding programs (Meuwissen *et al*., 2001; Forster and Thomas, 2005; Crossa *et al*., 2010; Reynolds *et al*., 2021; Gudi *et al*., 2022). And last but not least, CRISPR-Cas9 gene editing allows to introduce targeted modifications to genes of interest in an already stable background and has been used to increase nitrogen use efficiency or engineer wheat with fewer immunogenic gluten peptides (Zhang *et al*., 2021; Yu *et al*., 2024; Tiwari *et al*., 2024).

Traditional breeding methods are increasingly considered unsuitable for meeting the demands of a global population projected to reach 10 billion people by 2050 (Hickey *et al*., 2019; Godfray *et al*., 2010). Global yields have been stagnating since 1995 in some of the world's most productive regions, like France (Brisson *et al*., 2010; Charmet, 2011). In addition, the climate is changing more quickly than traditional methods can respond to, with rising temperatures and extreme weather events already causing significant year-to-year yield variability (Zampieri *et al*., 2017; Reynolds *et al*., 2021). Although the newer breeding approaches significantly accelerate the breeding time, original domestication and centuries of highly selective breeding have created a bottleneck, leaving modern wheat with a narrow genetic base, where "best × best" crossing strategies limit the introduction of novel alleles needed for resilience (Haudry *et al*., 2007; Charmet, 2011; van Ginkel and Ortiz, 2018; Reynolds *et al*., 2021).

The domestication of barley from its wild relative *Hordeum vulgare spp. spontaneum* began roughly at the same time as wheat somewhere around 10000 to 12000 years ago, also in the Fertile Crescent region (Zohary and Hopf 1993; Badr *et al*., 2000; Jiang *et al*., 2025). In contrast to wheat, multiple wild populations contributed to the domesticated gene pool in barley (Poets *et al*., 2015; Pankin and von Korff, 2017; Guo *et al*., 2025). The crop was spread into Europe around the 5th and 6th millennia BC with introduction to the Americas and Australia happening fairly recently in the 17th and 18th centuries (Liu *et al*., 2019; Hu *et al*., 2023).

Global barley production is estimated at 142 million tons annually, covering roughly 44 million hectares (FAOSTAT, 2025). It ranks as the fourth most produced cereal crop globally, with more than a third (49 million tonnes) being produced in the EU (FAOSTAT, 2025). Around 70% of barley production is used for animal feed, while 21% is used for malting and brewing and 6% as human food (Tricase *et al*., 2018).

Barley genome is diploid with a size of 5.1 Gb and small chromosome count (2n=14; HH), which makes it a good model organism for studying cereals (IBSC, 2012; Giraldo *et al*., 2019; Jiang *et al*., 2025). Barley domestication included development of non-brittle rachis similarly to wheat, as well as the evolution of six-rowed spikes, which can produce three times as many seeds as ancestral two-row genotypes (Komatsuda *et al*., 2007; Pourkheriandish and Komatsuda, 2007; Pourkheirandish *et al*., 2015; Jiang *et al*., 2025).

Barley breeding is similar to wheat, where conventional methods require many generations to achieve homozygotes, however identifying and selecting for specific homozygous recessive traits require fewer generations in barley because the phenotypes are immediately visible due to its diploid genome (Schmid and Thorwarth, 2014; Jiang *et al*., 2025).

Yields of barley have also been stagnating in the EU in the past 30 years. Moore and Lobell (2015) estimated in 2015, that higher temperatures and drier air had already reduced yields up to 2–4% in wheat and barley. Barley has better tolerance to abiotic stresses like drought compared with wheat, driven by faster leaf area development, extensive root system and earlier flowering time (López-Castañeda *et al*., 1995). This allows barley to escape terminal drought and complete its growth cycle before the rise in temperature in the later season (López-Castañeda *et al*., 1995). However, in favorable environments where soil water is not limited, bread wheat can produce higher grain yields and biomass than barley (Fischer and Wood, 1979; López-Castañeda *et al*., 1995).

2.6.1 What determines yield?

Yield in wheat and barley is a complex trait, emerging from the coordinated processes of photosynthesis, development of fertile reproductive structures and the rate and duration of grain filling under variable environments (Foulkes *et al*., 2011; Reynolds *et al*., 2012a). Mechanistically, yield determination can be split into source and sink physiology, where source activity is represented by the synthesis of assimilates in photosynthesis and remobilisation of already fixed carbon and nitrogen. Sink demand/activity, on the other hand, is represented by the capacity of growing organs to store those resources (Reynolds *et al*., 2012a).

In wheat and barley, total grain yield is determined first by the ear or head number per unit area, then grain number per ear or head, and finally individual grain weight (n heads x n grains x grain weight; Foulkes *et al*., 2011; AHDB, 2025a; AHDB, 2025b). The number of heads is determined from sowing until flag leaf emergence, grain number during stem elongation to anthesis and grain weight after anthesis (Figure 2; AHDB, 2025a and 2025b). Grain number per area

is more plastic and often more yield-defining component, as stress before anthesis limits maximum sink size, while grain weight is usually more conservative, but is vulnerable to post-anthesis heat or terminal drought limiting source duration (Foulkes *et al*., 2011; Serrago *et al*., 2013; Mirosavljevic *et al*., 2024).

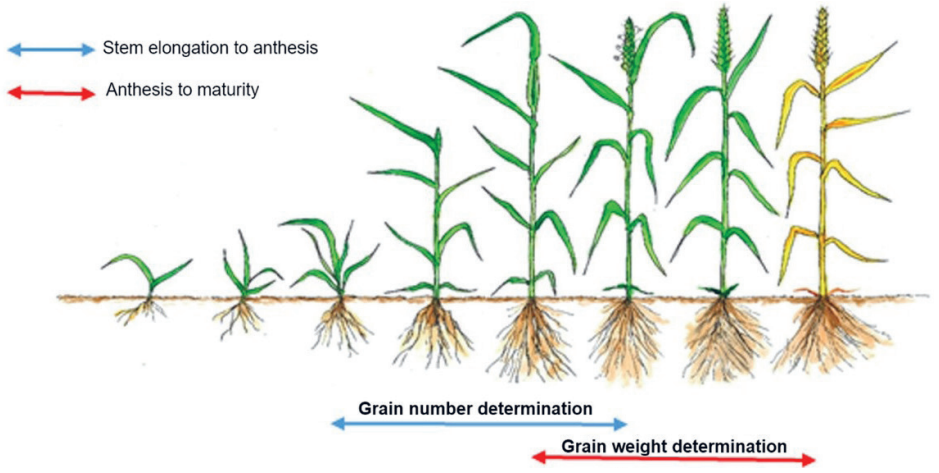

Figure 2. Grain number is determined during stem elongation until anthesis, while grain weight is determined during and after anthesis until maturity. Modified from Faralli and Lawson, 2020.

Historically, yield gains in wheat were driven by increasing harvest index and lodging resistance through semi-dwarfing that decreased stem elongation, improved standability under high nitrogen fertilisation and shifted dry matter partitioning toward grain, thus increasing the potential sink size (Foulkes *et al*., 2011; Aisawi *et al*., 2015; Nadolska-Orczyk *et al*., 2017). Another aspect breeders have selected for is phenology, where the timing of important stages, heading and anthesis, has shifted to a locally safer environmental window, (Hyles *et al*., 2020; Fernández-Calleja *et al*., 2021). From biotic stresses, disease resistance is the key selection criterion, as pathogens can limit both sink and source size and activity. For example, Fusarium head blight directly attacks the spikes, reducing grain numbers, while powdery mildew attacks the leaves diminishing photosynthetic source potential (Matarese *et al*., 2012; Gao *et al*., 2018; Yang and Luo, 2021). From a source perspective, breeders have selected for a canopy architecture with erect flag leaves, allowing better light distribution within the foliage, and "stay-green" trait that delays the onset of leaf senescence during the grain filling period, allowing the source to remain active under terminal heat stress (Lopes and Reynolds, 2012; del Pozo *et al*., 2016; Burgess *et al*., 2017; Kumar *et al*., 2023).

In favorable conditions, modern cultivars are typically limited by the number of grains and in this case, the potential source capacity can be up to 25% greater than the realised grain yield, indicating that the photosynthetic apparatus is underutilised (Giménez *et al*., 2025). However under abiotic stressors like drought, yield is mainly determined by photosynthesis and the efficiency of

remobilisation of carbohydrates, making source components the dominant factors in yield determination (Fischer, 2011). Crop yield is determined as a product of seasonally intercepted radiation, radiation use efficiency (i.e. photosynthesis) and harvest index. In modern cultivars, efficiency of solar radiation interception and the harvest index have already been bred close to their theoretical maxima, while total incident solar radiation depends on the environment (Monteith, 1977; Long *et al*., 2006; Foulkes *et al*., 2011; Reynolds *et al*., 2012b; Faralli and Lawson, 2019). The only remaining major route for significantly improving yield potential is to select for increased photosynthetic radiation use efficiency, which remains far from its theoretical optimal efficiency in C3 crops (Long *et al*., 2006; Zhu *et al*., 2008; Zhu *et al*., 2010; Faralli and Lawson, 2019).

Breeders use heritability analysis to estimate how much of the observed difference among lines is due to genetics rather than the environment, and thus how reliably selection of a trait is likely to work (Holland *et al*., 2003). It is done by testing genotypes in replicated field plots across several locations or years and then fitting statistical models that separate variation due to genotype (G), environment (E), G×E and residual error, where G×E interaction means that the genotype performs differently in different environments and residual error means the left-over unexplained noise (Smith *et al*., 2005). In practice, grain yield itself usually has lower heritability than simpler traits such as heading date, plant height or thousand-kernel weight, as yield depends on the weather and management and G×E interaction. This is why yield components and developmental traits are commonly bred for in early selection trials, while yield is selected for in advanced trials (Khadr, 1971; Holland *et al*., 2003; Lozada *et al*., 2020; Fischer and Rebetzke 2018). To quantify the stability of the performance of genotypes across different environments, breeders calculate the WAASB (Weighted Average of Absolute Scores from BLUPs) score (Olivoto *et al*., 2019). A low WAASB score indicates a stable genotype, that maintains consistent performance and deviates the least from its average across different environments, while unstable genotypes that are highly reactive to environmental effects have a high WAASB score (Olivoto *et al*., 2019). Together with yield data, breeders can identify genotypes that are both high yielding and broadly adapted (Pour-Aboughadareh *et al*., 2022).

2.6.2 Gas exchange as a breeding trait

Gas exchange traits, involving net CO_2 assimilation rate (A_{net}), water vapor transpiration and stomatal conductance, are increasingly recognised as important physiological traits for breeding varieties with improved radiation use efficiency and biomass, resulting in higher-yielding and more resilient crops (Long *et al*., 2006; Driever *et al*., 2014). As traditional yield components such as harvest index approach their theoretical limits, physiological traits related to carbon gain offer new strategies for increasing yield potential (Foulkes *et al*., 2011; Reynolds *et al*., 2012b; Roche, 2015).

Studies of historical wheat cultivars show that modern, high-yielding varieties have higher flag leaf photosynthetic rates than older varieties, suggesting that

breeding has already unintentionally selected for higher gas exchange (Fischer *et al*., 1998). At the same time, a significant variation of A_{net} exists within current cultivars and its wild relatives, providing a genetic pool for further improvements (Carmo-Silva *et al*., 2017; McAusland *et al*., 2020). Beyond leaves, photosynthesis in reproductive structures like wheat ears (spikes) can contribute 10% to 60% of final grain weight (Lawson and Milliken, 2023). Breeding for enhanced spike photosynthesis is a target with large potential for maintaining yield stability, particularly under stress, when leaves may senesce early (Molero and Reynolds, 2020).

Stomatal conductance has been associated with yield gains and leaf cooling through evapotranspiration and because it has higher heritability than yield, it has been proposed as an effective target for selection (Fischer *et al*., 1998; Reynolds *et al*., 2001; Faralli *et al*., 2024). The speed at which stomata open and close in response to fluctuating light is also a promising target in breeding: faster stomatal responses can reduce diffusional limitations on photosynthesis in sunlit leaves after shading while minimizing water loss during periods of shade (McAusland *et al*., 2016; Faralli *et al*., 2019; Lawson and Milliken, 2023).

Photosynthesis as a trait can be measured through survey measurements, screening a large number of plants or through CO_2- and light-response curves for mechanistic details (Walker *et al*., 2018; Faralli *et al*., 2024). By changing CO_2 concentration step-by-step and fitting the resulting A_{net} response curve to biochemical models, like the Farquhar von Caemmerer-Berry model, it is possible to estimate physiological parameters like the maximum rate of Rubisco carboxylation (V_{cmax}) and rate of electron transport (J_{max}; Faralli *et al*., 2024). Response curves help to determine, which photosynthetic process is rate-limiting, such as RuBP regeneration or Rubisco kinetic properties (Furbank *et al*., 2013; Driever *et al*., 2014). However, it generally takes 20–40 minutes per leaf for measuring the response curve to determine V_{cmax} and J_{max}, which is too slow for any modern breeding program that can have up to thousands of genotypes (Jackson *et al*., 1996; Busch *et al*., 2024). In contrast, survey measurements of A_{net} are significantly faster and typically take between 1 to 5 minutes per leaf but are less informative and only represent the current state of the leaf (Stinziano *et al*., 2017; Busch *et al*., 2024). It's possible to screen approximately 100 leaves per day with a single device, which is several fold faster than a few individuals per day possible with full response curves (Stinziano *et al*., 2017; Faralli *et al*., 2024). While survey measurements are the fastest way to directly screen gas exchange in large populations, their value for breeding relies on measurement timing and the ability to control for environmental noise. Measurements taken at the initiation of booting often show no correlation with yield, whereas measurements taken during grain filling are much stronger predictors of grain yield (Fischer *et al*., 1981; Reynolds and Pfeifer, 2000; Driever *et al*., 2014). Snapshot measurements are susceptible to errors from environmental effects, including sun/shade and change in humidity (Busch *et al*., 2024). As single leaf measurements are often unreliable, it is necessary to average multiple readings per plot or repeating the measurements over

several days to characterise a genotype accurately, further limiting its usefulness in modern breeding programs (Reynolds *et al*., 2001; Busch *et al*., 2024).

Field studies across different plant species have demonstrated that stomatal conductance positively correlates with photosynthetic capacity and net primary production (Roche, 2015; Hoshika *et al*., 2017). This relationship is however non-linear and subject to diminishing returns, because the biochemical fixation processes in the leaf are not able to absorb additional CO_2 indefinitely (Zhu *et al*., 2010; Busch *et al*., 2024). Despite this, maintaining open stomata is important under fluctuating light to prevent CO_2 losses, as stomata are much slower to respond compared with the photosynthetic machinery (Tinoco-Ojanguren and Pearcy, 1993; McAusland *et al.,* 2016). It needs to be noted that in field studies it's not necessarily the stomata that control the net assimilation rate, as both are responding simultaneously to the same environmental drivers such as light intensity or water availability (Bellasio, 2023).

Outside of mere selection in field trials, the photosynthetic apparatus, CO_2 diffusion barrier and stomatal responsiveness can be modified genetically with the aim of improving photosynthetic efficiency and crop yield. In tobacco (*Nicotiana tabacum*) by overexpressing the *Arabidopsis* pectin methyltransferase AtCGR3, cell wall porosity increased while wall thickness decreased resulting in a 28% increase in mesophyll conductance and 8% increase in photosynthetic CO_2 uptake in the field (Salesse-Smith *et al*., 2024). In rice (*Oryza sativa*) over-expressing the plasma membrane H^+-ATPase1 (OSA1), stomatal conductance was increased along with nutrient uptake, resulting in a 33% increase in grain yield (Zhang *et al*., 2021). Overexpressing maize-derived Golden2-like (GLK) transcription factors, key regulators of chloroplast development, increased chlorophyll levels and pigment-protein complexes in rice, resulting in 30–40% increase in vegetative biomass and grain yield (Yeh *et al*., 2022). In sorghum (*Sorghum bicolor*), gains in CO_2 assimilation and grain yield were accomplished through overexpressing the PSII component RieskeFeS subunit (Ermakova *et al*., 2023). Increasing Rubisco, the main CO_2 fixing enzyme, resulted in significant increase in photosynthesis and production of biomass in sorghum and sugarcane (Smith *et al*., 2025).

3. AIMS OF THE STUDY

Stomatal pores control photosynthetic CO_2 uptake from the atmosphere. At the same time, plants lose water as vapour through the stomata. To balance water loss with carbon gain, plants have evolved mechanisms that actively regulate stomatal apertures in response to air humidity, ambient CO_2 concentration, drought and other environmental cues. Detailed understanding of stomatal regulation predominantly comes from studies on *Arabidopsis thaliana*, the model object in plant genetics and physiology. To which extent these mechanisms are identical in economically important crops such as barley is not entirely known. Furthermore, the evolution of active stomatal regulation is still under debate. Understanding when specific stomatal regulation mechanisms emerged gives us insight into their function and their interplay, but also their use in breeding. Making informed and precise genetic modifications in crops allows us to generate plants with improved water use efficiency and/or photosynthesis. At the same time, there already exists a large natural variation of gas exchange traits in crops, which could be utilised in breeding. However, the current methodology does not allow high-throughput field screening of stomatal conductance and photosynthesis in parallel. The aim of this dissertation was to expand our understanding of stomatal regulation with the focus on crops and the following specific aims:

- To gain insight into the evolution of active stomatal regulation controlled by ABA, CO_2 and VPD by studying the stomatal responsiveness of species in the evolutionarily old genus *Equisetum*;
- To study the involvement of barley homologues of *A. thaliana* OST1 in determining gas exchange, stomatal density and grain yield of barley by disrupting the corresponding genes in the barley genome;
- To develop and validate a high-throughput screening method for *in situ* stomatal conductance and net assimilation rate measurements, and to use it in a field trial to assess its potential application in breeding studies;
- To characterise the grain yield potential and quality of 300 spring wheat genotypes from Norway and the Baltics to identify promising genotypes for future breeding.

4. MATERIALS AND METHODS

The materials and methods are described in the respective chapters in publications (I–IV) in detail.

4.1 Statistical analysis

Statistical analysis was performed with Statistica (v 7.1, Statsoft Inc., Tulsa, OK, USA). Differences between groups were analysed with one-way ANOVA, using Tukey HSD as post-hoc. Statistical analysis on linear regressions was performed using simple regression in GLM (General Linear Models).

4.2 Spring wheat field trial gas exchange measurements

Spring wheat (*Triticum aestivum*) was grown in the field in the scope of the Nordic-Baltic project NOBALWheat in METK (Centre of Estonian Rural Research and Knowledge, Jõgeva, Estonia). This project, including its agronomic practice and yield data was described previously by Jansone *et al*. (2024). From the 16 genotypes studied in this project, we selected 7 on the basis of their country of origin and contrasting date of release/background (EE – Hiie, Voore; LV – Robijs, 013-074; LT – DS-638-5-DH, DS-720-3-DH; NO – Runar). With Karal, a custom portable gas exchange device described in the manuscript, we measured the gas exchange of flag leaves on several days in the two weeks during early grain filling (Zadoks growth stage 60–75) for each genotype in 2021 and 2023, while we could only measure on one day during that period in 2022. Plots were measured once or twice a day, where a plot measurement consisted of 17 leaves measured at random. To reduce the diurnal variation of g_s and A_{net} and eliminate effect of the morning dew on g_s, measurements were conducted between 11:00 and 15:00 local time.

5. RESULTS AND DISCUSSION

5.1 Stomatal response to ABA application in *Equisetum* is conditional (I)

The presence and conditionality of stomatal ABA responses in evolutionarily older species such as ferns and their allies are still under debate. Stomatal responses to ABA in horsetails, the oldest extant land plant genus which has remained morphologically distinct for hundreds of millions of years, has remained unstudied (Elgorriaga *et al*., 2015; McAdam *et al*., 2025).

Here, we studied the stomatal responsiveness to exogenous ABA in three horsetail species (*E. arvense*, *E. pratense* and *E. sylvaticum*) using gas exchange measurements, repeated over three years. Average stable pre-treatment stomatal conductances of three studied horsetail species were relatively low compared with the model angiosperm *A. thaliana* in similar conditions (typically ~240 mmol m^{-2} s^{-1} at 250 μmol m^{-2} s^{-1} PPFD; Tulva *et al*., 2023): 37,7 (± 2,9 mmol m^{-2} s^{-1}) for *E. pratense*, 71,1 (± 5,4 mmol m^{-2} s^{-1}) for *E. sylvaticum* and 56,9 (± 3,0 mmol m^{-2} s^{-1}) for *E. arvense* when averaged over all years. Fern stomatal conductances are relatively similar, between 30–50 mmol m^{-2} s^{-1} (Hõrak *et al*., 2017). In 2019, no stomatal closure was detected after spraying with abscisic acid (50 μM) in any of the horsetail species (I, Figure 1A, B). However, in 2020, both *E. pratense and E. sylvaticum* closed their stomata in the same experimental setup (I, Figure 1A, B). When repeating the experiment again in 2023, *E. arvense* closed their stomata, but *E. pratense* did not (I, Figure 1A, B). Across all three years, all 3 studied species showed stomatal closure in response to ABA in one of the years, although its rate and magnitude was smaller than seen in angiosperms. Since the ABA responses were present in horsetails, albeit conditionally, this points at ABA-dependent stomatal signaling being present already in the last common ancestor of land plants. However, the ABA responses seem to be dependent on the environmental conditions, as previously indicated for ferns by Hõrak *et al*. (2017) and Plackett *et al*. (2021). It is difficult to pinpoint which conditions led to significant ABA responses in our study as the growth and gas exchange measurement conditions of leaves developed in the growth cabinet were identical in all years. Since the experimental plants were grown from rhizomes collected from nature, it's possible that "stress memory" from earlier growing conditions played a role, as there was variation in precipitation and relative humidity as also in timing of rhizome collection between the growing seasons (I, Figure 2A, C). On the other hand, differences in stomatal development in plants grown in 2023 could be related to stomatal ABA responsiveness. In that year *E. pratense* had significantly higher stomatal density than *E. arvense* (142.1 ± 4.1 and 112.7 ± 5.7 stomata/mm^2, respecively), while the values for their stomatal conductances were the opposite. This might have resulted in *E. pratense* absorbing significantly less ABA during the experiment with their numerous, but much more closed stomata. In the years when horsetail stomata were responsive

to ABA (2020 and 2023), the extent of stomatal closure in studied individuals positively and significantly correlated with initial stomatal conductance (Figure 3). Interestingly both *E. arvense* and *E. pratense* showed significant stomatal closure (VPD-treated g_s was 58% and 39% of initial g_s, respectively) in response to a sudden increase in air VPD in 2023 (0.84 ± 0.01 kPa to 2.22 ± 0.01 kPa; I, Figure 1E, F). Since in our experiments stomata of *E. pratense* in 2023 were able to further close in high VPD, despite its low initial g_s, but high stomatal density, suggests that ABA indeed might not have penetrated the stomatal pores sufficiently to induce the exogenous ABA response (Figure 3; I, Figure 1A, B, E, F). In order to fully understand the nature of the conditionality, experiments in contrasting controlled or monitored conditions are required. Even though the ABA responses seen here are inconsistent compared with angiosperms, McAdam *et al*. (2025) have called for a re-evaluation on stomatal regulation by ABA on horsetails and for more studies, involving field measurements, to clarify this. Gas exchange studies of horsetails in their natural environment, possibly enabling the capture of more individuals with more open stomata paired with VPD and ABA treatment, could help determine whether stomatal aperture is a prerequisite for ABA-dependent stomatal closure. To further test that horsetail, and in extension fern stomata are controlled by endogenous ABA signalling, molecular studies could be used, where for example ABA signalling components from horsetails are complemented one-by-one into *A. thaliana* respective knock-out backgrounds and their responses measured.

Horsetails have lower rates of g_s and A_{net} (3.5 to 5.5 µmol m^{-2} s^{-1} at 500 µmol m^{-2} s^{-1} PPFD; Kübarsepp *et al*., 2020). In other studies, A_{net} was higher for different horsetail species adapted to high light conditions and they were found to have rapid stomatal responses to light and VPD (Kübarsepp *et al*., 2020; McAdam *et al*., 2025). This makes *Equisetum* species highly competitive: they have an established presence in various environments and some of them, *E. arvense* included, are problematic weeds (Pratt *et al*., 2024; McAdam *et al*., 2025).

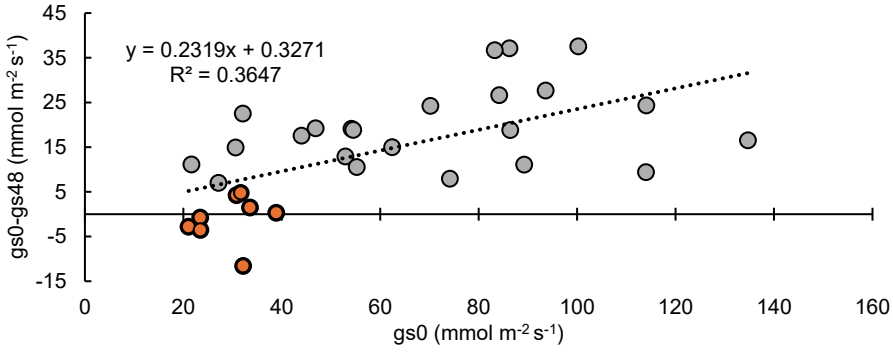

Figure 3. Linear regression between individual horsetail pretreatment stomatal conductances (gs0) and absolute closure values 48 after after spraying with abscisic acid (50 µM; gs0–gs48) in measurements conducted in 2020 and 2023. Values for *E. pratense* in 2023 are colored orange. Regression was statistically significant (GLM, $p<0.05$). n=31

5.2 AtSnRK2.6 homologues HvSnRK2.7 and HvSnRK2.9 are involved in ABA and VPD signalling in barley (II)

In *A. thaliana,* SnRK2 family consists of 10 members that act as master regulators of abiotic stress responses, where OST1/SnRK2.6 is the one central in stomatal closure signaling (Kulik *et al*., 2011; Yuan and Zhao, 2025). Similarily, there are 10 members in the SnRK2 family in barley (Chen *et al*., 2021). Phylogenetic tree analysis on the protein sequences of barley SnRK2s according to Chen *et al*. (2021) and *A.thaliana* SnRK2.6 showed that the closest homologues to AtSnRK2.6 are HvSnRK2.7 and HvSnRK2.9 (II, Figure S2). HvSnRK2.8 and HvSnRK2.10 could be excluded due to the absence of ABA responsive elements in their promotors (Chen *et al*., 2021).

HvSnRK2.7 and HvSnRK2.9 functional knock-outs in barley (Golden Promise) were generated using CRISPR-Cas9 resulting in a frameshift mutation due to large deletion in exon 1 of both genes (55bp and 50 bp respectively) and an addition of a single nucleotide insertion (20_21insT) in HvSnRK2.9 (II, Figure S3). Gas exchange measurements were made in two light conditions: low light (LL, LL, 260 μmol m^{-2} s^{-1} PPFD) and high light (HL, HL, 750 μmol m^{-2} s^{-1} PPFD). The averaged pre-treatment stomatal conductance in ABA and VPD experiments of the two double mutants L15 and L20 was significantly higher than that in wild-type (WT) plants both in LL (258 ± 11 mmol m^{-2} s^{-1}, 250 ± 18 mmol m^{-2} s^{-1} and 135 ± 7 mmol m^{-2} s^{-1}, respectively) and in HL (356 ± 17 mmol m^{-2} s^{-1}, 291 ± 19 mmol m^{-2} s^{-1} and 185 ± 12 mmol m^{-2} s^{-1}, respectively; Figure 4). The values of g_s of L15 and WT were significantly higher in HL compared with LL (LL g_s is 73% of HL g_s for both), whereas this effect was not significant in L20 (LL g_s was 86% of HL g_s; Figure 4). When high VPD was applied (from 0.97kPa to 2.08kPa), in 30 minutes g_s significantly decreased in WT at LL and HL (to 72% and 62% of initial g_s, respectively), but not in L15 and L20 in neither light intensities (II, Figure 3A, C). Similar results were obtained for ABA foliar spraying (20 μM), however L15 and L20 at HL did show ABA response (gspost to 88% and 85% of initial g_s, respectively), albeit the relative closure was smaller compared to WT (gspost to 53% of initial g_s; II, Figure 3B, D).

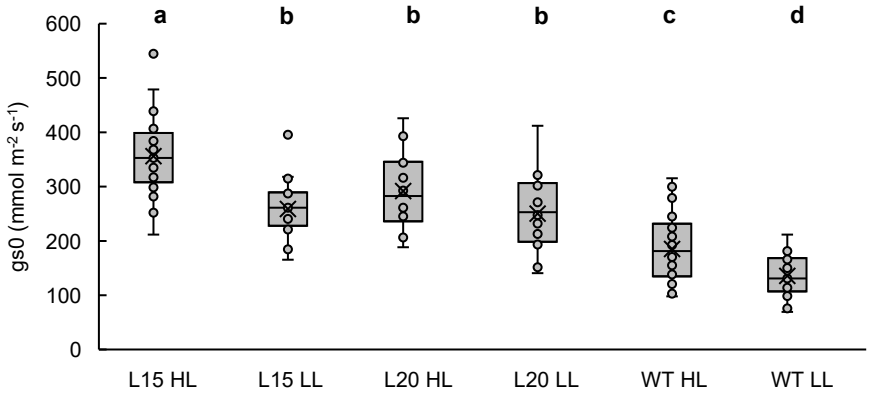

Figure 4. Box-and-whisker plots of barley pretreatment stomatal conductance values of *hvsnrk2.7/hvsnrk2.9* knock-out lines L15, L20 and wild-type (WT, Golden Promise) at low light (LL; 260 µmol m^{-2} s^{-1} PPFD) and high light (HL; 750 µmol m^{-2} s^{-1} PPFD). The box represents the interquartile range, while whiskers indicate the minimum and maximum. Horizontal line marks the median and "×" marks the mean. Outliers and inner points are represented as circles. Statistically significant groups are marked by letters (one-way ANOVA, *post-hoc* Tukey HSD; $p<0.05$). n=14–28

Higher g_s is frequently associated with higher CO_2 uptake, both across different species and different genotypes (Hetherington and Woodward, 2003; Jalakas *et al*., 2018; Zhang *et al*., 2021). When monitoring diurnal courses of the three genotypes in HL, there was a positive correlation between g_s and A_{net} detected during stomatal opening before noon (Fig 5; II, Figure 2A, B). However, grain yield harvested from these pot-grown plants did not show statistical differences between the genotypes, with total grain yield per plant for L15, L20 and WT being 22.1 ± 1.0 g, 23.9 ± 1.0 g and 21.1 ± 1.2 g, respectively. This was most likely the result of low light intensity in the greenhouse during grain filling due to experimental constraints (around 200 µmol m^{-2} s^{-1} PPFD), severely underutilising CO_2 diffusion potential. The positive effect of high g_s on CO_2 diffusion becomes relevant at higher light intensities: in wheat g_s values below ~550 mmol m^{-2} s^{-1} begun limiting A_{net} at 1500 µmol m^{-2} s^{-1} PPFD (Faralli *et al*., 2019). Growth experiments at field light intensities (above 1500 µmol $m^{-2}s^{-1}$; Zhen *et al*., 2022) are necessary to clarify, whether increased g_s results in higher grain yield. It must be noted however, that plants lacking OST1 homologues may lose more water and are more susceptible to drought stress and thus require artificial irrigation in dry seasons. Instead, they rather represent a model of how plant biomass production and yield might change in optimal conditions, not a real option to increase yields in the field. Furthermore, measuring grain yields in pots (3L here) is not representative of the field, as root growth space is limited. Here, we noted an unusually high number of productive tillers per plant, 49 ± 2.6, 39 ± 2.5 and 57 ± 2 for L15, L20 and WT, respectively, whereas in the field, average number of productive tillers can range from 2 to 15 for 2-row barley, depending on the plant density (Jalakas *et al*., 2018; Haaning *et al*., 2020).

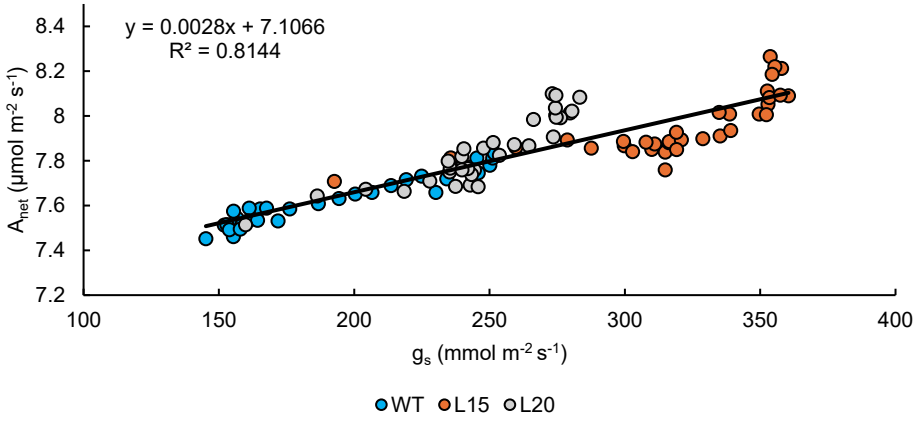

Figure 5. Linear regression between average stomatal conductance (g_s) and net assimilation rate (A_{net}) during the morning opening of barley (Golden Promise) *hvsnrk2.7/hvsnrk2.9* knock-out lines L15, L20 and wild-type. Regression was statistically significant (GLM, $p<0.05$). n=5–6

Complementing HvSnRK2.7 or HvSnRK2.9 into *A. thaliana ost1-3* background (the *ost1/snrk2.6* knock-out mutant) showed that both barley proteins are able to restore stomatal responsiveness to high VPD and ABA foliar spray (5 µM), however HvSnRK2.7 was better able to rescue baseline g_s compared with HvSnRK2.9 (II, Figure 1A, B). The difference might be due to 2.5x higher gene expression of HvSnRK2.7 compared with HvSnRK2.9, although it is not certain whether gene expression directly reflects the actual protein levels (II, Figure S5). Since the transcripts encoding barley SnRK-s were expressed 20- to 40-fold higher than the native *AtSnRK2.6* transcript in complementation lines, barley SnRK-s may not function efficiently in *A.thaliana* (II, Figure S5). OST1 expression has been known to increase during drought stress in *A. thaliana* (Huang *et al*., 2019). Previously, it has been shown that, the gene expression of HvSnRK2.7 (previously named HvSnRK2.5) increased consistently under drought stress in barley compared with HvSnRK2.9 (previously named HvSnRK2.4; Seiler *et al*., 2014). To clearly understand, whether HvSnRK2.7 and HvSnRK2.9 are functionally reduntant, complement each other or only one of them is necessary in ABA and VPD signalling in barley, barley single mutants of these genes need to be analysed.

5.3 Evaluation of the Nordic-Baltic spring wheat breeding material (III)

Wheat is the most grown cereal in the Nordic-Baltic region and breeding varieties suited for future climate predictions is essential for maintaining sustainable and high-quality grain for the food industry (Reynolds *et al*., 2021; FAOSTAT, 2025). Recently, yields have been fluctuating in this region, stressing the need for new varieties that can maintain stability across diverse and unpredictable environments

(Mäkinen *et al*., 2018; Wiréhn, 2018; Reynolds *et al*., 2021; Nóia Júnior *et al*., 2023). In NOBALWheat project, 300 spring wheat genotypes were grown in Estonia, Latvia, Lithuania and Norway in two consecutive seasons (2021 and 2022; LAMMC, 2021). Extreme weather episodes were captured in these 8 environments (4 locations × 2 years), including for example severe drought in Estonia in June, 2021 (13% of average rainfall) and 2.6 times the average rainfall in Lithuania in June, 2022 (III, Figure 1). Temperatures remained more stable across the locations, however June and July were significantly warmer everywhere in 2021 compared with 2022, while August was warmer in 2022 instead (III, Figure 2).

There was no significant relationship between grain yield and its stability across environments: higher yielding genotypes are not necessarily the more stable ones (III, Figure 5). Stability of several top-yielding genotypes (Kanyuk, Cornetto, Leidi) was below average, while the most stable genotype 10 kV 3 FKV 75 had below average yield (III, Figure 5). There were however some genotypes, that were high yielding and had relatively high stability (DS-674-9-DH, DS-762-3-DH, DS-14 and DS-10-18-DH; III, Figure 5). Unlike grain yield, there was a significant negative relationship between protein content and stability, as genotypes with the highest protein content (SHA3/CBRD and Sport) were among the most unstable (III, Figure 6). Protein content was strongly influenced by weather, with drier than average conditions during grain filling leading to higher protein content. Thus breeding for high protein risks reduced stability, which makes more balanced genotypes like Cervino and Tjalve valuable for future breeding programs. Genotypes with the highest protein content also had shorter than average growing periods. Growing period stability itself was highest for genotypes with an average growing period length of ~105 days, with really short or long growing periods showing the most instability (III, Figure 9).

Grain yield is generally negatively related with protein content and this was also the case here (III, Figure S2; Simmonds, 1995). In favourable conditions starch accumulation outcompetes nitrogen assimilation in wheat, causing lower concentration of protein by weight, as starch synthesis is more efficient than nitrogen assimilation (Triboi *et al*., 2006). Stress increases protein content through reduction in grain yield and thousand-kernel-weight, as plants have less time to accumulate starch during grain filling. As a result the highest protein contents were observed in genotypes grown under drought stress in the Baltics in 2021 (III, Figure 4B). However, it was possible to select several high yielding genotypes with above average protein content (DS-661-11-DH, DS-655-5-DH, DS-661-14-DH and Daugana).

To conclude, this study revealed promising genotypes for Nordic-Baltic region, which produce stable and relatively high yields; genotypes with different combinations of yield quantity and quality traits can additionally be selected for (III, Table 3).

5.4 Methodology for high-throughput simultaneous stomatal conductance and net assimilation field measurements (IV)

Low throughput of IRGA (infrared CO_2 and H_2O gas analyser) based gas exchange devices limits their application in breeding, requiring 1–5 minutes per leaf in survey measurements (Stinziano *et al*., 2017; Busch *et al*., 2024). Although porometers can take snapshot stomatal conductance measurements under 10 seconds in parallel with fluorometry, direct CO_2 uptake can currently only be measured with IRGA based devices. Thus far an IRGA based system that can match porometer speeds has been lacking. This was the key motivation for construction of a portable gas exchange device for field measurements. The total inner volume of this system named Karal was ~17ml, majority of which was comprised by the IRGA cell (14.5 ml). Constructing the chamber with a laminar airflow allowed to keep the chamber volume very small (0.85 cm^3). This approach does not require fans for turbulent air mixing inside the chamber, which otherwise would need a larger internal cavity, subsequently increasing signal detection time. In addition, the air stream drawn from the reference container for leaf exposures was fully mixed and stable, minimising any fluctuations in CO_2 and H_2O concentration that would introduce errors (IV, Figure 3A, C). Experimentally, it was possible to detect a stable CO_2 and H_2O concentration at the end of the 8 second exposure after clamping the leaf (IV, Figure 3B, D). Using a single IRGA and collecting, storing and measuring the reference air before each measurement cycle also resolved the necessity of frequent matching of two IRGA's that is standard for commercial devices (Li-Cor, 2026). A small part of the gas from the previous sample still remains in the analyser, even when 8 seconds have passed and this becomes relevant when air streams with very different CO_2 and H_2O concentrations are measured one after another, for example reference measurement immediately followed by leaf exposure. The first leaf needs to be discarded from the measurements because of this, although we opted for discarding the first three leaves as a precautionary measure. The device was designed to integrate several leaves into one measurement, meaning one datapoint is an average of multiple leaf measurements.

5.4.1 Validation of the Karal device against commercial devices through parallel measurements

Validation of the Karal methodology was done by carrying out parallel gas exchange experiments with two Karal devices against LI-6800 (Li-Cor, USA) and LI-600 (Li-Cor, USA) on silver birch (*Betula pendula*), tilia (*Tilia cordata*) and sorghum (*Sorghum bicolor*) leaves in similar conditions. Before measurements, LI-6800 chamber conditions (CO_2, relative humidity and block temperature) were set similar to the ambient environment. Leaves were measured in sets of 5 and roughly at the same sun angle within minutes from each other with all devices. In

all three species there were no significant differences between A_{net} rates measured by two Karals and the LI-6800 (IV, Table 2). The slope of the regression between LI-6800 and the Karals in these experiments was 0.93 (Figure 6A). In stomatal conductance values, there were significant differences between the devices when measuring sorghum and birch, however the largest differences were between LI-600 and LI-6800 measurements (IV, Table 2). Karal values were similar to each other in all measurements, and more similar to LI-6800 measurements (1 statistically different value observed) compared with LI-600 measurements (3 statistically different values observed; IV, Table 2). Stomatal conductances measured by LI-600 porometer were on average higher compared with Karal measurements (1.38 and 1.23 times compared to K1 and K2, respectively) and with LI-6800 (Figure 6B). Values of g_s measured with LI-6800 were on average lower compared with Karals (0.88 and 0.78 times compared with K1 and K2, respectively; Figure 6B). Measurements on NOBALWheat spring wheat plots conducted over 5 days in 2021 summer, comparing Karal and LI-6400 (Li-Cor, USA) yielded similar results, with flag leaf A_{net} rates matching closely (Karal A_{net} 1,05 times the LI-6400 value), but LI-6400 measured lower g_s values (0.69 times lower than Karal; IV, Figure 4A, C). However, the thermocontrol was not used in LI-6400, resulting in significantly higher leaf temperatures in the chamber (IV, Figure 4B). Higher g_s values measured by the LI-600 porometer could partly be explained by the higher default boundary layer conductance (g_b) of chamber (2900 mmol m^{-2} s^{-1}, compared with 2250 mmol m^{-2} s^{-1} of Karal and LI-6800), as the higher the g_b, the more local VPD is increased, thus resulting in measurement of increased transpiration and calculated stomatal stomatal conductance (Aphalo and Jarvis, 1993). In addition, porometers have been documented to overestimate g_s values by 50–100%, compared with IRGA based gas exchange systems: in these g_s ranges measured here the mean overestimation was reported to be up to 30% (Figure 6B; Rizzo and Bailey, 2025; Rizzo *et al*., 2026).

The measurement speeds for Karal were comparable with LI-600. Although Karal measures a single leaf in 8 seconds, the time it takes to move from leaf to leaf, as well as the initial airing and reference measurement procedure (40s and 60s, respectively) adds time to the overall “plot” measurement. As a result, a collection of data for 20 leaves took little over 6 minutes to complete with Karal (just over 18 seconds per leaf), compared with just under 6 minutes with LI-600 (17 seconds per leaf due to measuring both sides separately), whereas with LI-6800 it took 30 minutes (1.5 minutes per leaf). Measuring spring wheat flag leaves, LI-6400 took on average just over 3 minutes per leaf. Compared with LI-6800 and LI-6400 Karal was 5 and 10 times faster, respectively. Airing and reference cycle lenghts in this configuration were conservatively long: shortening these would allow for even further reduction in plot measurement time.

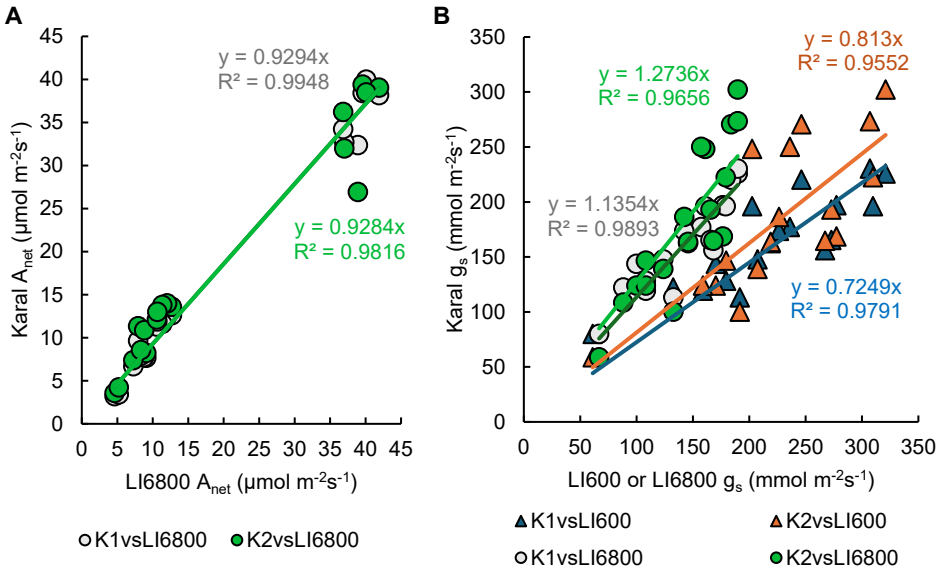

Figure 6. Linear regressions between Karal and Li-Cor devices (LI-6800 or LI-600) of measured net assimilation (A_{net}; A) and stomatal conductance (g_s; B). Intercept was fixed at 0. Regressions were statistically significant (GLM, $p<0.05$). n=18, of which each point consisted of 5 leaf measurements.

5.4.2 Spring wheat flag leaf normalised net assimilation measured over three seasons was correlated with grain yield

Karals were tested by conducting field gas exchange survey in the frame of the NOBALWheat project. Stomatal conductance and net assimilation rates during early grain filling (Zadoks growth stages 60–75) were measured in seven spring wheat genotypes in the field in three consecutive seasons (2021, 2022 and 2023). Gas exchange measurements can be conducted only on dry leaves, which posed a limitation on the frequency of the measurements during early grain filling period. As a result, measurements were conducted on 5 and 7 days in 2021 and 2023, respectively, while only one day could be measured in the two week window immediately after anthesis in 2022. Furthermore, measurements were made in a heterogenous environment, where light intensity (intermittent cloud shading) differed within and between days, while humidity and temperature differed between days. In order to compare genotypes, measured A_{net} values were normalised to incident PAR intensity. This normalisation was not done for g_s, because A_{net} responds instantaneously to changing light, while g_s has a significant lag time (Faralli *et al*., 2019; Eyland *et al*., 2021; Shrestha *et al*., 2026). Normalisation of A_{net} to incident PAR does not reflect the quantum yield, which is calculated from the linear slope of the light response curve, as it also includes the A_{max} portion of the PAR vs A_{net} regression (Busch *et al*., 2024). This dataset did not allow to infer quanum yield and A_{max} separately.

There was a significant variation between the genotypes in their measured values of stomatal conductance and normalised net CO_2 assimilation rate. On average, DS-638-5-DH had the highest g_s and normalised A_{net} values, while Hiie had the lowest g_s and Runar the lowest normalised A_{net} values (Figure 7).

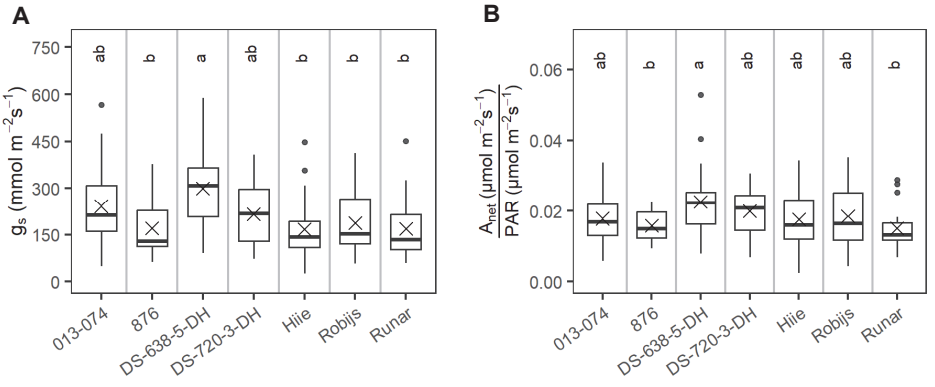

Figure 7. Box-and-whisker plots of 7 spring wheat genotpyes measured in the field with Karals over 3 seasons (2021, 2022 and 2023) within 2 weeks after anthesis grown under 150 kg ha^{-1} nitrogen fertilisation in 5x1m plots. (A) Plot measurements of stomatal conductance (g_s) of the flag leaves in 7 genotypes. (B) Plot measurements of normalised net assimilation (A_{net}/PAR) of the flag leaves in 7 genotypes. The box represents the interquartile range, while whiskers indicate the minimum and maximum. Horizontal line marks the median and "×" marks the mean. Outliers are represented as points. Statistically significant groups are marked by letters (one-way ANOVA, *post-hoc* Tukey HSD; $p<0.05$). n=18–33

Measured stomatal conductance values positively correlated with grain yield in 2021 and 2022, but not in 2023 (Figure 8A). Correlations between yield and A_{net} or transpiration were even weaker, being present in only 2022 (data not shown). However, yield was most strongly correlated with normalised A_{net} in 2021, 2022 (Figure 8B). Although the number of genotypes selected for this study was limited, a significant variation in measured gas exchange traits was detected as well significant correlations of g_s and normalised A_{net} with yield in 2021–2022, showing Karals potential applicability in field phenotyping and breeding. There was a marked variation in precipitation patterns among the years: 2022 was generally favourable, whereas in 2021, drought developed after anthesis, and in 2023, drought started early, lasting until anthesis. These contrasting conditions may explain the year-specific relationships between gas exchange traits and yield. If drought happens early in development, when sink size is determined like in 2023, increased source activity during grain filling does not translate into higher yield due to sink limitation. In contrast, under favourable conditions and when drought coincides with grain filling, genotypes with increased stomatal conductance and CO_2 uptake may benefit from greater assimilate supply in terms of yield production (Figure 8A, B; Reynolds *et al*., 2011). These findings demonstrate that

Karal-based gas exchange phenotyping can be considered a promising complementary tool for breeding. Using the high throughput capacity of Karals to measure leaves from different canopy layers together with LAI estimation could enable scaling to canopy level photosynthesis and transpiration, currently only possible with whole-plant chambers (Niinemets, 2012; Song and Zhu, 2018).

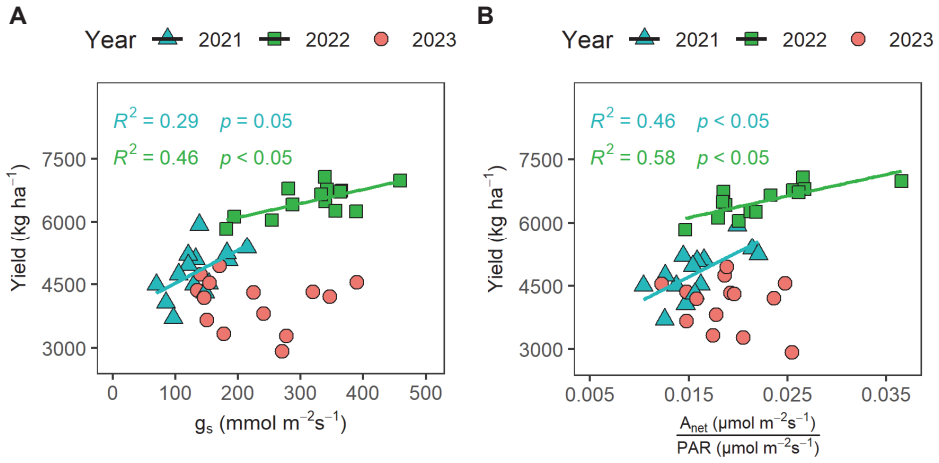

Figure 8. Linear regressions between gas exchange traits and yield measured in the field with Karals over 3 seasons (2021, 2022 and 2023) within 2 weeks after anthesis. Plants were grown under 150 kg ha^{-1} nitrogen fertilisation in 5x1m plots. (A) Linear regressions between stomatal conductance (g_s) and yield, and (B) normalised net assimilation (A_{net}/PAR) and yield, where solid colored lines indicate in which years the regressions were statistically significant (GLM, p<0.05).

6. CONCLUSIONS

Main conclusions based on the work presented here are the following:

- Horsetail stomata can exhibit stomatal responsiveness to ABA, with significant stomatal closure observed in all studied horsetails (*E. arvense, E. pratense* and *E. sylvaticum*). Relative closure was somewhat smaller in horsetails compared with wild-type barley for example, while using higher ABA spraying concentrations for horsetails (50 μM vs 20 μM). These findings contribute to a wider debate, regarding the origin of active ABA-dependent stomatal regulation.
- Horsetail stomata respond to VPD with extensive closure, even when ABA response is absent.
- In barley, protein kinases HvSnRK2.7 and HvSnRK2.9 are involved in stomatal ABA and VPD responses similarily to OST1 in *A. thaliana*.
- Barley HvSnRK2.7 was more effective than HvSnRK2.9 in restoring wild-type like stomatal conductance and stomatal VPD and ABA response phenotypes, when complemented into *ost1-3* background of *A. thaliana*.
- Higher stomatal conductance of barley *hvsnrk2.7/hvsnrk2.9* double mutants is correlated with increased CO_2 uptake.
- Yields of pot grown barley *hvsnrk2.7/hvsnrk2.9* double mutants did not benefit from increased CO_2 assimilation under relatively low light conditions in the greenhouse, potentially requiring higher light intensities to capture the photosynthetic gains into yield.
- Grain yields of studied genotypes was not correlated with yield stability across various environments. Stable, but high yielding genotypes were identified and selected for Nordic-Baltic breeding programs or for agricultural production in this region.
- Protein content was negatively correlated with its stability, as high grain protein genotypes generally showed higher unstability of this trait. Above average quality, but high stability genotypes were identified and selected for breeding programs.
- Novel gas exchange methodology, Karal, had fast signal acquisition speed and enabled measurement of stomatal conductance and net assimilation in parallel. Comparisons with commercial systems showed strong correlation between net assimilation values, but slightly increased/decreased stomatal conductance depending on the device compared against.
- Measurement speeds with Karal were comparable to those of porometers, which measure only stomatal conductance, but were 5–10 times faster than those of IRGA-based gas exchange systems, which also measure net assimilation.

- Gas exchange traits of seven spring wheat genotypes were characterised in the field and significant variation in gas exchange traits was detected between the genotypes using Karals.
- Significant correlations between grain yield and stomatal conductance or normalised net assimilation rate measured with Karals during grain filling were detected for years when crop yields were potentially source limited.
- Karals were able to identify variation between genotypes and correlations with yield showing their potential application in field studies and breeding programs.

Overall, this work contributed to our understanding of stomatal regulation in low air humidity and in response to stress hormone abscisic acid; served to identify promising wheat genotypes with higher than average yield quantity and quality traits and introduced a new practical tool for gas exchange phenotyping.

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SUMMARY IN ESTONIAN

Gaasivahetustunnuste evolutsioon ja aretuspotentsiaal taimedes

Maismaataimede gaasivahetus käib läbi taimelehtede pinnal asetsevate õhulõhede, mis moodustuvad kahest sulgrakust. Läbi nende mikroskoopiliste pilude omastavad taimed fotosünteesi käigus atmosfäärist süsihappegaasi. Fotosünteesi käigus seovad taimed süsihappegaasi kasvamise jaoks ning sellest moodustub saak. Läbi õhulõhede taimed ka transpireerivad vett, mis on vajalik toitainete mullast kätte saamiseks. Taimedel on arenenud võime õhulõhede avatust reguleerida, et säästa vett ebasoodsates tingimustes. Kui vett on vähe või õhk on kuiv, siis taimed vähendavad õhulõhede avatust, et vältida närbumist, kuid sellega nad piiravad ka süsihappegaasi pääsemist taime. Heades tingimustes hoiavad taimed õhulõhesid avatuna, et maksimaalselt süsihappegaasi omastada. Süsihappegaasi omastamise ja transpiratsiooni tasakaal erineb taimeliikide vahel ning ka liigisiseselt. Kliima muutusega ennustatakse rohkem ebakorrapärased sademete perioode ja madalamat suhtelist õhuniiskust tõusva temperatuuri tõttu, mis vähendavad põllumajanduslikku saagikust. Seeõttu on õhulõhede regulatsiooni uurimine vajalik, et tagada toidukindlus ka edaspidi.

Õhulõhede avatuse kontroll on molekulaarsel tasemel keerukas protsess ning selle väljaarenemine ja peenhäälestamine on toimunud sadu miljoneid aastaid. Hariliku müürlooga peal on näidatud, et õhulõhede sulgumine põuas ja suhtelise õhuniiskuse langemisel toimub peamiselt läbi taimehormoon abstsiishappe (ABA), mis omakorda kontrollib proteiin-kinaasi OST1 aktiivsust. OST1 aktivatsioon käivitab omakorda allavoolu asetsevate komponentide aktivatsiooni ja vee väljavoolu sulgrakust, mistõttu sulgrakud kaotavad turgori ja pilu nende vahel kahaneb. Millal see protsess evolutsiooni käigus täpselt välja arenes, on veel ebaselge. Lisaks on vähe teada, kas sama valk osaleb õhulõhede regulatsioonis nii mudeltaimes kui põllumajanduslikult olulistes taimedes, näites odras.

Esmalt uuriti töös ABA rolli õhulõhede regulatsioonis kolmes Eestis kasvavas osjaliigis mitme aasta vältel. Osjad on evolutsiooniliselt iidsed taimeliigid, mis on püsinud morfoloogiliselt muutumatuna sadu miljoneid aastaid ja seetõttu kutsutakse neid tihti elavateks fossiilideks. Kuigi algeliste taimede genoomides on ABA signaaliraja komponendid valdavalt olemas, on tulemused nende õhulõhede reaktsioonides ABA-le vastakad. Osad on näidanud, et vanematel taimedel puudub ABA poolt tingitud õhulõhede sulgumine ning nende põuas ja madalas õhuniiskuses toimuv õhulõhede sulgumine on passiivne, läbi üleüldise turgori languse. Teisalt on neis evolutsiooniliselt vanades liikides näidatud katseliselt ABA-le reageerimist ja seda, et vastuse olemasolu on sõltunud kasvutingimustest. Käesolevas töös leiti, et kolmel osjaliigil oli vähemalt ühel aastal mõõdetud statistiliselt oluline õhulõhede sulgumine ABA-ga pritsides. ABA-toimelise sulgumise olemasolu võis sõltuda nii kasvutingimustest kui ABA taimelehte pääsemisest. Sulgumist indutseerinud ABA kontsentratsioon oli kümme korda kõrgem sellest, mis põhjustab harilikus müürloogas sulgumist. Õhu-

niiskuse langusele reageerisid osjade õhulõhed isegi siis, kui ABA-le sulgumisvastus puudus. Need tulemused viitavad, et abstsiishappest tingitud õhulõhede regulatsioon võis olla varajane omadus taimede evolutsioonis ja sarnaselt varasematele uuringutele, sulgumisvastuse olemasolu võib vajada eelhäälestust. Evolutsiooniliselt vanade liikide õhulõhede ABA-vastuste olemuses selguse saamiseks on vaja teostada katseid nii täpselt kontrollitud kui ka looduslikes tingimustes,

Odras uuriti Arabidopsise OST1 proteiin-kinaasi homoloogide HvSnRK2.7 ja HvSnRK2.9 rolli õhulõhede reageerimisel abstsiishappele ja õhuniiskusele. Odra vastavate geenide viimisel müürlooga *ost1*-defitsiitsesse liini selgus, et HvSnRK2.7 taastab paremini metsiktüübi fenotüübi võrreldes HvSnRK2.9 valguga, kuid erinevused võisid tuleneda ka sellest, et SnRK2.7 geeni ekspresseeriti suuremal hulgal. CRISPR-Cas9 meetodiga loodud odra *hvsnrk2.7/hvsnrk2.9* kaksikmutantidel olid kõrgemad õhulõhede juhtivused võrreldes metsiktüübiga ning nende õhulõhede vastused abstsiishappele ja madalale õhuniiskusele olid häiritud. Õhulõhede suuremal avatusel oli positiivne seos fotosünteesiga kõrgemas valguses, kuid terasaagis see ei väljendunud. Selle põhjuseks ilmselt oli madal valgus saagi moodustumise ajal, mistõttu polnud väiksemast süsihappegaasi diffusioonibarjäärist fotosünteesi jaoks kasu.

Lisaks uuriti 300 Põhja- ja Baltimaade suvinisugenotüübi saagikust ja selle kvaliteedinäitajaid kahel aastal neljas asukohas (Eestis, Lätis, Leedus ja Norras). Aina enam varieeruvate sademe- ja põuaperioodide taustal on sordiaretuses oluline hinnata stabiilsusnäitajaid. Kahe aasta jooksul ilmnes väga suur varieeruvus keskkonnatingimustes katsepaikade vahel, mis võimaldas hinnata sortide saagikust ja saagi kvaliteeti ning nende keskkonnatundlikkust. Selgus, et saagikuse ja saagi stabiilsuse vahel pole seost, kuid oli võimalik identifitseerida stabiilsed, võrdlemisi kõrget saaki andvad genotüübid. Samas selgus, et mida kõrgem oli valgusisaldus, seda ebastabiilsem oli antud tunnus keskkondade ning aastate vahel. Siiski oli võimalik selekteerida keskmisest kõrgema valgusisaldusega ning kõrge valgusisalduse stabiilsusega genotüübid. Uuring identifitseeris aretusprogrammide ja põllumajandustootmise jaoks kohalikud genotüübid, millel on head saagi suuruse ning kvaliteedi näitajad koos püsiva stabiilsusega erinevates keskkonnatingimustes.

Viimaks loodi põllul taimede gaasivahetustunnuste kiireks mõõtmiseks uus metoodika ja seade, nimega Karal. Seade võimaldas samaaegset õhulõhede juhtivuse (väljendab õhulõhede avatust) ja fotosünteesi kiiremat mõõtmist, kui hetkel kommertsiaalselt saadaolevad gaasivahetusseadmed võimaldavad. Paralleelsed katsed Karalite ja kommertsiaalsete seadmetega näitasid, et fotosünteesi mõõtetulemused on väga sarnased, kuid mõõdetud õhulõhede juhtivuse väärtustes esineb erinevusi sõltuvalt võrreldud seadmega. Mõõtes Karaliga põllul seitset suvinisu sorti aastatel 2021–2023, ilmnes sortide vahel suur varieeruvus õhulõhede juhtivuses ja fotosünteesis. Gaasivahetustunnustel oli oluline seos saagiga 2021 ja 2022 aastal. 2023 aastal polnud gaasivahetustunnustel seost saagiga, mis võis tuleneda põua ajastusest: põud enne terade loomist määrab, kui palju on võimalik fotosünteesi produkte talletada. Kuna Karal võimaldas eelkõige tuvastada gaasivahetustunnustes genotüüpide vahel olulisi erinevusi, kuid ka seoseid

saagiga on võimalik seadet potentsiaalselt rakendada sordiaretuses gaasivahetustunnuste selekteerimiseks.

Kokkuvõttes panustas käesolev töö õhulõhede regulatsiooni molekulaarse mehhanismi mõistmisse erinevatel liikidel, sordiaretusse kasutamiseks sobivate genotüüpide identifitseerimisse ning põllul gaasivahetustunnuste kiire mõõtmise metoodikasse.

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