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Identification of key regulators of stomatal CO_2 signalling via O_3-sensitivity

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Author's contribution

I The author performed the gas-exchange experiments and commented on the manuscript.

II The author participated in the design of the study, performed a part of the gas-exchange experiments and participated in the writing and commenting of the manuscript.

III The author participated in the planning and design of the study, performed most of the experiments and wrote the manuscript.

IV The author participated in the design of the study, performed a part of the gas-exchange experiments and participated in the writing and commenting of the manuscript.

V The author participated in the conception and design of the study, performed a few experiments and wrote the manuscript.

ABBREVIATIONS

ABA – abscisic acid, a plant hormone that is a key regulator of stomatal responses
ABI1, ABI2 – ABA INSENSITIVE 1 and 2, protein phosphatases of type 2C, negative regulators of ABA signalling
BLUS1 – BLUE LIGHT SIGNALING 1, protein kinase involved in blue light signal transduction
C24 – an *A. thaliana* accession from Portugal
C_a – ambient CO_2 concentration
CA1, CA4 – β-carbonic anhydrases that catalyse the interconversion between CO_2 and bicarbonate
CHLH – CHLOROPLASTIC MG^{2+} CHELATASE SUBUNIT H, a protein involved in guard cell ABA signalling
C_i – intercellular CO_2 concentration
Cl^- – chloride ions
Col-0 – an *A. thaliana* accession from Germany, most commonly used as wild-type
CPK – calcium-dependent kinase, Ca^{2+}-sensing protein kinase
Cvi-0 – an *A. thaliana* accession from Cape Verde islands
GHR1 – GUARD-CELL HYDROGEN PEROXIDE RESISTANT 1, a leucine-rich receptor-like kinase that facilitates SLAC1 activation
GORK – GATED OUTWARDLY-RECTIFYING K^+ CHANNEL, major guard cell K^+ efflux channel
GSNO – S-nitrosoglutathione, an NO donor
HAB1 – HYPERSENSITIVE TO ABA 1, protein phosphatase type 2C, regulator of ABA signalling
HT1 – HIGH LEAF TEMPERATURE 1, protein kinase, key regulator of stomatal CO_2 signalling
KAT1, KAT2 – POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1, 2, voltage-dependent potassium uptake channels
MAPKKK – mitogen-activated protein kinase kinase kinase
MPK – mitogen-activated protein kinase
NIL – near-isogenic line
NO – nitric oxide, a mediator of guard cell signal transduction
NRT1.1 – NITRATE TRANSPORTER 1.1, guard cell nitrate importer involved in stomatal opening
OST1 – OPEN STOMATA 1, key kinase that activates the SLAC1 anion channel, also known as SRK2E
OST2 – OPEN STOMATA 2, plasma membrane H^+-ATPase required for stomatal opening, also known as AHA1
PATROL – PROTON ATPASE TRANSLOCATION CONTROL 1, protein that controls the location of H^+-ATPase in CO_2 signalling
PHOT1, PHOT2 – PHOTOTROPIN 1, 2, blue light receptors
PIP2;1 – PLASMA MEMBRANE INTRINSIC PROTEIN 2;1, an aquaporin

PP2A – protein phosphatase type 2A
PP2C – protein phosphatase type 2C, regulators of stomatal signalling
PP2CA – PROTEIN PHOSPHATASE 2CA, protein phosphatase type 2C, regulator of stomatal ABA signalling
PRSL1 – PP1 REGULATORY SUBUNIT2-LIKE PROTEIN 1, a regulator of blue light signalling
PYR/PYL/RCAR – PYRABACTIN RESISTANCE 1/PYR1-like/ REGULATORY COMPONENT OF ABA RECEPTOR – ABA receptor proteins
QTL – quantitative trait locus
QUAC1 – QUICK-ACTIVATING ANION CHANNEL 1, rapid-type guard cell anion channel, also known as ALMT12
RH – relative air humidity
RHC1 – RESISTANT TO HIGH CO_2 1, membrane transport protein implicated in stomatal CO_2 signalling
RIL – recombinant inbred line
ROS – reactive oxygen species, reactive particles that participate in guard cell signal transduction and regulation of stress responses
RTD – rapid transient decrease of stomatal conductance in response to short-term O_3 pulse
Rubisco – ribulose-l,5-bisphosphate carboxylase-oxygenase
SA – salicylic acid, plant stress hormone
SD – standard deviation
SEM – standard error of the mean
SLAC1 – SLOW ANION CHANNEL 1, the key anion channel that needs to be activated for stomatal closure
SLAH3 – SLAC1 HOMOLOGUE 3, guard cell anion channel that participates in stomatal closure
SnRK2 – Sucrose-non-fermenting kinase 2, a class of protein kinases that incorporates several regulators of ABA responses, including OST1
Te – Tenela, an *A. thaliana* accession from Finland
WUE – water use efficiency, ratio of the carbon assimilation to transpiration, reflects the water loss for the production of a unit of biomass

1. INTRODUCTION

Plants are the basis of life on Earth, producing organic matter from carbon dioxide (CO_2) and water, and releasing oxygen that the aerobic life forms need. From the perspective of humankind, plants are the producers of food and raw materials for different industries. Thus, deep understanding of how plants work is required for growing and breeding plant varieties that are more productive, flexible or resistant to various abiotic and biotic stresses.

Efficient uptake of CO_2 for photosynthesis and controlled water use are essential for plants to thrive in changing environmental conditions. Plant gas-exchange with the environment occurs via stomatal pores, formed by specialized guard cells that regulate the aperture of each pore. Adequate guard cell responses to various environmental and endogenous signals result in optimal stomatal apertures for efficient CO_2 uptake with controlled water loss. Stomatal opening and closure are driven by transport of ions across guard cell membranes, leading to changes in water potential and turgor pressure. During stomatal opening, ions and water enter the guard cells, causing guard cells to swell and move apart. This results in increased stomatal aperture and also higher stomatal conductance at the plant level. During stomatal closure, ions and water leave the guard cells, causing guard cells to shrink and stomatal aperture to decrease. Stomatal opening is induced by light, low CO_2 concentration and increased air humidity. Stomatal closure is triggered by darkness, elevated CO_2 concentration, reduced air humidity, air pollutants such as ozone (O_3) and pathogens. Endogenous signalling molecules involved in stomatal closure include the plant hormone abscisic acid (ABA), nitric oxide (NO), reactive oxygen species (ROS) and Ca^{2+} ions.

Guard cell regulation has been an attractive field of research for decades. This has partly been due to practical reasons as guard cells reside in the epidermis that can be easily separated from the plant. Thus, stomatal responses can be studied *in vitro* and monitored directly with the help of a microscope. More importantly, as guard cells control plant water loss, stomatal regulation is an attractive target for engineering crop plants that would have higher water use efficiency (WUE), better drought tolerance or increased responsiveness to various environmental factors. As the atmospheric CO_2 concentration has been steadily increasing for more than a 100 years and continues to grow, understanding plant responses to elevated CO_2 is becoming increasingly important. Thus, considerable effort has been devoted to stomatal research and there is growing data on the molecular mechanisms of stomatal regulation. Whereas vast progress has been made in studies of stomatal ABA signalling over the recent years, considerably less is known of guard cell CO_2 signalling.

This thesis describes the identification of a core CO_2 signalling module in guard cells via several studies that all stemmed from the O_3-sensitivity of different natural variants or mutants of *Arabidopsis thaliana*. The evolution of stomatal responsiveness to ABA and CO_2 was also addressed by studying stomatal responses in ferns. Together, the data presented in this thesis bring valuable insight into the molecular mechanisms and evolution of stomatal CO_2 signalling and provide interesting perspectives for further research on stomatal movements and plant water use.

2. REVIEW OF LITERATURE

2.1. Ion channels in stomatal closure

Stomatal closure occurs in response to darkness, reduced air humidity, elevation in CO_2 concentration, air pollutants (O_3), pathogens and hormones (ABA). Stomatal closure is triggered by export of ions from the guard cells, resulting in loss of turgor pressure and closure of the stomatal pore. Slow-type (S-type) anion channels have been known to play a key role in this process for a long time. Guard cell protoplast electrophysiology assays led to the identification of two types of anion currents across the membrane of these cells – slow-type (S-type) currents that showed very slow voltage-dependent activation and deactivation and did not inactivate, and rapid-type (R-type) currents that were activated rapidly by depolarization, deactivated by hyperpolarization and inactivated during prolonged stimulation (Schroeder and Keller, 1992). A slow-type anion channel (SLAC) that was active already at very low membrane potential values, was suggested to be the main contributor to stomatal closure (Linder and Raschke, 1992). Further evidence for the role of S-type anion channels in stomatal regulation was obtained via identification of slow-type anion channel blockers (Schroeder et al., 1993). The Cl^- channel blocker 5-nitro-2,3- phenylpropylaminobenzoic acid (NPPB) was shown to efficiently inhibit S-type anion currents. NPPB also abolished stomatal closure induced by combination of ABA and malate (Schroeder et al., 1993). Anthracene-9-carboxylic acid (9-AC) and niflumic acid, also inhibitors of S-type anion channels, blocked ABA-induced stomatal closure and reversed ABA-induced inhibition of stomatal opening while inhibition of S-type anion channels resulted in increased stomatal opening under various KCl concentrations (Schwartz et al., 1995). These data indicated an important role for S-type anion channels in the regulation of stomatal closure and also suggested cross-regulation of stomatal closure and opening pathways. The guard cell S-type anion currents have been shown to be activated by ABA (Pei et al., 1997; Raschke et al., 2003; Roelfsema et al., 2004; Levchenko et al., 2005), indicating a direct role for this hormone in the regulation of S-type anion channel activity in guard cells.

The gene encoding the central guard cell S-type anion channel SLOW ANION CHANNEL 1 (SLAC1) was identified independently in two screens − one for O_3-sensitivity (Vahisalu et al., 2008) and another for CO_2-insensitivity (Negi et al., 2008). Plants deficient in SLAC1 function had severely impaired responses to CO_2 elevation, darkness, reduced air humidity, O_3 and ABA. SLAC1 is selective towards nitrate ions but is also permeable to chloride and, to a lesser extent, malate ions (Schmidt and Schroeder, 1994; Geiger et al., 2009; Chen et al., 2010). In addition to SLAC1, its homologue SLAC1 HOMOLOGUE 3 (SLAH3) is also expressed in guard cells and was shown to be a nitrate-dependent channel stimulated by ABA (Geiger et al., 2011). Moreover, SLAH3 could complement the impaired stomatal CO_2-response of *slac1-2* mutants (Negi et al., 2008). These data indicate that in addition to SLAC1,

SLAH3 also contributes to stomatal closure. The three-dimensional structure of SLAC1 and its homologues has been modelled based on the crystal structure of the SLAC1 homologue TehA from *Haemophilus influenzae* (Chen et al., 2010). SLAC1 is a trimer formed of SLAC1 subunits each harbouring 10 transmembrane helices, five of which form the central pore that is gated by a conserved phenylalanine residue. SLAC1 pore seems to lack anion-binding site and the permeability of SLAC1 to various anions corresponds to the hydration energies of anions – SLAC1 is more permeable towards the anions with lower hydration energy (Chen et al., 2010).

The R-type anion channels mediate quick and voltage-dependent anion efflux from the guard cells. Voltage-dependent anion channels in the plasma membrane of guard cells activated at membrane potentials around -80 mV and had maximal activity at -40 mV in *Vicia faba* (Keller et al., 1989). These channels were permeable to nitrate, chloride and malate (Keller et al., 1989). The voltage-dependent anion channels showed increased activity at higher cytoplasmic Ca^{2+} levels (Hedrich et al., 1990) and were inhibited by anion channel blockers (Marten et al., 1992). The activation of the channels was shifted to more hyperpolarized potentials in the presence of extracellular malate (Hedrich and Marten, 1993). Thus, malate was suggested to act as a CO_2 sensor for the guard cells that could promote the activation of the voltage-dependent anion channels and stomatal closure. In accordance with this, malate was able to induce stomatal closure and malate levels were higher in plants subjected to increased atmospheric CO_2 concentration (Hedrich et al., 1994). Similar to S-type anion currents, the R-type anion currents were enhanced by ABA in whole guard cells (Raschke et al., 2003; Roelfsema et al., 2004; Levchenko et al., 2005) as well as in isolated guard cell protoplasts (Levchenko et al., 2005), indicating that ABA is a common activator of both types of guard cell anion channels.

A gene encoding a guard cell R-type anion channel QUICK-ACTIVATING ANION CHANNEL 1/ALUMINUM-ACTIVATED MALATE TRANSPORTER 12 (QUAC1/ALMT12) was first identified as important for guard cell responsiveness to darkness and ABA, but was initially classified as a transporter different from the S-type and R-type anion channels (Sasaki et al., 2010). However, the same gene was identified in a search for an aluminium-activated malate transporter (ALMT) that would be expressed in a guard-cell specific manner (Meyer et al., 2010). Plants deficient in ALMT12 had impaired stomatal responses to darkness and elevated CO_2 and decreased R-type anion currents in the presence of malate in the guard cells, indicating that ALMT12 was a malate-sensitive guard cell R-type anion channel (Meyer et al., 2010). Thus, ALMT12 was named QUAC1 (QUICK-ACTIVATING ANION CHANNEL 1). The cytosolic C-terminus of QUAC1 was shown to be involved in the voltage-dependent deactivation (closure) of the R-type channel as the QUAC1 C-terminally fused to YFP remained open at hyperpolarized membrane potentials (Mumm et al., 2013). A glutamate residue in the C-terminus was also required for QUAC1 activity in *Xenopus laevis* oocytes. As the R-type anion currents were inhibited by 40% when compared to wild type in the guard cells of *almt12* (Meyer et al.,

2010), it is likely that in addition to QUAC1, there are other R-type anion channels in *A. thaliana*.

The continued efflux of anions via the S-type anion channels enables subsequent depolarization-induced efflux of K^+ ions. Outward K^+ currents induced by depolarization of the membrane potential to values more positive than -40 mV were recorded in *V. faba* guard cell protoplasts (Schroeder et al., 1987) and these currents did not inactivate during prolonged stimulation by depolarized membrane potential (Schroeder, 1988). The gene encoding the major guard cell K^+ outward-rectifying channel was isolated in a search for a depolarization-activated K^+ channel expressed in guard cells of *A. thaliana* (Ache et al., 2000). The activation of the GATED OUTWARDLY-RECTIFYING K^+ CHANNEL (GORK) shifted to more depolarized potentials in the presence of higher concentration of extracellular K^+ ions, indicating K^+-sensitivity of the channel (Ache et al., 2000). Guard cell protoplasts of plants deficient in GORK lacked depolarization-induced outward K^+ currents and the respective currents were clearly decreased in plants expressing the dominant negative versions of GORK (Hosy et al., 2003), further supporting the identity of GORK as the major guard cell K^+ outward-rectifying channel. Plants deficient in GORK had high fresh weight loss from detached leaves, increased transpiration and impaired stomatal closure in response to darkness (Hosy et al., 2003), indicating an important role for GORK and efficient K^+ efflux in stomatal closure.

The coordinated activation of anion and potassium channels leads to loss of guard cell turgor and thus stomatal closure. Activation of guard cell ion channels is regulated by various environmental and endogenous stimuli and involves complex signal transduction cascades. The regulation of ion channel activation in ABA- and CO_2-induced stomatal closure will be discussed in the following chapters.

2.2. Stomatal ABA signalling

ABA is a plant hormone with diverse functions. ABA was first extracted from cotton fruit (Ohkuma et al., 1963) and named abscisic acid because it was associated with induction of abscission. Later, it was shown to be a regulator of seed development and dormancy, germination and growth, various stress responses and stomatal regulation (Cutler et al., 2010, and references therein). ABA levels in plants increase in response to drought stress and ABA induces stomatal closure as well as inhibits stomatal opening. Thus, ABA is the key hormone in stomatal regulation and great effort has been devoted to unravelling the components and mechanisms of ABA signal transduction in guard cells.

2.2.1. Identification and characterization of the core ABA signalling pathway

Over the recent years, significant progress has been made in understanding stomatal ABA response, and the core ABA signalling pathway involving ABA receptors, protein phosphatases and kinases that regulate the anion channel SLAC1, has been identified. This chapter will give an overview of the unravelling of the guard cell ABA signalling and presents a model of how it is presently understood.

2.2.1.1. The PP2C protein phosphatases and OST1 kinase are involved in ABA responses

An important role for protein phosphatases in ABA signalling was discovered early. The *A. thaliana* dominant ABA-insensitive mutants *abi1-1* and *abi2-1* were isolated already in 1984 (Koornneef et al., 1984) and later the corresponding genes were shown to encode type 2C protein phosphatases ABA-INSENSITIVE 1 (ABI1) (Leung et al., 1994; Meyer et al., 1994) and ABA-INSENSITIVE 2 (ABI2) (Leung et al., 1997; Rodriguez et al., 1998). ABI1 and ABI2 act as negative regulators of ABA signalling as demonstrated by enhanced ABA responses of *abi1-1* and *abi2-1* revertants and loss or decrease of phosphatase activity in proteins encoded by respective alleles (Gosti et al., 1999; Merlot et al., 2001). Other PP2Cs have also been shown to be involved in ABA signalling as negative regulators. HYPERSENSITIVE TO ABA 1 (HAB1) transcript is highly induced by ABA and *hab1-1* plants display ABA-hypersensitivity (Leonhardt et al., 2004), while HAB1 overexpression results in reduced sensitivity to ABA (Saez et al., 2004). PROTEIN PHOSPHATASE 2CA (PP2CA) also appears to contribute to stomatal ABA signalling as illustrated by hypersensitivity to ABA in *pp2ca* mutants and reduced ABA-induced stomatal closure in plants overexpressing PP2CA (Kuhn et al., 2006).

One of the first identified positive regulators of stomatal ABA signalling was the ABA-activated serine-threonine protein kinase (AAPK) from *V. faba* (Li et al., 2000). The corresponding kinase in *A. thaliana*, OPEN STOMATA 1 (OST1), was found via a screen for low leaf temperature in drought-stressed plants (Merlot et al., 2002; Mustilli et al., 2002). Independently, OST1 was identified as an ABA-activated member of subfamily 2 of SUCROSE NON-FERMENTING1 (SNF1)-related protein kinases (SnRK2) and named SUCROSE NON-FERMENTING1 RELATED PROTEIN KINASE 2E (SRK2E) (Yoshida et al., 2002). Plants with dysfunctional OST1/SRK2E had disturbed stomatal responses to ABA and reduced air humidity. The serine 175 in the activation loop of OST1 was shown to be essential for kinase activity both in recombinant OST1 purified from *Escherichia coli* and OST1 immunoprecipitated from *A. thaliana* protoplasts (Belin et al., 2006; Boudsocq et al., 2006). OST1/SRK2E C-terminal domain was shown to interact with the PP2C ABI1 (Yoshida et al., 2006) and be important for functional stomatal regulation (Belin et al., 2006), while its role in ABA signal transduction remained elusive at this point.

2.2.1.2. The hunt for ABA receptors

The receptors for ABA were long sought after by plant biologists and several proteins have been proposed to act as ABA sensors over the years. For example, the chloroplastic Mg-chelatase subunit H (CHLH/ABAR) was shown to bind ABA and mediate ABA signalling and thus suggested to be an ABA receptor (Shen et al., 2006; Wu et al., 2009). However, further analyses of the function of CHLH indicated that while it has a role in ABA signal transduction, the Mg-chelatase H subunit is not an ABA receptor (Tsuzuki et al., 2011). An ABA-binding G protein-coupled receptor (GPCR) GCR2 has also been reported as an ABA receptor and *gcr2* mutants were shown to be ABA-insensitive in seed germination and stomatal closure (Liu et al., 2007). However, independent analysis of plants deficient in GCR2 and its homologues revealed functional ABA-responses in seed germination and development (Gao et al., 2007; Guo et al., 2008) and GCR2 was shown not to bind ABA in an independent assay (Risk et al., 2009), suggesting that GCR2 is not an ABA receptor. GPCR-type G proteins GTG1 and GTG2 have also been proposed to be ABA receptors, based on their binding of ABA and impaired ABA-responses in *gtg1 gtg2* double mutants (Pandey et al., 2009). Elsewhere, the *gtg1 gtg2* double mutants were shown to respond normally to ABA (Jaffé et al., 2012), suggesting that these proteins were not involved in ABA perception.

In 2009, several research groups identified a family of ABA receptors via different strategies. In one case pyrabactin, a selective agonist of ABA identified in a chemical genetics screen (Zhao et al., 2007; Park et al., 2009), was used to isolate pyrabactin resistant plants, which led to the identification of a START family of proteins that act as ABA receptors (Park et al., 2009). The proteins were named PYRABACTIN RESISTANCE 1 (PYR1) and PYL1 to PYL13 (PYR1-like). In a different approach, a yeast two-hybrid screen was used to find interactors of protein phosphatase ABI2, which led to the identification of REGULATORY COMPONENT OF ABA RECEPTOR 1 (RCAR1), a member of the same START family of ABA receptors (Ma et al., 2009). A yeast two-hybrid screen for interactors of the PP2C HAB1 also identified PYL5, PYL6 and PYL8, members of the same ABA receptor family (Santiago et al., 2009b). A search for the proteins interacting with ABI1 *in planta,* via protein complex purification and mass spectrometric analysis, also resulted in the identification of the PYR/PYL/RCAR family proteins (Nishimura et al., 2010). The PYL/PYR/RCAR proteins bound ABA, interacted with PP2Cs and suppressed PP2C activity in the presence of ABA (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009b; Nishimura et al., 2010; Szostkiewicz et al., 2010). ABA-induced stomatal closure and inhibition of light-induced stomatal opening were absent in the *pyr1 pyl1 pyl2 pyl4* quadruple mutant of ABA receptors (Nishimura et al., 2010).

The molecular mechanism of ABA perception by ABA receptors was soon revealed in several independent crystal structure analyses. The structure of PYR1 and the PYR1-ABA complex visualized ABA-binding to the receptor and

indicated that PYR1 formed a homodimer (Nishimura et al., 2009; Santiago et al., 2009a); the same was true for PYL2 (Yin et al., 2009). Analysis of PYL2, PYL2 in complex with ABA and the PYL2-ABA-HAB1 complex indicated that binding of ABA to the ABA receptor protein PYL2 induces a conformational change in PYL2 that enables subsequent binding of the complex to the HAB1 active site, thus inhibiting the phosphatase activity of HAB1 (Melcher et al., 2009). The PP2C acts as a co-receptor of ABA in this process, as upon the binding of PYL2-ABA complex to HAB1, ABA forms a contact with the PP2C as well (Melcher et al., 2009). As the amino acids involved in the binding of ABA to the receptor and binding of the ABA-receptor complex to the PP2C are conserved in other members of the PYR/PYL/RCAR family, the mechanism of ABA perception is likely the same for all ABA receptors of this family (Melcher et al., 2009). Indeed, at the same time, similar results were obtained from the crystal structure analysis of PYL1 bound with ABA and the PYL1-ABA-ABI1 complex (Miyazono et al., 2009) as well as analysis of crystallized PYL2, ABA-bound PYL2 and the PYL1-ABA-ABI1 complex (Yin et al., 2009).

2.2.1.3. Emergence of the core ABA signalling pathway

At the same time as the ABA receptors were discovered, the kinase OST1 was shown to interact with SLAC1 and ABI1 in *X. laevis* oocytes (Geiger et al., 2009) and activate SLAC1 anion channel in oocytes (Lee et al., 2009b; Geiger et al., 2009), while ABI1 and ABI2 (Geiger et al., 2009) and also PP2CA inhibited this activation (Lee et al., 2009b). Interestingly, PP2CA interacted with both SLAC1 and OST1 and inhibited SLAC1 phosphorylation by OST1 *in vitro* independent of its phosphatase activity (Lee et al., 2009b). ABI1 was shown to interact with OST1 and dephosphorylate OST1 *in vitro* (Umezawa et al., 2009). HAB1 also interacted with OST1 via the C-terminal domain of OST1 *in planta* and dephosphorylated OST1 *in vitro,* putatively at pS175 (Vlad et al., 2009), whereas it did not inhibit OST1-induced activation of SLAC1 in oocytes (Geiger et al., 2009).

Shortly thereafter, the ABA signalling pathway was reconstituted *in vitro*. PYR1 was shown to inhibit OST1 inactivation by ABI1 in the presence of ABA, whereas this inhibition was absent when the *abi1-1* version of ABI1 that cannot bind to PYR/PYL/RCAR proteins was used in this assay (Umezawa et al., 2009). Similarly, the reconstitution of the core ABA signal transduction pathway was achieved in *A. thaliana* protoplasts and *in vitro* (Fujii et al., 2009). ABA-triggered induction of ABA-responsive genes via activation of the transcription factor ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2/ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING PROTEIN 2 (ABF2/AREB1) in protoplasts deficient in three SnRK2 kinases (Fujii and Zhu, 2009) was achieved by addition of OST1 kinase, suppressed by further addition of protein phosphatase ABI1 and restored, when ABA receptor PYR1 was combined with OST1 and ABI1. Other ABA receptors from the PYR/PYL/RCAR

family could also function in this reconstituted pathway, albeit with different efficiencies, and in addition to ABI1, the PP2C HAB1 also worked in this system (Fujii et al., 2009). Expression of an ABA receptor was shown to disrupt interaction of PP2Cs (ABI1, ABI2 and HAB1) with the OST1 kinase. The pathway could also be reconstituted *in vitro*. A scheme of the core ABA-signalling pathway is shown in Figure 1.

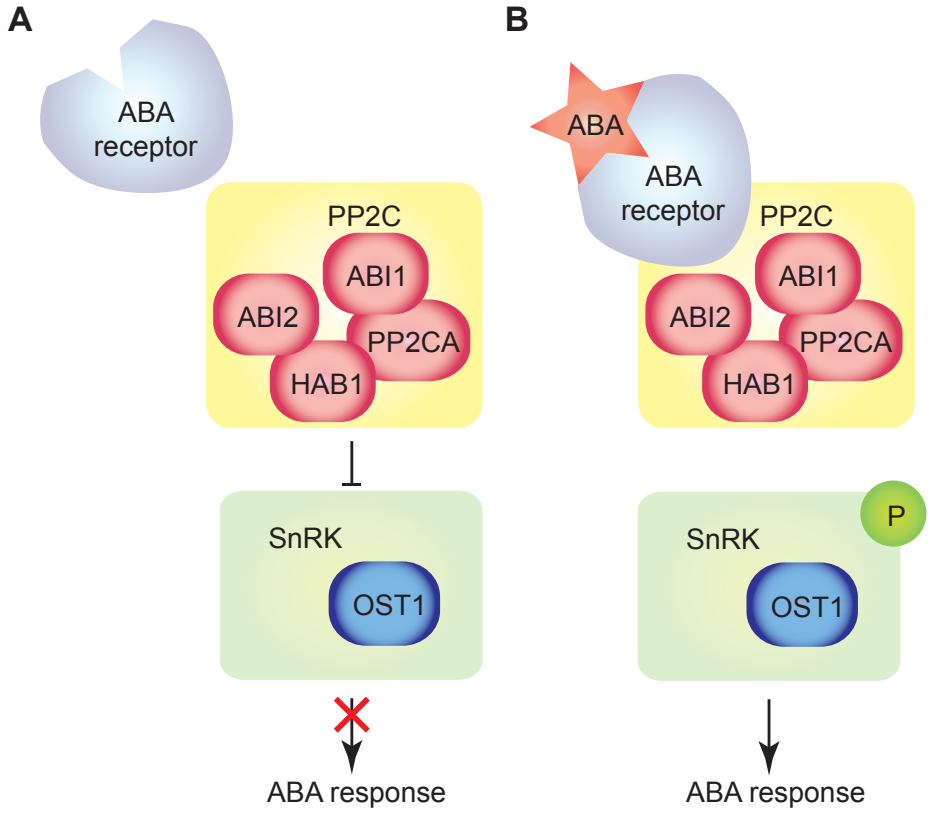

Figure 1. The core ABA signalling pathway. (A) In the absence of ABA, the PP2Cs are active and dephosphorylate the SnRK-type kinases, thus impeding the activation of ABA response by the kinases. (B) In the presence of ABA, the ABA receptor, PP2C and ABA form a complex that blocks the phosphatase activity of the PP2Cs. SnRK-type kinases autophosphorylate and activate ABA responses. The key PP2Cs and a SnRK involved in stomatal regulation are indicated by red and blue rounded rectangles, respectively.

As the core ABA signalling pathway had been reconstituted, further research followed to elaborate the roles and molecular mechanisms of ABA receptors, PP2C-s and OST1 in anion channel regulation. As the PYR/PYL/RCAR ABA receptors form a large family, different expression pattern, ABA-sensitivity, PP2C preference and contribution to guard cell ABA responsiveness for differ-

ent receptor proteins in this family would be expected. For example, PYR10 has been shown to be capable of regulation of PP2C activity even in the absence of ABA (Hao et al., 2011), although it was later shown to be ABA-dependent (Li et al., 2015). Some of the ABA receptors have been shown to form dimers (Yin et al., 2009), whereas PYL10 forms a monomer in solution (Hao et al., 2011). PYL13, an ABA-independent inhibitor of PP2Cs, has been suggested to function as a negative regulator of other ABA receptors by forming heterodimers with them, thus reducing their capacity to inhibit PP2C activity (Li et al., 2013; Zhao et al., 2013). Quantitative differences between the ABA-sensitivity of different receptors have also been documented (Szostkiewicz et al., 2010). Constitutive activation of a single ABA receptor has been shown to efficiently activate ABA responses at the plant level (Mosquna et al., 2011), indicating that while the specific characteristics of different ABA receptors likely contribute to the fine-tuning of ABA-responses, full activation of a single receptor is sufficient to induce ABA-dependent processes.

The molecular mechanisms of PP2C and SnRK2 interaction have also been investigated. ABI1 was shown to dephosphorylate the phosphoserine 175 in the activation loop of OST1, which is positioned into the catalytic site of ABI1 during the interaction of the two proteins (Xie et al., 2012). The OST1-HAB1 interaction interface is very similar to the ABA receptor-PP2C interface (Soon et al., 2012), but the catalytic activity of HAB1 was not required for the inhibition of OST1 (Soon et al., 2012), suggesting that the steric blocking of the SnRK2 active site by the PP2C active site is enough to inhibit SnRK2 activity. Importantly, PP2Cs have been shown to directly dephosphorylate SLAC1 (Brandt et al., 2015) as well as OST1 (Umezawa et al., 2009; Vlad et al., 2009), indicating that the phosphatases can inhibit the activation of the major anion channel at different levels.

The OST1 kinase is central in ABA responses and its regulation has been thoroughly studied. OST1 has been shown to phosphorylate serines (S) in positions 59, 86, 113 and 120 in the N-terminal part of SLAC1 *in vitro* and the S120 was important for O_3-induced stomatal closure (Vahisalu et al., 2010). In addition to SLAC1, OST1 also activated the R-type anion channel QUAC1 in oocytes (Imes et al., 2013), indicating that the kinase controls anion efflux mediated by both types of anion channels. Crystal structure studies of OST1 kinase indicated that OST1 can form a partially active structure in unphosphorylated state but autophosphorylation of serine 175 (S175) in the activation loop is required for full activation (Ng et al., 2011). Plants deficient in OST1 had impaired stomatal closure in response to elevated CO_2, reduced air humidity and O_3 (Merilo et al., 2013), indicating that OST1 was not only important for ABA signalling but also for stomatal response to CO_2 and other environmental factors. OST1 overexpression caused hypersensitivity to ABA both in ABA-induced stomatal closure and inhibition of stomatal opening and resulted in increased ABA-induced activation of S-type anion channel currents in guard cell protoplasts (Acharya et al., 2013). This suggests that the amount of OST1 kinase is rate-limiting in ABA-responses.

ABA triggered an aquaporin PLASMA MEMBRANE INTRINSIC PROTEIN2;1 (PIP2;1) dependent increase in guard cell osmotic water permeability (Grondin et al., 2015). Furthermore, OST1 was shown to phosphorylate the aquaporin PIP2;1 at serine in position 121 *in vitro* and plants deficient in PIP2;1 or *pip2;1* mutants expressing a version of PIP2;1 with serine 121 substituted with alanine were shown to have impaired ABA-induced stomatal closure (Grondin et al., 2015). Thus, PIP2;1 phosphorylation by OST1 was proposed to be required for increased water permeability of guard cells and efficient ABA-induced stomatal closure. However, ABA-induced stomatal closure was recently shown to be intact in the *pip2;1* mutants (Wang et al., 2016), suggesting that the role of PIP2;1 in guard cell ABA response is not essential and could potentially be replaced by other aquaporins.

OST1 was shown to interact with other SnRK-type kinases SnRK2.2, SnRK2.3 and SnRK2.8 and also with several type 2A protein phosphatase (PP2A) regulatory subunits (Waadt et al., 2015). The *pp2a* double mutants with decreased levels of the regulatory subunits of PP2As had impaired stomatal closure in response to ABA, suggesting that PP2As are positive OST1-interacting regulators of ABA signalling in the guard cells (Waadt et al., 2015). OST1 was shown to interact with the chloroplastic Mg^{2+} chelatase subunit H and plants deficient in CHLH had decreased ROS and NO production in response to ABA, as did the *pyr1 pyl1 pyl2 pyl4* quadruple mutant of ABA receptors (Liang et al., 2015). OST1 was suggested to act downstream of CHLH in ABA signal transduction in manner similar to PYR/PYL ABA receptors (Liang et al., 2015).

Recently, a mechanism for OST1 inactivation was also proposed. In the presence of NO donors, such as S-nitrosoglutathione (GSNO), the cysteine 137 close to the OST1 catalytic site was S-nitrosylated, resulting in inhibition of OST1 activity (Wang et al., 2015). The nitrosylation was enhanced by ABA and plants deficient in S-nitrosoglutathione reductase 1 (GSNOR1), which resulted in higher levels of NO, had impaired ABA-induced stomatal closure that could be partially rescued by expression of an OST1 version with cysteine to serine substitution at position 137 (Wang et al., 2015). This suggests that the production of NO enables negative feedback in the regulation of ABA-signalling.

In addition to the core ABA-signalling pathway, there are other proteins contributing to guard cell ABA signal transduction. For example, the leucine-rich receptor-like kinase (LRR-RLK) GUARD-CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1) has been shown to be essential for stomatal responses to ABA and hydrogen peroxide (Hua et al., 2012). Moreover, GHR1 phosphorylated SLAC1 N-terminal fragment *in vitro* and activated SLAC1 in oocytes (Hua et al., 2012), suggesting a prominent role for GHR1 as a regulator of SLAC1. A scheme of ABA signal transduction in guard cells is shown in Figure 2.

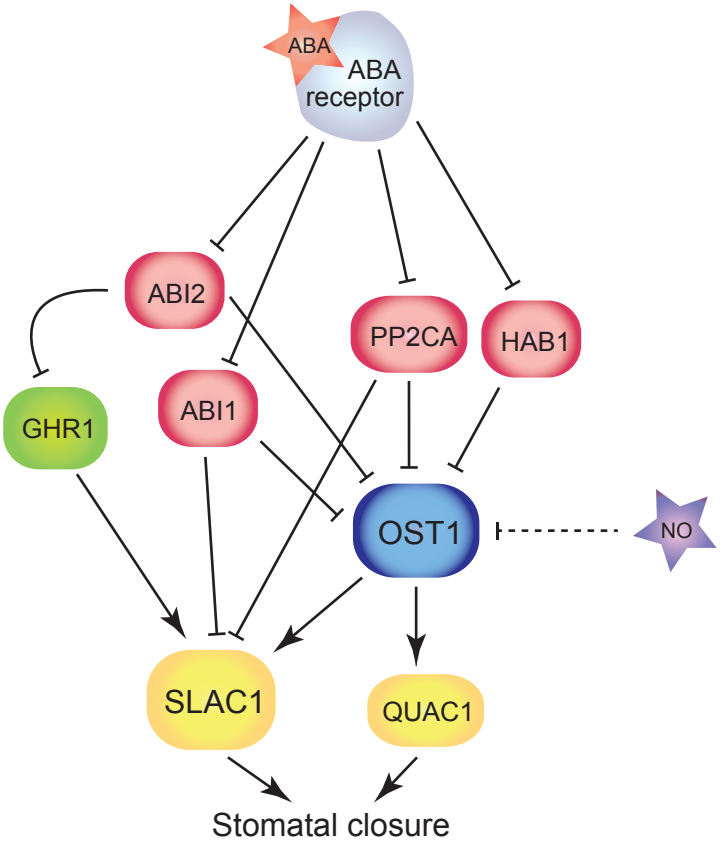

Figure 2. Guard cell ABA signalling pathway. ABA is bound by the ABA receptors and the ABA-receptor complex binds the PP2Cs ABI1, ABI2, PP2CA and HAB1, thus releasing OST1, GHR1 and SLAC1 from inhibition by the PP2Cs. OST1 autophosphorylates and activates SLAC1 and QUAC1 by phosphorylation, triggering stomatal closure. GHR1 also contributes to the activation of SLAC1. OST1 has been proposed to be negatively regulated by NO.

The identification of the core ABA-signalling pathway gives fundamental basis for potential applications in the field to improve crop water use efficiency. For example, loss of several ABA receptors results in increasing stomatal conductance and decreasing ABA-sensitivity in higher order mutants, especially in the *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* sextuple mutant (Gonzalez-Guzman et al., 2012). This indicates that the ABA receptors provide an attractive target for engineering plants with higher water use efficiency. For example, overexpression of PYL5 (Santiago et al., 2009b) and PYL13 (Zhao et al., 2013) resulted in increased drought tolerance. Engineering of ABA receptors to perceive agrochemicals that could be used to induce stomatal closure in crops when desired has been suggested as a tool to control plant water use efficiency (Park et al., 2015). Indeed, a modified version of the PYR1 ABA receptor that senses the agrochemical man-

dipropamide was identified and its expression was shown to lead to mandipropamide-induced stomatal closure in Arabidopsis and tomato and drought tolerance in Arabidopsis (Park et al., 2015). *A. thaliana* plants overexpressing the ABA receptors PYL4 or PYL12 had high growth rates and increased WUE (Yang et al., 2016), further supporting the idea that modification of the expression and sensitivity of ABA receptors could be applied for generating plants with increased WUE. Alteration of the regulation and expression of other components of the ABA signalling pathway may also provide an opportunity for developing water-saving plants.

2.2.2. Ca^{2+}-dependent protein kinases in ABA signalling

Ca^{2+}-dependent protein kinases (CDPKs or CPKs) are Ca^{2+}-sensing proteins characterized by a kinase domain, autoinhibitory domain and a calmodulin-like domain, which harbours the Ca^{2+}-binding EF-hand motifs (Cheng et al., 2002; Harper et al., 2004). At low Ca^{2+} levels, the autoinhibitory domain is bound to the kinase domain, rendering the kinase inactive; upon Ca^{2+}-binding to the EF-hands, a conformational rearrangement occurs and the kinase becomes active. There are 34 CPKs in *A. thaliana* and they participate in the regulation of various processes in the cell (Cheng et al., 2002). Several CPKs have also been shown to contribute to ABA signal transduction and SLAC1 activation in guard cells.

CPK3 and CPK6 were shown to be involved in ABA signalling and S-type anion channel activation, as stomatal closure and S-type anion currents in response to ABA were impaired in the *cpk3 cpk6* double mutants (Mori et al., 2006). This was supported by the enhanced drought tolerance of plants overexpressing CPK6 (Xu et al., 2010). CPK6 interacted with SLAC1 (Geiger et al., 2010) and activated SLAC1 in oocytes (Brandt et al., 2012; Scherzer et al., 2012) as well as phosphorylated the serine at position 59 (S59) in SLAC1 (Brandt et al., 2012). Furthermore, SLAC1 activation by CPK6 was suppressed by the ABI2 phosphatase and rescued in the additional presence of the ABA receptor PYR1 and ABA in the oocytes (Brandt et al., 2012). The activity of CPK6 towards SLAC1 was shown to be suppressed by dephosphorylation of SLAC1, and not CPK6, by the PP2Cs ABI1 and PP2CA (Brandt et al., 2015). No significant CPK3-induced SLAC1 activation was achieved in oocytes with wild-type CPK3 (Brandt et al., 2012; Scherzer et al., 2012), but SLAC1 was activated by a version of CPK3 that lacked the Ca^{2+}-binding EF-hand motifs (Scherzer et al., 2012). The homologue of SLAC1, SLAH3 was also activated by both CPK6 and CPK3 in oocytes (Scherzer et al., 2012). Furthermore, CPK3 activity was dependent on Ca^{2+}-concentration, whereas the activity of CPK6 was not largely affected by higher Ca^{2+} levels. CPK5 could also activate SLAC1 in oocytes (Brandt et al., 2015), indicating that this kinase may also contribute to guard cell signal transduction.

CPK4 and CPK11 have been implicated in various ABA responses, including stomatal regulation (Zhu et al., 2007). Namely, the *cpk4* and *cpk11* single and *cpk4 cpk11* double mutants showed impaired ABA-induced stomatal closure, whereas plants overexpressing CPK11 had enhanced closure response and increased drought tolerance. Similarly, CPK10 has been suggested to function in ABA signalling and drought responses, as the *cpk10* mutants had impaired ABA-induced stomatal closure and increased drought sensitivity (Zou et al., 2010). However, in a different study, the *cpk10* mutant and the *cpk4 cpk11* double mutant were shown to close their stomata in response to ABA (Hubbard et al., 2012). Thus, the role of CPK4, CPK10 and CPK11 in guard cell ABA responses remains controversial and further research on their function is required.

CPK21 and CPK23 interacted with SLAC1 and ABI1 in oocytes, where SLAC1 was activated by CPK23 and the CPK23-induced SLAC1 activation could be suppressed by the phosphatases ABI1 and ABI2, but not by HAB1 and HAB2 (Geiger et al., 2010). Both CPK23 and CPK21 phosphorylated SLAC1, whereas only the latter showed a major Ca^{2+}-dependent increase in activity (Geiger et al., 2010). CPK21 also phosphorylated the SLAC1 homologue SLAH3 in a Ca^{2+}-dependent manner and activated it in oocytes, where the activation was suppressed by ABI1 (Geiger et al., 2011). The *in vitro* addition of the ABA receptor PYL9 to the mixture containing the N-terminus of SLAH3 or SLAC1, CPK21 or CPK23 and ABI1 resulted in ABA-dependent phosphorylation of SLAH3 by CPK21 or SLAC1 by CPK23, respectively (Geiger et al., 2010, 2011), indicating that the two S-type anion channels are activated by similar mechanisms in the guard cells. Intriguingly, the *cpk23* single mutant had smaller stomatal aperture and increased drought tolerance (Ma and Wu, 2007), which is opposite to what would be expected in the absence of a SLAC1-activating protein. Later, both the *cpk21* and *cpk23* mutants were shown to have normal stomatal conductance and responses to elevation of CO_2 concentration, reduction in air humidity, O_3 and darkness were modestly impaired in *cpk23* but not *cpk21* (Merilo et al., 2013), indicating that CPK23 is more important for fast stomatal responses to various stimuli.

Since there is a large number of CPKs in *A. thaliana* and many of them have been implicated in stomatal responses, there is likely a functional redundancy between different CPKs. Thus, higher order mutants of several CPKs should be studied to unravel possible stomata-related phenotypes. This approach has shown that the *cpk5 cpk6 cpk11 cpk23* quadruple mutant has impaired ABA-induced stomatal closure and lacks ABA-induced S-type anion channel activation (Brandt et al., 2015), further supporting a role for these CPKs in guard cell signal transduction. Further research of plants lacking various combinations of CPKs is necessary to understand the role of CPKs in stomata. As some CPK-encoding genes, such as *CPK21* and *CPK23*, lie close to each other in the genome (Hrabak et al., 2003), traditional genetic analysis cannot always be applied and genome editing tools, such as the CRISPR-Cas9 system, could be useful in addressing the role of CPKs in stomatal regulation. The current model for the function of CPKs in ABA signal transduction and anion channel activation is summarized in Figure 3.

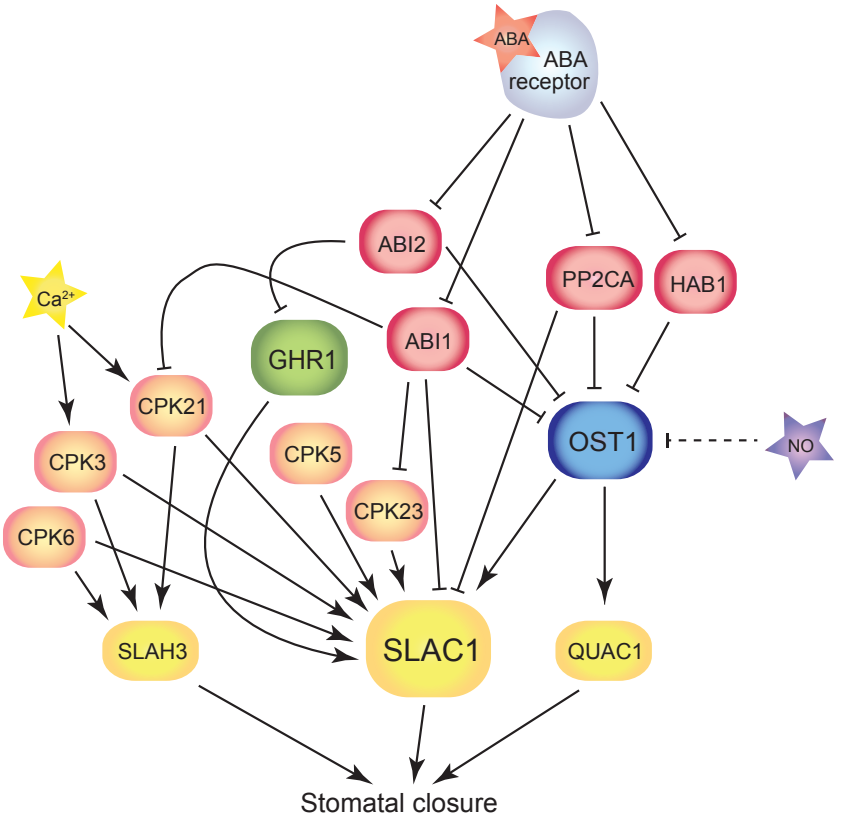

Figure 3. CPKs in ABA signal transduction and anion channel activation. CPKs activate the SLAC1 and SLAH3 anion channels, thus contributing to stomatal closure. CPK3 and 21 are sensitive to changes in Ca^{2+}-concentration, CPK6 and 23 have high basal activity irrespective of the Ca^{2+} concentration. CPK3, 6 and 21 are capable of activating both SLAC1 and SLAH3; CPK5 and CPK23 have been shown to activate SLAC1. The PP2C ABI1 can inhibit the activity of CPK21 and 23.

2.2.3. MAP kinases and stomatal ABA signalling

Mitogen-activated protein kinases (MPKs) play important roles in various signal transduction pathways in plant cells (Colcombet and Hirt, 2008; Rodriguez et al., 2010). Several MPKs have been shown to be strongly expressed in guard cells and contribute to guard cell signalling in response to both abiotic and biotic factors (for review see Lee et al., 2016). To date, only a few MAP kinases have been linked with stomatal ABA signalling.

MPK9 and MPK12 were shown to be preferentially and highly expressed in guard cells (Jammes et al., 2009). Plants harbouring point mutations in these kinases (*mpk9-1*, *mpk12-1*, and *mpk12-2*) displayed wild-type stomatal response to ABA (Jammes et al., 2009), but *mpk12-1* and *mpk12-2* had reduced WUE and *mpk12-1* had increased stomatal conductance (Des Marais et al., 2014). The

mpk9-1 mpk12-1 double mutants had impaired ABA-induced stomatal closure (Jammes et al., 2009; Montillet et al., 2013) and impaired inhibition of stomatal opening by ABA (Jammes et al., 2009). Similar impairment of ABA-induced stomatal closure was present in plants where the expression of MPK9 and MPK12 was silenced by RNAi. The activation of S-type anion channels in response to ABA was also impaired and water loss was increased in *mpk9-1 mpk12-1* double mutants (Jammes et al., 2009).

A point mutation in *MPK12*, resulting in the substitution of glycine 53 with arginine (G53R) in the respective protein, was shown to cause decreased WUE in the Cvi-0 ecotype and in the near isogenic line (NIL) obtained via crossing a locus associated with the decreased WUE from Cvi-0 to the L*er* ecotype (Des Marais et al., 2014). The NIL had higher stomatal conductance than the L*er* wild-type that could be complemented by expression of L*er*-MPK12 in the NIL, indicating that the decreased WUE in the NIL could be caused by increased stomatal conductance due to the G53R substitution in MPK12 (Des Marais et al., 2014). The NIL had larger guard cells, but stomatal closure in response to ABA was functional in these plants, whereas ABA-induced inhibition of stomatal opening was partially impaired (Des Marais et al., 2014).

In addition to a role in stomatal ABA signalling, MPK12 is also a negative regulator of auxin signalling. MPK12 was shown to interact with a MAP kinase phosphatase INDOLE-3-BUTYRIC ACID-RESPONSE5 (IBR5) in a yeast two-hybrid assay and dephosphorylation by IBR5 led to the inactivation of MPK12 (Lee et al., 2009a). Plants deficient in IBR5 (*ibr5-3*) had decreased sensitivity to auxin in root growth, while suppression of MPK12 expression via RNAi led to auxin-hypersensitivity in root growth. Moreover, auxin could activate MPK12 purified from the *A. thaliana* protoplasts (Lee et al., 2009a). These data indicate an important regulatory function for MPK12-IBR5 kinase-phosphatase pair in auxin signal transduction. However, the potential role of IBR5 in guard cells has not been studied to date.

Similar to plants with deficient MPK12 function, silencing of MPK3 in the guard cells of *A. thaliana* resulted in impaired inhibition of stomatal opening by ABA, whereas stomatal closure in response to ABA was intact in these plants (Gudesblat et al., 2007). Thus, a role for MPK3 in stomatal ABA signalling cannot be excluded. Recently, MAP kinase kinase kinase 18 (MKKK18) was shown to be regulated by the ABI1 protein phosphatase (Mitula et al., 2015). Plants deficient in MKKK18 function (*mkkk18-1* and *mkkk18-2*) had increased stomatal aperture and impaired stomatal closure in response to ABA whereas plants overexpressing MKKK18 showed ABA-hypersensitive stomatal closure (Mitula et al., 2015). MKKK18 interacted with ABI1 but not ABI2, and ABI1 inhibited MKKK18 kinase activity (Mitula et al., 2015). MKKK18 also interacted with OST1 (Tajdel et al., 2016). These data indicate a role for MKKK18 in guard cell ABA signal transduction and suggest that ABI1 and OST1 could be common nodes of the core ABA signalling pathway and guard cell MAP kinase signalling.

In *Nicotiana tabacum*, silencing of MPK4 and MPK4L (MPK4-like) caused increased stomatal aperture and high O_3-sensitivity (Gomi et al., 2005;

Yanagawa et al., 2016), indicating a role in stomatal responses for these kinases. Furthermore, CO_2-induced stomatal closure was impaired in *N. tabacum* plants with silenced MPK4 (Marten et al., 2008). The *A. thaliana* MPK4 has strong expression in guard cells (Petersen et al., 2000; Rodriguez et al., 2010), but so far it has been proposed to function in stress responses (Petersen et al., 2000; Brodersen et al., 2006). The *mpk4* mutants are dwarfed due to high salicylic acid content (Petersen et al., 2000) and thus studies of stomatal regulation in these plants are hindered. Whether MPK4 contributes to stomatal signalling in Arabidopsis as well as in tobacco remains unclear.

Thus, the roles of MAP kinase signalling cascades in the regulation of stomatal responses are largely unknown at present and remain a promising research direction that should help to understand stomatal responses to ABA as well as other stimuli. A scheme of ABA signal transduction and anion channel activation together with the putatively involved proteins from MAP signalling cascades is shown in Figure 4.

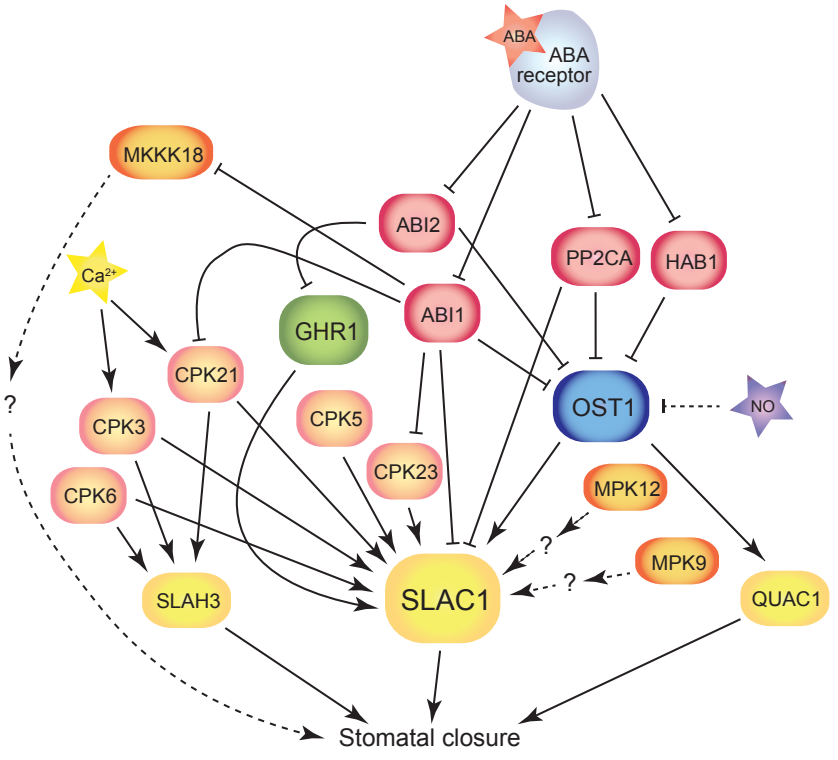

Figure 4. MPKs in ABA signal transduction and anion channel activation. MPK9 and 12 have been implicated in stomatal ABA responses and S-type anion channel activation via currently unknown mechanisms. MKKK18 is inhibited by the PP2C ABI1 and required for efficient ABA-induced stomatal closure, but the exact role of MKKK18 in ABA signal transduction remains to be characterized.

2.3. Stomatal CO_2 signalling

Atmospheric CO_2 concentration affects stomata and thus plant gas exchange at different levels. On one hand, the density of stomatal pores on plant surfaces is decreased in an atmosphere of increased CO_2 concentration (Woodward, 1987; Woodward and Kelly, 1995), as sufficient CO_2 uptake can be achieved via a smaller number of stomata. However, the response of stomatal density to changes in CO_2 concentration is developmental and hence time-consuming. On the other hand, CO_2 directly regulates the size of stomatal aperture, enabling a fast response to changing CO_2 concentration and thus greater plasticity in the control of WUE. Stomata open in response to decreased CO_2 concentration to gain enough CO_2 for photosynthesis and close in response to an increase in CO_2 concentration to preserve water, as sufficient CO_2-uptake is possible at lower stomatal conductance. Albeit CO_2 is a substrate for photosynthesis and an important regulator of stomatal responses, the mechanisms of stomatal CO_2 signalling are far less clear than those of guard cell ABA signalling. This chapter gives an overview of the studies addressing the mechanisms of stomatal responsiveness to CO_2. The role of photosynthesis and mesophyll in relation to guard cell CO_2 responses is discussed and the molecular components of CO_2 signalling identified to date are introduced.

2.3.1. The role of photosynthesis and mesophyll in CO_2 signalling

The underlying mechanisms of perception of changes in the atmospheric CO_2 concentration by plants require further clarification. Stomatal response to a rise in the CO_2 concentration could occur via perception of increased intercellular CO_2 concentration (C_i), or alternatively via perception of ambient CO_2 concentration (C_a) or concentration of CO_2 in the stomatal pore. The first hypothesis is supported by experiments by Mott (1988), where stomata did not respond to changes in the concentration of CO_2 at the surface of the leaf when C_i was held constant. On the other hand, several studies analysing various parameters of photosynthesis and stomatal conductance in transgenic plants where expression of a protein essential for efficient photosynthesis has been suppressed, suggest that changes in C_i alone do not drive changes in stomatal conductance. For example, stomatal conductance was unaltered in tobacco plants with decreased ribulose-l,5-bisphosphate carboxylase-oxygenase (Rubisco) content, despite the higher C_i of these plants (Stitt et al., 1991; Lauerer et al., 1993). Similarly, tobacco plants with suppressed expression of the Rieske FeS subunit, a component of the cytochrome *bf* complex, also displayed decreased photosynthesis but unaltered stomatal conductance, although the C_i/C_a ratio increased in the transgenic plants (Price et al., 1998). These data suggest that a change in C_i is not necessarily accompanied by a change in stomatal conductance. The same appears to be true for stomatal responses to CO_2: tobacco plants with reduced Rubisco content had decreased photosynthesis, whereas stomatal conductance and stomatal response to CO_2 were similar to wild-type plants (von Caemmerer

et al., 2004). Stomatal conductance was mostly unaltered and stomatal closure in response to elevated CO_2 was completely functional in transgenic tobacco plants with reduced levels of sedoheptulose-1,7-bisphosphatase despite the increased C_i of these plants (Lawson et al., 2008). Similar results have been obtained in other species as well. For example, in potatoes with suppressed chloroplastic fructose-1,6-bisphosphatase levels, photosynthesis decreased and the C_i increased, but leaf conductance was not lower compared to wild-type potatoes at neither ambient nor elevated CO_2 (500 ppm) (Muschak et al., 1999). Thus, instead of the C_i, perception of C_a or CO_2 concentration in the stomatal pore could be of key importance in stomatal responses to elevated CO_2 concentration. Alternatively, coordinated perception of both C_i and C_a could be important for efficient CO_2 responsiveness. Collectively, these data also suggest that photosynthesis is not directly coupled with stomatal conductance and stomatal CO_2 responses.

Nevertheless, photosynthetic metabolites or proteins related to photosynthesis may be involved in CO_2 signal transduction leading to stomatal movements. For example, stomatal conductance correlated with photosynthetic capacity in cotton, maize and *Eucalyptus pauciflora,* which led the authors to suggest that a photosynthetic metabolite may be involved in stomatal response to elevated CO_2 concentration (Wong et al., 1979). Stomatal response to C_i was shown to be altered in plants with blocked photosynthetic electron transport, suggesting that at least a part of stomatal CO_2 response depends on the latter (Messinger et al., 2006). The role of guard cell chloroplasts in stomatal responses has also been analysed. Guard cell, but not mesophyll cell chloroplast zeaxanthin content was shown to positively correlate with stomatal aperture, whereas zeaxanthin levels were higher in lower CO_2 concentrations and lower at elevated CO_2 levels (Zhu et al., 1998). In the presence of DTT, an inhibitor of violaxanthin de-epoxidase, accumulation of zeaxanthin and stomatal opening in response to decreased CO_2 concentration were inhibited. Thus, these data support a role for guard cell chloroplast zeaxanthin as a mediator of stomatal CO_2-responses (Zhu et al., 1998).

On the other hand, guard cell chloroplasts may not be essential for stomatal CO_2 responses in all plants, as illustrated by a species of *Paphilopedium* that lacks chloroplasts in guard cells, but displays stomatal closure in response to elevated CO_2 concentration (Nelson and Mayo, 1975). This is also supported by the presence of CO_2-induced stomatal responses in *V. faba* plants treated with norflurazon that inhibits carotenoid biosynthesis and causes a lack of functional chloroplasts in guard cells and mesophyll cells (Roelfsema et al., 2006). These data suggest that neither mesophyll nor guard cell photosynthesis are essential for stomatal CO_2 responses. Furthermore, stomatal responses to changes in CO_2 concentration occur in *A. thaliana* plants that lack chlorophyll in the stomata due to guard-cell specific expression of a chlorophyllase (Azoulay-Shemer et al., 2015). Nevertheless, guard cell chlorophyll appears to be required for normal guard cell turgor and shape as a subset of chlorophyll-less stomata displayed a thin-shaped morphology and were constitutively closed (Azoulay-Shemer et al., 2015). However, starch biosynthesis in the guard cells, but not mesophyll cells, was shown to be necessary for efficient CO_2-induced stomatal closure via analy-

sis of plants deficient in starch biosynthesis either in all cells or only in mesophyll cells (Azoulay-Shemer et al., 2016). The former showed partially impaired CO_2-induced stomatal closure, while stomata of the latter closed in response to elevated CO_2. Stomatal opening in response to low CO_2 was also impaired in completely starch-deficient plants, but it was enhanced in plants where starch was present in guard cells but not mesophyll cells (Azoulay-Shemer et al., 2016). Thus, although guard cell chloroplasts are not essential for stomatal CO_2 responsiveness, metabolites related to guard cell photosynthesis, such as starch and sugars, could still contribute to stomatal CO_2 responses.

Another interesting point of debate in the research on CO_2-induced stomatal responses has been the question whether guard cell responses to CO_2 are autonomous or is a contribution from the mesophyll required for the stomatal responses to occur. Swelling in response to low CO_2 in isolated guard cell protoplasts of *Commelina communis* (Fitzsimons and Weyers, 1983; Weyers et al., 1983) and shrinking in response to elevated CO_2 in guard cell protoplasts of *V. faba* (Gotow et al., 1982) suggested a guard cell autonomous mechanism of low CO_2-induced stomatal opening and high CO_2-induced stomatal closure. Stomata in the isolated epidermis of *C. communis* had different aperture after incubation at different CO_2 concentrations (Webb et al., 1996), also suggesting inherent CO_2-responsiveness of guard cells. In accordance with this, stomata closed in response to elevated CO_2 in the epidermal peels of *V. faba*, although there was no stomatal opening in response to low CO_2 (Brearley et al., 1997). In *A. thaliana*, both stomatal closure in response to increased CO_2 (Young et al., 2006; Hu et al., 2010; Chater et al., 2015) as well as opening in response to treatment with CO_2-free air (Mustilli et al., 2002; Merlot et al., 2007) have been observed in experiments conducted in epidermal peels. Thus, there is considerable evidence for guard cell autonomous responses to changes in CO_2 concentration.

Nevertheless, a role for a mesophyll-derived signal in stomatal CO_2 responses has been argued. Stomatal responses to changes in CO_2 concentration have been studied in the leaves and isolated epidermises of different species. Stomata in the leaves but not in isolated epidermises of *Tradescantia pallida* and *Pisum sativum* responded to changes in CO_2 concentration (Mott et al., 2008). However, when the epidermis from *T. pallida* or *P. sativum* was placed on exposed mesophyll tissue from either the same or a different species, responsiveness to CO_2 was restored. Similar results were obtained with *C. communis*: in red light, stomatal opening in response to low CO_2 and closure in response to high CO_2 were clearly stronger in leaf segments and epidermal strips placed on mesophyll when compared to isolated epidermal strips (Fujita et al., 2013). However, in white light the stomatal opening in response to low CO_2 occurred in epidermal strips, albeit to a smaller extent than in leaf segments and epidermal strips placed on mesophyll, whereas stomata did not close in response to high CO_2 in isolated epidermis (Fujita et al., 2013). These experiments suggest that the role of mesophyll in stomatal response to high CO_2 and low CO_2 could be different and depend on light signalling. A comparison of stomatal CO_2-responsiveness in leaves and epidermal peels in *A. thaliana* also showed that stomatal response to

increased CO_2 was clearly stronger in intact leaves than in isolated epidermis (Young et al., 2006; Mott, 2009). Together, these data suggest that a signal from mesophyll is important for efficient stomatal CO_2-responses, especially for CO_2-induced stomatal closure.

The nature of the proposed mesophyll signal remains vague at this point. When water was added to *T. pallida* epidermis grafted onto mesophyll, stomata rapidly closed (Sibbernsen and Mott, 2010). A similar response occurred in the leaves of *T. pallida*, *Helianthus annuus*, and *Oenothera caespitosa* when the mesophyll was flooded with water, KCl solution or silicon oil. When mesophyll and epidermis in the grafts of *T. pallida* were separated by a polyethylene plastic wrap with the aim to block all liquid- and vapour-phase signals, stomata did not open (Sibbernsen and Mott, 2010). However, when an air-permeable hydrophobic filter was placed between the epidermis and mesophyll, stomata opened and were able to respond adequately to the increase and decrease of CO_2 concentration. When the filter was kept at 100% relative water content, stomata did not open. These data were interpreted as suggestive of a vapour-phase mesophyll-derived signal that is involved in stomatal responses to light and CO_2 (Sibbernsen and Mott, 2010). Stomata opened in response to low CO_2 and closed in response to high CO_2 in epidermal strips placed on mesophyll in both red and white light (Fujita et al., 2013). The same was true when the epidermal strips were separated from mesophyll by a cellophane spacer permeable to small molecules in the liquid. However, when a polyethylene spacer that was expected to block all signals was used, stomata did not respond to changes in CO_2 concentration in red light. Stomatal opening in response to low CO_2 in epidermal peels separated from the mesophyll by a polyethylene spacer occurred in white light, whereas stomatal closure in response to high CO_2 was completely impaired (Fujita et al., 2013). These data suggest that a signal from mesophyll is indeed required for efficient response to high CO_2, but may not be that important for low CO_2-induced stomatal opening. Based on these data, the mesophyll signal involved in CO_2-responses was proposed to be aqueous (Fujita et al., 2013), not gaseous as suggested previously (Sibbernsen and Mott, 2010). However, it has been pointed out that as cellophane is also permeable to gases, the vapour-phase nature of the signal cannot be excluded (Mott et al., 2014). An electrically charged electrode was used to produce negative air ions and the effect of these on stomata in isolated epidermis of *T. pallida* was studied (Mott et al., 2014). Negative ions created by the electrode induced stomatal closure in isolated epidermis. Stomatal closure in response to elevated CO_2 in intact leaves of *T. pallida* was accompanied by increased blue colour intensity in the epidermis that decreased together with lowering of the CO_2 concentration. This was suggested to be caused by changes in the pH of epidermal cells brought about by changed pH of the mesophyll that was signalled to guard cells via a vapour-phase ion, such as the hydronium ion (Mott et al., 2014).

Considerable effort has been made to unravel the links between photosynthesis and stomatal CO_2 responses and the contribution of mesophyll to the behaviour of guard cells. The evidence so far is ambiguous and there are more ques-

tions than answers. It is likely that although photosynthesis and C_i do not always march hand-in-hand with stomatal CO_2-responses, they are linked via for example photosynthetic metabolites. Although the guard cells can respond autonomously to changes in CO_2 levels at least to some extent, several lines of evidence indicate that a contribution from the mesophyll is required for fast and efficient stomatal responses to changes in CO_2 concentration. Further research is needed to elucidate the interactions between photosynthesis and stomatal conductance and the role of mesophyll in guard cell CO_2 responses.

2.3.2. CO_2 signalling in guard cells

The CO_2-signal transduction in the guard cells is proposed to be mediated by the bicarbonate anion (HCO_3^-). This is supported by impaired stomatal CO_2-responses in plants deficient in the β-carbonic anhydrases βCARBONIC ANHYDRASE 1 (CA1) and βCARBONIC ANHYDRASE 4 (CA4) and the ability of a human carbonic anhydrase αCAII to complement this deficiency (Hu et al., 2010). Similar impairment in high CO_2-iduced stomatal closure and low CO_2-induced stomatal opening was also present in rice plants lacking the chloroplastic βCA1 (Chen et al., 2017). Bicarbonate, rather than CO_2, activated S-type anion currents in guard cell protoplasts (Xue et al., 2011). In the presence of the anion channel SLAC1 and a SLAC1-activating kinase (OST1, CPK6 or CPK23), bicarbonate could enhance S-type anion currents also in *X. laevis* oocytes (Wang et al., 2016). Thus, SLAC1 was proposed as the protein that perceives bicarbonate while kinases that phosphorylate SLAC1 were required for channel activation.

Stomatal CO_2 signalling appears to be tightly coupled with ABA signalling as several proteins involved in ABA perception and signal transduction also function in CO_2-induced stomatal closure. Studies of plants lacking functional anion channel SLAC1 (Negi et al., 2008; Vahisalu et al., 2008), protein kinase OST1 (Xue et al., 2011), PP2C phosphatases and ABA receptors (Merilo et al., 2013; Chater et al., 2015) indicate a role for these proteins in CO_2 signalling as these plants show impaired CO_2-induced stomatal closure. However, recent research indicates that while functional SLAC1 regulation is essential for both ABA and CO_2 signal transduction, SLAC1 activation is likely to occur via different mechanisms in these cases. While SLAC1 N- and C-termini were shown to be important for functional stomatal response to ABA, they were not required for CO_2-induced stomatal closure (Yamamoto et al., 2016). Instead, two tyrosine residues in the transmembrane domain of SLAC1 were found to be important for CO_2-induced stomatal closure.

In addition to the S-type anion channel SLAC1 and its regulators, R-type anion channel QUAC1/ALMT12 also seems to be involved in CO_2 signalling as *almt12* plants show partially impaired CO_2-induced stomatal closure (Meyer et al., 2010). The malate importer ARABIDOPSIS THALIANA ATP-BINDING CASSETTE B14 (AtABCB14) has also been implicated in stomatal response to

elevated CO_2 concentration as the *atabcb14* mutants and overexpressors had enhanced or decreased CO_2-induced stomatal closure, respectively (Lee et al., 2008). Changes in Ca^{2+} concentration have been shown to be important for CO_2-induced stomatal closure in *C. communis* (Webb et al., 1996) and in *A. thaliana* (Hubbard et al., 2012), indicating that Ca^{2+} is another common component of ABA- and CO_2-induced signal transduction in guard cells. However, the stomatal responses to CO_2 in plants deficient in Ca^{2+}-dependent protein kinases have not been thoroughly characterized yet. The response to elevated CO_2 concentration is mildly impaired in the *cpk23* mutants (Merilo et al., 2013), but further research is required to characterize the role of CPKs in stomatal CO_2 signalling.

Few CO_2-specific regulators of stomatal closure are known to date. The protein kinase HIGH LEAF TEMPERATURE 1 (HT1) was identified in a screen for CO_2-insensitive mutants and shown to act as a negative regulator of stomatal CO_2 signalling, as illustrated by constitutively low stomatal conductance and lack of CO_2-responses in *ht1-2* plants (Hashimoto et al., 2006). Additional recessive alleles of *HT1* that showed high leaf temperature in low and ambient CO_2 concentration and harboured point mutations or deletions of amino acids predicted to be essential for kinase activity, were isolated recently (Hashimoto-Sugimoto et al., 2016). In addition to the novel recessive alleles, a dominant allele of HT1 with arginine to lysine substitution at position 102 (R102K) was shown to cause very open stomata and loss of stomatal responses to CO_2, while the kinase activity of HT1(R102K) was similar to HT1. Previously predicted classification of HT1 as a Raf-1-type Ser/Thr kinase (Ichimura et al., 2002) was also confirmed in *in vitro* kinase assays (Hashimoto-Sugimoto et al., 2016). HT1 has been shown to phosphorylate OST1 and inhibit SLAC1 activation by OST1 in oocytes in a kinase activity dependent manner (Tian et al., 2015). However, the HT1 used in the study was a version of HT1 annotated in TAIR database that lacked 45 amino acids from the N-terminus when compared to the full-length HT1 used in Hashimoto et al. (2006) and Hashimoto-Sugimoto et al. (2016). Thus, further studies with full-length HT1 would help to elucidate the interplay of HT1 and OST1 in CO_2 signalling.

A plasma membrane transporter protein RESISTANT TO HIGH CO_2 1 (RHC1) has also been suggested to be involved in stomatal CO_2-responses (Tian et al., 2015). Plants deficient in RHC1 were shown to have impaired stomatal closure in response to increased CO_2 concentration and RHC1 interacted with CA4 and HT1 in *A. thaliana* protoplasts. Based on reconstitution of CO_2 signalling in oocytes, RHC1 was proposed as a bicarbonate-sensing protein that counteracts the negative effect of HT1 on SLAC1 activation (Tian et al., 2015). However, as the expression of RHC1 alone in oocytes also induced ion currents that were not dependent on bicarbonate or OST1 (Wang et al., 2016), the function of RHC1 as a potential bicarbonate sensor requires further study. A role for the CO_2-permeable aquaporin PIP2;1 in CO_2 signalling has also been proposed as PIP2;1 interacted with CA4 (Wang et al., 2016). The co-expression of PIP2;1, CA4, OST1 and SLAC1 in oocytes in the presence of extracellular bicarbonate

enhanced SLAC1 activation compared to SLAC1 activation induced by OST1 alone.

Thus, some components specific to CO_2 signal transduction in the guard cells have been identified, but many are shared between ABA- and CO_2-response. A model based on the literature discussed in this chapter, highlighting components unique to CO_2 signalling and in common with ABA signalling, is presented in Figure 5.

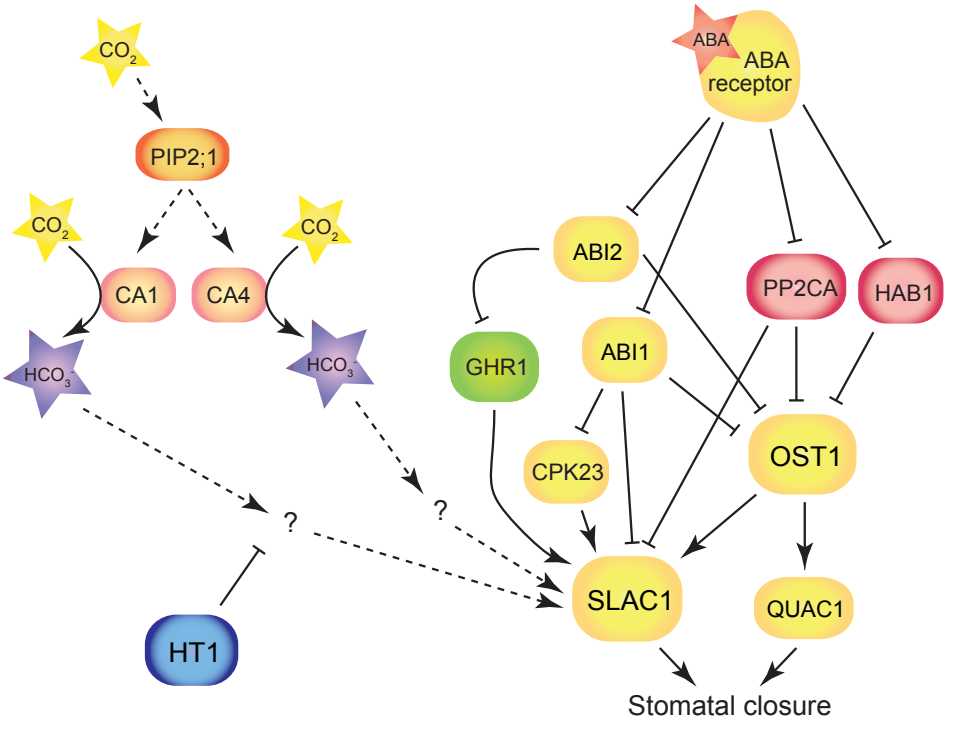

Figure 5. Guard cell ABA and CO_2 signalling. The components of ABA signalling pathway that have been shown to be important for CO_2 signal transduction as well are shown in yellow. In the CO_2-specific branch, the aquaporin PIP2;1 has been proposed to facilitate CO_2 uptake. The carbonic anhydrases CA1 and 4 catalyse the conversion of CO_2 to bicarbonate that activates SLAC1 via yet to be characterized mechanisms. HT1 acts as an inhibitor of SLAC1 activation and stomatal closure in response to CO_2.

2.4. The evolution of stomatal ABA and CO_2 signalling

Stomatal responses to drought-induced ABA and atmospheric CO_2 concentration are closely linked to plant WUE. Thus, the origin and evolution of stomatal ABA and CO_2 signalling provide an attractive subject of research that has given rise to interesting debates among plant biologists over the past years. Stomata emerged early in the evolution of land plants. Stomatal fossils date back more

than 400 million years and display morphology similar to guard cells as they are known today (Edwards et al., 1992, 1998). The most basal land plant group with stomata are mosses (Figure 6).

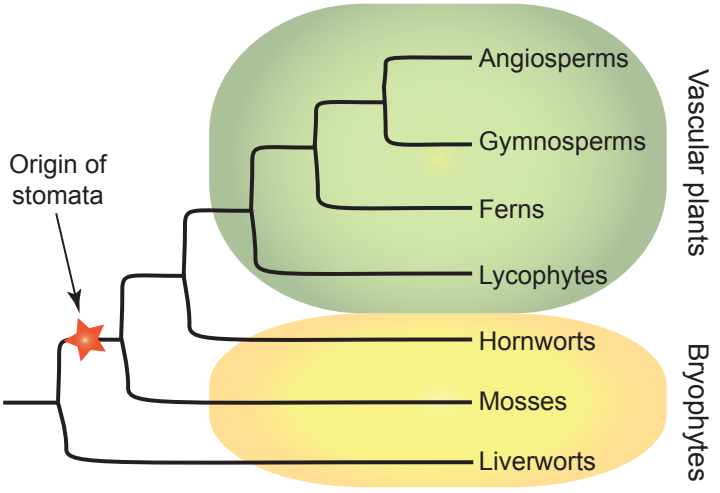

Figure 6. The origin of stomata. Based on McAdam and Brodribb (2012b) and Chater et al. (2013).

An analysis of stomatal responsiveness to ABA in several fern and lycophyte species led to the hypothesis that these plant groups completely lack ABA-induced stomatal responses and are capable of only hydropassive stomatal regulation that is controlled by leaf water content (Brodribb and McAdam, 2011). Furthermore, high drought-induced ABA levels failed to inhibit stomatal opening upon rehydration in ferns and a lycophyte, which was interpreted as insensitivity to endogenous ABA in these plants (McAdam and Brodribb, 2012). On the other hand, as the ABA concentration strongly increased in response to drought in these species (McAdam and Brodribb, 2012a), it is appealing to hypothesize that this plant hormone participates in the control of transpiration and stomatal aperture also in ferns and lycophytes. In accordance with this, concentration-dependent ABA-induced stomatal closure has been found in the mosses *Physcomitrella patens* and *Funaria hygrometrica* (Chater et al., 2011), the lycophyte *Selaginella uncinata* (Ruszala et al., 2011) and the ferns *Polystichum proliferum* and *Nephrolepis exaltata* (Cai et al., 2017).

Homologues for the genes that belong to the core ABA signalling pathway in *A. thaliana* are present in mosses, lycophytes and ferns (Hanada et al., 2011; Cai et al., 2017). Furthermore, the OST1 variants from *P. patens* (Chater et al., 2011) and *Selaginella moellendorffii* (Ruszala et al., 2011) have been shown to restore ABA-responsiveness in the *A. thaliana ost1* mutant, suggesting a conserved role for OST1 in stomatal regulation across the evolutionary tree of

plants. The *P. patens* OST1 orthologues and others from the alga *Klebsormidium nitens* and the liverwort *Marchantia polymorpha* were capable of activating *A. thaliana* SLAC1 in oocytes but not the SLAC1 from the respective species, with the exception of *P. patens* SLAC1, which was activated by one of the *P. patens* OST1 versions (Lind et al., 2015). These data indicate that while OST1 and SLAC1 are conserved from algae to angiosperms, the mechanisms of SLAC1 activation by OST1 are different in species that predate mosses. The presence of the key angiosperm ABA signal transduction components in mosses, lycophytes and ferns does not necessarily mean that they would regulate stomatal aperture in these plant groups as in angiosperms. For example, an OST1 homologue from the fern *Ceratopteris richardii* was recently shown to control sex determination (McAdam et al., 2016).

The jury is still out on the evolutionary origin of stomatal CO_2 responses. Brodribb et al. (2009) carried out an analysis of stomatal responses to low (100 ppm) and high (600 ppm) atmospheric CO_2 concentration in 15 species across the evolutionary tree of vascular land plants. All the analysed species showed stomatal opening in response to low CO_2, but the opening response was stronger in angiosperms and conifers than in ferns and a lycophyte *Selaginella pallescens*. However, of the studied species, only angiosperms displayed strong high CO_2-induced stomatal closure, whereas the response was marginal in conifers, ferns and *S. pallescens*. Therefore, angiosperms had the highest WUE at 600 ppm of CO_2 (Brodribb et al., 2009). These experiments led the authors to the conclusion that high CO_2-induced stomatal closure was specific to angiosperms. Further analysis of various plant species by Brodribb and McAdam (2013) supported this hypothesis as stomatal responses to changes in CO_2 concentration were absent in all the studied species of gymnosperms, ferns and lycophytes but present in all angiosperm species, when experiments were carried out in darkness. However, in the range of CO_2 concentrations from 100 to 400 ppm, all the tested species showed high CO_2-induced stomatal closure and low CO_2-induced stomatal opening in light (Brodribb and McAdam, 2013). Above-ambient CO_2 concentration (600 ppm) caused a significant decrease in stomatal conductance, when compared to ambient 400 ppm, only in angiosperms in light. The authors suggested that the universal CO_2-responsivness in light under sub-ambient transitions of CO_2 concentrations was related to photosynthesis but the unique responsiveness of angiosperms to above-ambient CO_2 concentrations was related to Ca^{2+}-dependent stomatal signalling (Brodribb and McAdam, 2013). They reasoned that the latter enabled a more efficient control of water use in angiosperms. Altogether, these studies support a hypothesis whereby high CO_2-induced stomatal closure is a distinct feature of angiosperms that enabled more efficient control of water use and thus contributed to the expansion of flowering plants.

On the other hand, there are several examples of stomatal closure in response to elevated CO_2 occurring in non-angiosperm species. For example, stomata of the fern *Phyllitis scolopendrium* have been shown to close in response to elevated CO_2 concentration (Mansfield and Willmer, 1969). More recently, stomata

in the epidermis of the lycophyte *S. uncinata* were also shown to close in response to increased CO_2 and to open in response to low CO_2 (Ruszala et al., 2011). Stomata of the mosses *P. patens* and *F. hygrometrica* also responded to elevated CO_2, suggesting that CO_2-induced stomatal closure was acquired early in the evolution of stomata as it is present already in mosses (Chater et al., 2011). This is also supported by the presence of homologues of genes important for CO_2 signalling in *A. thaliana*, such as *HT1* and carbonic anhydrases, in the genome of the moss *P. patens* (Chater et al., 2013; O'Donoghue et al., 2013). Recently, stomatal responses to CO_2 were analysed in three fern species and compared with the angiosperm *A. thaliana* (Franks and Britton-Harper, 2016). Stomata of all the studied species closed in response to elevated atmospheric CO_2 concentration (Franks and Britton-Harper, 2016), suggesting that responsiveness to above-ambient CO_2 concentration is a universal trait of vascular plants that may have been lost in some plant species.

Interestingly, when stomatal closure in response to elevated CO_2 has been observed in lycophytes and mosses, the magnitude of the response has been smaller than in angiosperms (Chater et al., 2013). Stomatal closure in response to CO_2 in ferns also appears to be slower when compared to the angiosperm *A. thaliana* (Franks and Britton-Harper, 2016). This suggests that even when stomatal response to high CO_2 is present already in mosses, lycophytes and ferns, the underlying molecular details and the consequent kinetics of the response can be different from angiosperms. Moreover, research of stomatal CO_2-responses in ferns has revealed remarkable differences between species – the stomatal conductance of some ferns even increased in response to elevated CO_2 concentration, while there was some degree of stomatal closure or no response in others (Creese et al., 2014). Thus, differences between species and experimental approach may affect the results; and interpretation of these results in the evolutionary context should be cautious. Further research is required to clarify the controversies and to unravel the origins of stomatal CO_2-responses.

2.5. Stomatal opening

Stomata open in response to light, low CO_2 concentration and increased air humidity. The molecular mechanisms of stomatal opening are less well known than those of stomatal closure. The key requirement for efficient stomatal opening is the activation of the plasma membrane H^+-ATPase OPEN STOMATA 2 (OST2/AHA1) (Assmann et al., 1985; Merlot et al., 2007) that leads to proton efflux, membrane hyperpolarization and subsequent activation of voltage-dependent K^+ uptake channels (Schroeder et al., 1987), including POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1) (Schachtman et al., 1992) and KAT2 (Pilot et al., 2001). Nitrate ions are imported into the guard cells via NITRATE TRANSPORTER 1.1 (NRT1.1) (Guo et al., 2003). Ion flux into guard cells results in water influx and increased turgor, leading to stomatal opening.

Blue light is a strong signal for stomatal opening that induces the activation of H^+-ATPase by phosphorylation of its C-terminus and subsequent binding of 14-3-3 protein (Kinoshita and Shimazaki, 1999). The photoreceptors PHOTOTROPIN 1 (PHOT1) and PHOT2 perceive the blue light signal (Kinoshita et al., 2001) and are activated by autophosphorylation (Inoue et al., 2008). The activated phototropins phosphorylate the kinase BLUE LIGHT SIGNALING 1 (BLUS1), which is required for blue light induced activation of H^+-ATPase (Takemiya et al., 2013a; Takemiya and Shimazaki, 2016). The downstream targets of BLUS1 remain unknown at present. The phosphatase PP1 has also been shown to act as a positive regulator of blue light response that is required for H^+-ATPase activation downstream of phototropins (Takemiya et al., 2006). The localization of the PP1 to cytoplasm is regulated by PP1 REGULATORY SUBUNIT2-LIKE PROTEIN 1 (PRSL1), which is also necessary for efficient blue light induced H^+-ATPase activation (Takemiya et al., 2013b).

Red light also causes stomatal opening but at higher photon flux densities than blue light. The response to red light has been proposed to be mediated via reduced CO_2 concentration due to its use for photosynthesis in mesophyll in the presence of red light (Roelfsema et al., 2002). This is supported by experiments where red light induced stomatal opening was not present in albino leaf patches that lacked chlorophyll in the mesophyll but not in guard cells (Roelfsema et al., 2006). On the other hand, red light induced stomatal opening was observed when the C_i was kept constant (Messinger et al., 2006), indicating that change in CO_2 concentration is not a prerequisite for red light response. In further support of this, suppression of photosynthesis, leading to increased C_i, did not disturb stomatal responsiveness to red light (Baroli et al., 2008). However, the HT1 kinase that is required for stomatal CO_2 responses (Hashimoto et al., 2006) is also required for red light induced stomatal opening (Matrosova et al., 2015), supporting the hypothesis that stomatal red light and CO_2 signalling are related.

The molecular mechanisms of low CO_2-induced stomatal opening remain poorly understood. Very few mutants that have impairments in low CO_2-induced stomatal opening have been isolated to date. For example, plants lacking PROTON ATPASE TRANSLOCATION CONTROL 1 (PATROL), a protein that regulates the translocation of the H^+-ATPase OST2 in the plasma membrane, have impaired low CO_2-induced stomatal opening (Hashimoto-Sugimoto et al., 2013). Low CO_2-induced stomatal opening in darkness was slower than in wild type also in plants lacking the nitrate transporter NRT1.1 (Guo et al., 2003). Plants deficient in HT1 lack both low CO_2-induced stomatal opening and high CO_2-induced stomatal closure (Hashimoto et al., 2006) and plants deficient in the carbonic anhydrases CA1 and CA4 also show strong impairment in low CO_2-induced stomatal opening (Hu et al., 2010). The role of these components in the signal transduction pathway that leads to H^+-ATPase activation and stomatal opening in response to a decrease in CO_2 concentration is unknown at this point. It is likely that light-induced and low CO_2-induced stomatal opening pathways share some components, similar to signalling cascades leading to ABA- and high CO_2-induced stomatal closure (Figure 5). Thus, a thorough char-

acterization of responses to low CO_2 in plants deficient in light-induced stomatal opening may unravel proteins that function in both pathways. A general model of stomatal opening is shown in Figure 7.

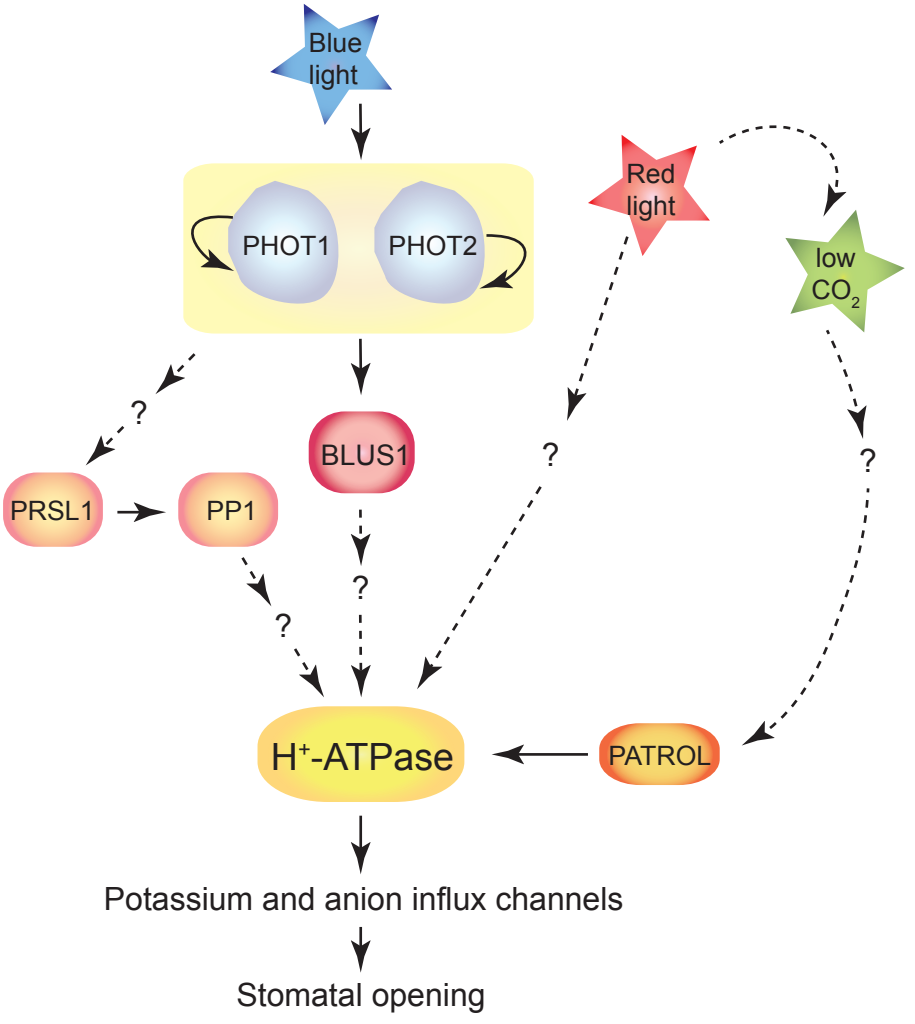

Figure 7. Stomatal opening. The activation of H^+-ATPase is required for stomatal opening. Blue light induces the autophosphorylation of the PHOT1 and 2 receptors that phosphorylate the BLUS1 kinase. Blue light induced H^+-ATPase activation occurs by yet to be characterized mechanisms involving the phosphatase PP1 as a positive regulator, the localization of which is controlled by PRSL1. PATROL controls the plasma membrane localization of the H^+-ATPase and is important for low CO_2-induced stomatal opening. The molecular mechanisms of red light and low CO_2-induced stomatal opening remain to be unravelled.

3. AIMS OF THE STUDY

The general aim of the current thesis was to elucidate the molecular mechanisms of plant stomatal regulation via analysis of several highly O_3-sensitive plant lines. The specific aims were as follows:

- identify the causes for O_3-sensitivity and O_3-tolerance in the *A. thaliana* Tenela and C24 accessions, respectively
- identify the mutations causing O_3-sensitivity and impaired stomatal responses to CO_2 in the *A. thaliana* Cvi-0 accession, and *suu* and *rcd7* mutants isolated in an O_3-sensitivity screen
- characterize the role of MPK12, HT1 and GHR1 in stomatal CO_2 signalling and propose a model for stomatal CO_2-signal transduction that incorporates the obtained knowledge
- investigate the effect of growth conditions on the stomatal responses to ABA and CO_2 in different fern species in order to gain insight into the evolution of stomatal responses

4. MATERIALS AND METHODS

The experimental procedures used in preparation of manuscripts I-V have been described in the materials and methods sections of the corresponding articles or manuscripts. The methodology used to obtain previously unpublished data presented in this thesis is described here.

4.1. Plant growth

Plants were grown in glass-covered pots in 4:2:3 peat:vermiculite:water mixture as described before (Kollist et al., 2007). Plants were grown in growth cabinets (AR-66LX, Percival Scientific, IA, USA and MCA1600, Snijders Scientific, Drogenbos, Belgia) at 70% relative air humidity, 12/12 h light/dark cycle with ~100-150 µmol m^{-2} s^{-1} light, at 23°C during day and 19°C at night. Plants were watered once a week and 24-30 days old plants were used for experiments.

4.2. Gas-exchange measurements

Gas-exchange experiments were carried out with a temperature-controlled custom-built gas exchange device analogous to the one described before (Kollist et al., 2007). Plants were allowed to stabilize in the measurement cuvettes at ~70% relative air humidity, 150 µmol m^{-2} s^{-1} light intensity and ~400 ppm CO_2. When stomatal conductance had stabilised, CO_2 concentration was either elevated to 800 ppm or reduced to 100 ppm, and change in stomatal conductance was monitored for 58 minutes.

4.3. Data analysis

Stomatal opening and closure rate were calculated by the formula $\frac{gst_{t_2}-gst_{t_1}}{t_2-t_1}$, where gst_{t_1} is pre-treatment stomatal conductance and gst_{t_2} is the stomatal conductance $t_2 - t_1$ minutes after the onset of treatment. The change in CO_2 concentration was applied at $t_1 = 0$ minutes; $t_2 = 18$ minutes for Figure 20 and $t_2 = 10$ minutes for Figure 23. All the statistical analyses were carried out with Statistica (StatSoft Inc., Tulsa, OK, USA), all effects were considered significant at α=0.05. One-way ANOVA with Tukey unequal N *post hoc* test was used to identify statistically significant differences between different groups.

5. RESULTS AND DISCUSSION

5.1. Natural variation as a tool to identify the mechanisms of ozone-sensitivity (I, II)

A. thaliana is a cosmopolite plant that grows in most of the countries in the Northern hemisphere (Hoffmann, 2002). In various environments, *A. thaliana* has evolved to develop different characteristics, yielding ecotypes – variations of the species from different conditions that have been subject to different selection pressure and genetic drift. To date, thousands of natural variants of this model angiosperm have been isolated and the genome of more than a thousand has been sequenced (Alonso-Blanco et al., 2016). The natural variation of the thale cress is a valuable resource for genetic analysis, enabling the mapping of genetic loci that are linked with particular phenotypic traits (Koornneef et al., 2004). For example, natural variation of *A. thaliana* has been used to identify the genetic determinants of cold tolerance, flowering time, salt tolerance, light responses etc. (Alonso-Blanco et al., 2009; Lefebvre et al., 2009).

Ozone, that protects life on Earth from the UV-radiation when present in the stratosphere, is an air pollutant in the troposphere. Increasing tropospheric O_3 concentrations have been shown to cause decreased yields of various crop plants (Avnery et al., 2011). Ozone exposure induces programmed cell death in plants that is manifested in lesions in plant leaves (Kangasjärvi et al., 2005). Identification of the molecular mechanisms of O_3-induced cell death would benefit to the development of O_3-tolerant cultivars of crop plants.

5.1.1. Stomatal behaviour is a cause of the different O_3-sensitivity of Tenela and C24

Various accessions of *A. thaliana* have different O_3-sensitivity (Brosché et al., 2010). Tenela (Te) is an O_3-sensitive ecotype from Finland, whereas C24 is an O_3-tolerant ecotype from Portugal (Brosché et al., 2010). Quantitative trait loci (QTL) mapping and RNA-sequencing were used to assess the underlying mechanisms of O_3-sensitivity and -tolerance in Te and C24, respectively (I). A recombinant inbred lines (RIL) population had been created from a cross between Te and C24, and had been used for mapping the QTLs involved in freezing tolerance (Meissner et al., 2013). The O_3-sensitivity of this population was assessed and an O_3-hypersensitive RIL line, CT101, was isolated (I, Fig. 1a). As stomatal malfunctioning is one possible cause of O_3-sensitivity (Brosché et al., 2010), water loss of detached leaves of the RIL lines was also determined. The O_3-induced leaf damage and water loss, indicative of stomatal function, were linked with QTLs on chromosomes 2 and 3 (I, Fig. 1b and 1c). The QTLs identified via analyses of O_3-induced leaf damage and water loss partly overlapped, indicating a central role for stomata in the O_3-sensitivity of the studied plant lines. Thus, the behaviour of the stomata in O_3-tolerant C24 and O_3-sensitive Te and CT101 in response to O_3-treatment was further characterized.

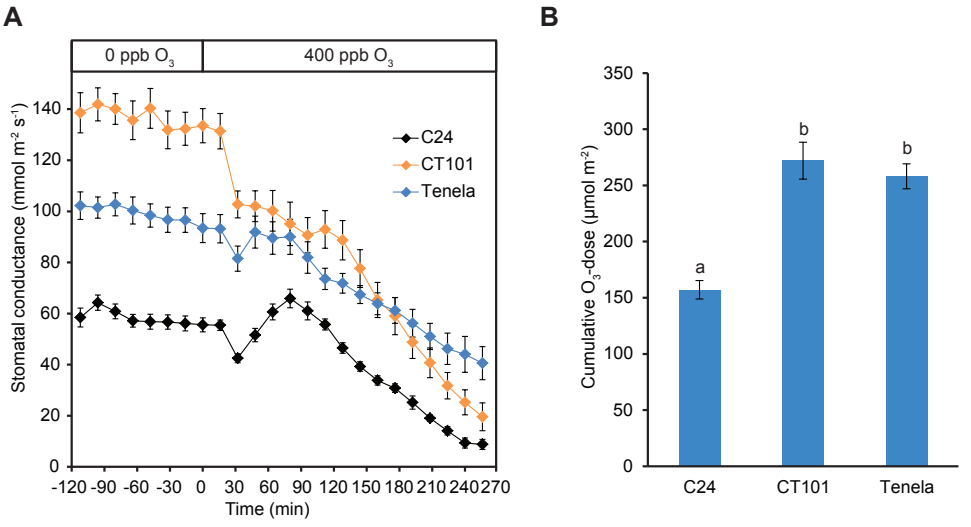

Figure 8. Stomatal response of C24, CT101 and Te to prolonged O_3 and cumulative O_3-dose received. (A) Mean ± SEM stomatal conductance, 400 ppb of O_3 was applied at time point zero (n = 10-11). (B) Mean ± SEM cumulative O_3-dose received by the studied plant lines in the experiment presented in (A). Different letters mark statistically significantly different groups (One-way ANOVA, Tukey unequal N *post hoc* test). The figure is based on data presented in I, Fig. 2.

Stomatal conductance and O_3-uptake rate as well as cumulative O_3-dose within two hours of exposure to 400 ppb of O_3 were measured in C24, Te and CT101. As expected, the stomatal conductance corresponded to O_3-sensitivity: the O_3-hypersensitive CT101 and the O_3-tolerant C24 had the highest and lowest stomatal conductance, respectively (Figure 8A). Interestingly, in response to O_3-treatment, Te and C24 displayed a two-stage stomatal response where a short-term rapid transient decrease (RTD) in stomatal conductance was followed by a steady decline upon prolonged O_3-treatment. This two-step stomatal response to prolonged O_3-exposure also occurs in the Col-0 ecotype (Vahisalu et al., 2008). The rapid O_3 response has been suggested to function as a mechanism of fast defence that reduces O_3 uptake via stomata (Kollist et al., 2007). This short-term stomatal closure may grant the plant the time necessary for the activation of defence mechanisms that would protect the plant upon prolonged O_3 exposure. The response to O_3 in the most O_3-sensitive CT101 had different kinetics – a rapid non-transient decrease in stomatal conductance was followed by a steady but sharp decline after ~1.5 hours of slow stomatal closure (Figure 8A). As CT101 suffered from the most prominent O_3-induced leaf damage upon six-hour O_3-exposure, it is conceivable that the sharper drop of stomatal conductance in CT101 when compared to C24 and Te could be due to faster initiation of cell death in CT101. However, the cumulative O_3-dose received by CT101 and Te was similar (Figure 8B), suggesting that stomatal behaviour alone is not sufficient to account for the higher O_3-sensitivity of CT101 when compared to Te.

On the other hand, the O_3-dose received by CT101 and Te was clearly higher than that in C24 (Figure 8B), indicating that the O_3-tolerance of C24 could be at least partly due to lower cumulative O_3-dose received during the O_3-exposure. Interestingly, the ecotype Est-1 isolated from Estonia also had low stomatal conductance, but was more O_3-sensitive than C24 (Brosché et al., 2010). Thus, although stomata are major entry points for O_3 and more open stomata as well as dysfunctional stomatal regulation have been associated with increased O_3-sensitivity (Kangasjärvi et al., 2005, II, III, IV), stomatal behaviour is not the sole determinant of O_3-sensitivity.

5.1.2. Different changes in gene expression in Tenela and C24 upon O_3-treatment

In order to gain further insight into O_3-induced changes in gene expression, transcriptome analysis by RNA-sequencing was carried out in O_3-treated (2h) and non-treated C24, Te and CT101. In all the lines, genes encoding proteins related with defence and stress responses, with membrane-localization and with kinase and transferase activity had increased expression levels, while genes related with energy metabolism and hormone responses had decreased expression levels (I, Fig. 3b and 3c), indicating a trade-off between defence responses and growth.

Previously, salicylic acid (SA), a stress hormone, has been shown to be present at higher levels in C24 than in other *A. thaliana* accessions (Bechtold et al., 2010), indicating constitutive activation of defence responses. Generally, higher SA levels lead to greater O_3-sensitivity (Overmyer et al., 2003; Kangasjärvi et al., 2005), but elevated SA levels have been suggested to be necessary for activation of antioxidant systems and protective function during the O_3-induced lesion formation (Kangasjärvi et al., 2005). Thus, the higher level of SA could contribute to the O_3-tolerance of C24 as this could lead to faster activation of protective defence mechanisms. In accordance with this, genes related with activation of SA and defence signalling had increased expression in untreated C24 when compared to Te and CT101 (I, Fig. 4a). Interestingly, in C24 the expression of genes related to chloroplast function was not decreased (I, Fig. 4a). This could explain the lack of growth and development deficiencies in C24 when compared to spontaneous cell death mutants that also have constitutively activated defence and SA-signalling (Bruggeman et al., 2015). The O_3-sensitivity of Te and CT101 was reduced by treatment with SA prior to O_3-exposure (I, Fig. 4b and 4c), indicating an important role for SA-signalling in O_3-tolerance. It is possible that the protective effect of SA is at least partly conveyed via stomatal regulation as SA has been shown to induce stomatal closure (Miura and Tada, 2014) and elevated endogenous SA levels have been associated with lower stomatal apertures and increased drought tolerance (Okuma et al., 2014).

In order to gain further insight into the molecular mechanisms of O_3-responses, genes with major expression differences in C24 when compared to Te

and CT101, were identified. These included for example the SA biosynthetic genes *EPSPS1 (ENOLPYRUVYL SHIKIMATE PHOSPHATE SYNTHASE 1)* and *PAL2 (PHENYLALANINE AMMONIA-LYASE 2)* and genes related to antioxidant metabolism, *PDX1.1 (PYRIDOXINE BIOSYNTHESIS 1.1)* and *PDX1.2* that participate in pyridoxine biosynthesis and *MDHAR3 (MONODEHYDRO-ASCORBATE REDUCTASE 3)* that is involved in recycling of oxidized ascorbic acid (I, Fig. 6b). The SA biosynthesis genes *EPSPS1* and *PAL2* had higher O_3-induced expression levels in Te and CT101 than in C24 (I, Fig. 6b), indicating higher *de novo* SA biosynthesis in the O_3-sensitive plant lines. Indeed, O_3-induced SA levels were higher in Te than in C24 (Brosché et al., 2010). These data suggest that in the C24 accession, further induction of SA biosynthesis is not necessary for efficient defence response. In accordance with this, whereas SA treatment protected the O_3-sensitive Te and CT101 from excessive leaf damage, it did not affect the O_3-sensitivity of the C24 ecotype (I, Fig. 4b and 4c). The pyridoxine biosynthesis genes were induced in Te and CT101 upon O_3-treatment, whereas *MDHAR3* expression was higher in C24 both in untreated and O_3-treated plants (I, Fig. 6b). This suggests that different antioxidants may be used in ROS scavenging in these plants with different O_3-sensitivity.

5.1.3. The O_3-sensitivity of Cvi-0 has stomata-related as well as unrelated causes

In addition to Tenela and C24, other thale cress accessions have been used to assess the underlying mechanisms of O_3-sensitivity. The most commonly used *A. thaliana* ecotype Col-0 from Germany is relatively tolerant to O_3-treatment, whereas Cvi-0 is a highly O_3-sensitive ecotype from Cape Verde islands (Rao and Davis, 1999). The mapping of Cvi-0 O_3-sensitivity led to the identification of two loci on the lower ends of chromosomes 2 and 3 (Brosché et al., 2010). In order to find the candidate genes that control O_3-sensitivity, two near isogenic lines (NIL) were generated by back-crossing. Col-S (Col-0 ozone sensitive), was obtained via eight generations of back-crossing Cvi-0 with Col-0 and selection of O_3-sensitive individuals. Cvi-T (Cvi-0 ozone tolerant) was generated via six generations of back-crossing Col-0 with Cvi-0 and selection of O_3-tolerant individuals. These NILs, Col-0, Cvi-0 and RILs were used to map the QTLs underlying Cvi-0 O_3-sensitivity to a 90 kb region on chromosome 2 and ~18 Mbp region on chromosome 3. The QTLs were isolated by back-crossing Col-S with Col-0, yielding the NILs Col-S2 and Col-S3. Both of the obtained NILs were less O_3-sensitive than Col-S (Figure 9A), indicating that both these QTLs contribute additively to the O_3-sensitivity of Col-S and Cvi-0. Interestingly, only the Col-S2, but not Col-S3, had higher stomatal conductance than Col-0 (Figure 9B). These data suggested that the QTL on chromosome 2 affected O_3-sensitivity via stomatal conductance and/or regulation, whereas that on chromosome 3 regulated O_3-sensitivity via a different mechanism, potentially involving SA-, antioxidant- or ROS-signalling. These data further corroborate the strong rela-

tionship of stomatal regulation and O_3-sensitivity, while they also show that in addition to more open stomata, there are other causes for increased O_3-induced leaf damage.

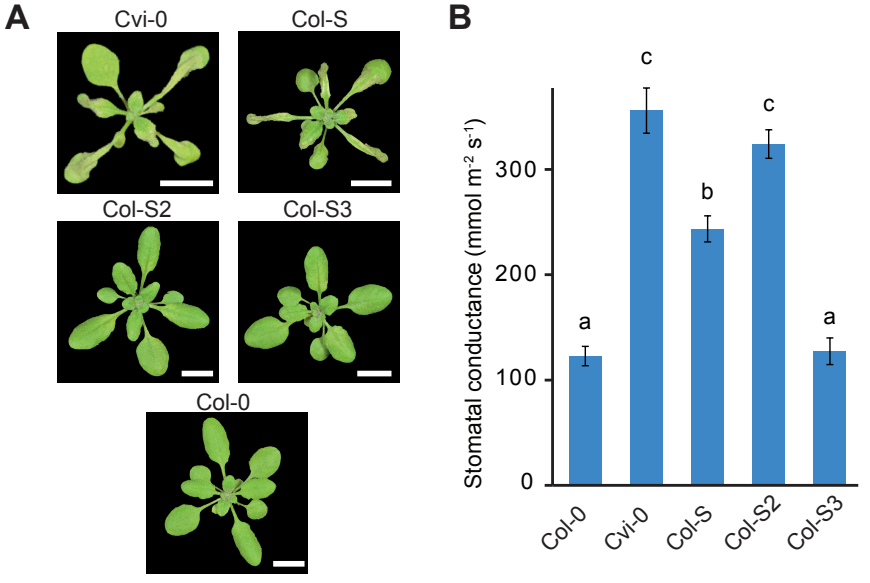

Figure 9. O_3-sensitivity and stomatal conductance in Cvi-0 and NILs. (A) Leaf damage induced by 6 h of O_3 exposure (350 ppb). Scale bars are 1 cm. (B) Mean ± SEM stomatal conductance of Cvi-0 and NILs, different letters mark statistically significantly different groups (One-way ANOVA, Tukey unequal N *post hoc* test, n = 7-12). The figure is modified from II, Fig. 1a and 1b.

5.2. From ozone sensitivity to stomatal CO_2 signalling (II, III, IV)

Stomata are the major entry gateways for O_3 and stomatal regulation is tightly linked with O_3-sensitivity (Brosché et al., 2010, I, II, Figure 9). This enables the use of O_3 as a tool to identify mutants with deficient stomatal regulation as stomatal malfunctioning can be visualised through O_3-induced leaf damage (Kangasjärvi et al., 2005; Overmyer et al., 2008). The identification of SLAC1, the central guard cell S-type anion channel, via an O_3-sensitivity screen, is a beautiful example of the benefits of this approach in the study of guard cell signalling pathways (Vahisalu et al., 2008). Analysis of several O_3-sensitive mutants and mapping of the stomata-related QTL involved in the O_3-sensitivity of the Cvi-0 ecotype led to the identification of a core guard cell CO_2 signalling pathway, which is the focus of this thesis.

5.2.1. MPK12 is a component of stomatal CO_2 signalling

We set out to identify the stomata-related aspect of O_3-sensitivity in Cvi-0, Col-S and Col-S2 highlighted above. Within the candidate region of chromosome 2, there was one gene encoding a mitogen-activated protein kinase that was strongly and preferentially expressed in the guard cells – *MPK12* (Jammes et al., 2009). The Cvi-0 *MPK12* had a point mutation leading to glycine to arginine substitution at position 53 of the protein (MPK12 G53R). Thus, we considered the mutation in *MPK12* as a likely candidate for the O_3-sensitivity and abnormally high stomatal conductance of Col-S2. At the same time, we isolated a T-DNA knock-out line for a calcium-sensing receptor (CAS), *cas-2*, that had high stomatal conductance and impaired CO_2-induced stomatal closure that were not present in *cas-1* and *cas-3* plants (II; Fig. 1c), indicating an additional mutation in *cas-2* plants. Back-crossing of *cas-2* to Col-0 revealed that the T-DNA insert in *CAS* was not related to the impairment in CO_2-sensitivity, whereas another line without the T-DNA in *CAS* but with increased stomatal conductance and impaired response to high CO_2 concentration, was isolated and named *cis* (*CO_2-insensitive*). The stomata of both *cis* and Col-S2 closed more slowly than those of Col-0 in response to increased CO_2 concentration (II, Fig. 1d). The mapping of the locus responsible for the impaired CO_2-response in *cis* identified a deletion of *MPK12* as well as *BYPASS2*, a neighbouring gene (II, Fig. 1e). Thus, *cis* was renamed *mpk12-4*. The identification of a deletion spanning two genes in an established T-DNA line for another gene highlights the importance of using more than one T-DNA knock-out line in assessing the function of a gene of interest. If multiple knock-out lines are not available then back-crossing the T-DNA line under investigation would be a useful strategy to avoid arriving at false conclusions on the function of the gene of interest. For example, previous analyses of plant lines with a T-DNA insertion in *MPK12* had concluded that deletion of *MPK12* would be lethal (Jammes et al., 2009; Lee et al., 2009a). However, the normal growth and development of the *cis* plants (II, Fig. 1a) indicates that additional mutations or T-DNA insertions in essential genes could have been the cause of lethality in these plant lines.

In order to gain further insight into the role of MPK12 in stomatal regulation, we characterized the stomatal behaviour of Col-S2 harbouring the point mutation corresponding to G53R substitution in MPK12, *mpk12-4* that completely lacks *MPK12* and a T-DNA knock-out line for *MPK12*, *mpk12-3*. All these plants had increased day-time stomatal conductance, leading to decreased WUE (II, Fig. 2a and 2b). To verify that the increased stomatal conductance in the tested mutants was due to the dysfunction or lack of MPK12, complementation lines were generated. The high stomatal conductance of Cvi-0 and Col-S2 was reduced by the expression of wild-type MPK12 (II, Fig. 2c and 2d), and the increased stomatal conductance of *mpk12-4* was complemented by the expression of wild-type MPK12, but not MPK12 G53R (II, Fig. 2e). These data indicate that loss of MPK12 function is the underlying cause of increased stomatal conductance and decreased WUE in Col-S2, *mpk12-3* and *mpk12-4*. Further-

more, the point mutation leading to G53R substitution in MPK12 is sufficient to cause the impairments. Similarly, the same mutation in the Cvi-0 *MPK12* was identified as the underlying cause of the decreased WUE in the Cvi-0 ecotype (Des Marais et al., 2014).

As the stomatal closure in response to increased CO_2 concentration was impaired in Col-S2 and *mpk12-4* (II, Fig. 1c and 1d), we assessed the stomatal behaviour in response to various other treatments in MPK12-deficient plants. Stomatal closure in response to high CO_2 concentration was moderately impaired in Col-S2 and *mpk12-4* (Figure 10A), whereas stomatal opening in response to low CO_2 concentration was severely impaired in Col-S2, *mpk12-3* and *mpk12-4* (Figure 10B). These data indicate a more prominent role for MPK12 in low CO_2-induced stomatal opening than in high CO_2-induced stomatal closure, albeit the kinase apparently contributes to both of these processes. As guard cell CO_2 signalling is tightly linked with ABA signalling (Xue et al., 2011; Merilo et al., 2013; Chater et al., 2015) and *mpk9-1 mpk12-1* double mutants as well as plants with simultaneously silenced *MPK9* and *MPK12* expression had previously been shown to have impaired stomatal responses to ABA (Jammes et al., 2009), we assessed ABA-induced stomatal closure and ABA-induced inhibition of light-induced stomatal opening in Col-S2, *mpk12-3* and *mpk12-4*. Interestingly, ABA-induced inhibition of light-induced stomatal opening was impaired in all the studied MPK12-deficient plant lines (II, Fig. 3b), whereas ABA-induced stomatal closure was present in all the lines (II, Fig. 3d). Similar results were obtained for the NIL line containing an introgression harbouring *MPK12* from the Cvi-0 genome in the L*er* background, where stomata closed in response to ABA-treatment but also opened in light in the presence of ABA (Des Marais et al., 2014), indicating impaired ABA-induced inhibition of stomatal opening. Plants deficient in carbonic anhydrases have impaired stomatal responses to CO_2 (Hu et al., 2010) and bicarbonate has been shown to activate the S-type anion channel currents in guard cell protoplasts (Hu et al., 2010; Xue et al., 2011) as well as enhance SLAC1 activation in oocytes (Tian et al., 2015; Wang et al., 2016), indicating that bicarbonate is a mediator of guard cell CO_2 signalling. Thus we assessed the effect of bicarbonate on S-type anion channel activity in the guard cell protoplasts of Col-S2 and *mpk12-4.* Bicarbonate-induced S-type anion channel activation was impaired in both Col-S2 and *mpk12-4* (II, Fig. 3e), suggesting that intact MPK12 is required for efficient anion channel activation in response to increased CO_2 concentration. Together these data indicate that MPK12 has a prominent role in the regulation of guard cell CO_2 signalling.

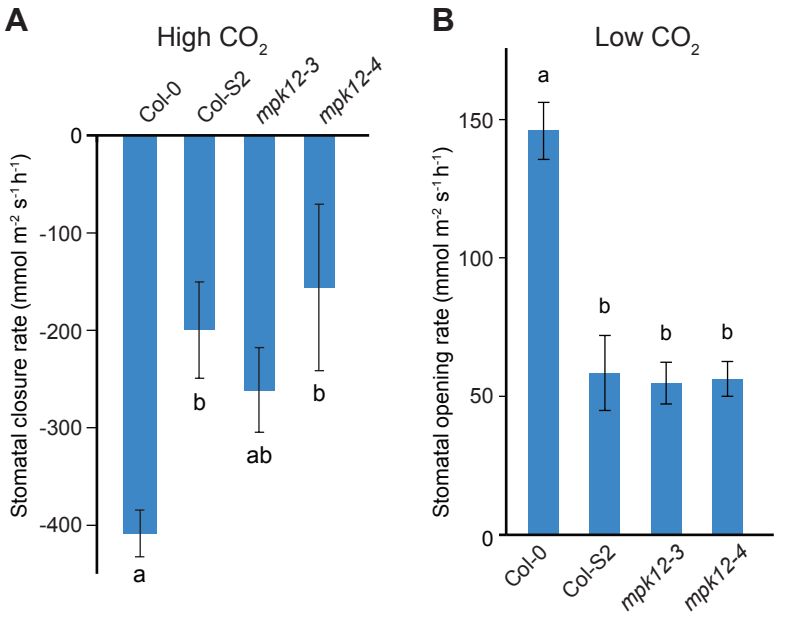

Figure 10. CO_2-responses of the Col-S2 and *mpk12* mutants. (A) Mean ± SEM stomatal closure rate in response to elevation of CO_2 concentration from 400 ppm to 800 ppm. (B) Mean ± SEM stomatal opening rate in response to reduction of CO_2 concentration from 400 ppm to 100 ppm. In (A) and (B), n = 12-13 and different letters mark statistically significantly different groups (One-way ANOVA, Tukey unequal N *post hoc* test). The figure is modified from II, Fig. 3a and 3c.

5.2.2. A novel dominant mutation in HT1 – a component of stomatal CO_2 signalling

An O_3-sensitivity screen was carried out to identify genes involved in ROS-signalling and programmed cell death (Overmyer et al., 2000). As O_3 enters the leaves through stomata, several plants with deficient stomatal control were isolated when selecting plants with increased O_3-induced leaf damage (Kangasjärvi et al., 2005; Overmyer et al., 2008; Vahisalu et al., 2008). One of the isolated plant lines, *rcd7* (*radical-induced cell death 7*) suffered major O_3-induced leaf damage and had increased stomatal conductance. Mapping of the O_3-sensitivity of the *rcd7* plant line revealed that the original line had two mutations related with the O_3-sensitivity and stomatal phenotypes (III, IV). The two mutations were separated by back-crossing the *rcd7* mutant with the Col-0 wild-type. The subsequently obtained mutant lines were named *rcd7* and *suu*, which means "mouth" in Estonian and Finnish, due to the very high stomatal conductance of the latter plants (III, Fig. 1b). The identification of the mutation in *rcd7* (IV) will be described in the following subchapter.

The *suu* plants were highly O_3-sensitive (Figure 11A and 11B) and had very open stomata (Figure 11C) as well as increased fresh weight loss from detached

leaves (III, Fig. 1c). The mutation underlying the phenotypes was dominant, as indicated by the high fresh weight loss of plants from the F1 generation of the cross between *suu* and Col-0 wild-type (III, Fig. 1c). The *suu* plants lacked stomatal closure response to elevated CO_2-concentration (III, Fig. 2a) and thus fresh weight loss in combination with stomatal closure in response to high CO_2 were used for mapping the mutation in *suu*. An F2 mapping population generated from the cross of *suu* and L*er* was used to identify 34 individuals with wild-type fresh weight loss and normal CO_2-induced stomatal closure (III, Supplemental Fig. 1a). Subsequent rough mapping by using 21 SSLP (simple sequence length polymorphism) markers led to a candidate list consisting of 66 genes that included *HT1*. As HT1 is a central regulator of stomatal CO_2-responses (Hashimoto et al., 2006), this was the first candidate gene to be studied in detail.

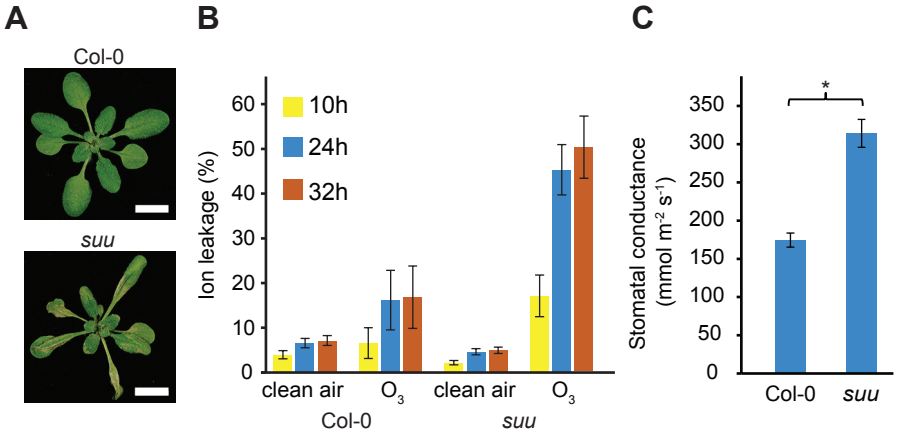

Figure 11. The O_3-sensitivity and stomatal conductance of *suu*. (A) Leaf damage induced by 6 h of O_3 exposure (350 ppb). Scale bars are 1 cm. (B) Mean ± SD ion leakage from O_3-treated (350 ppb for 6 h) and control (clean air) plants 10, 24, and 32 h after the beginning of O_3 exposure (n = 4). O_3-treated *suu* plants were statistically significantly different from the wild type in all time points (One-way ANOVA with Tukey HSD *post hoc* test). (C) Mean ± SEM stomatal conductance; asterisk indicates statistically significant difference (Student's t-test). The figure is modified from III, Fig. 1a, 1b and 1d.

The *HT1* gene in *suu* plants had a point mutation leading to an alanine-to-valine substitution at position 109 of the HT1 protein (A109V). The expression of HT1 A109V controlled by the native promoter [ProHT1:HT1 A109V] caused severe O_3-sensitivity (III, Fig. 1d), increased stomatal conductance and loss of stomatal responsiveness to CO_2 in the Col-0 wild-type (III, Fig. 2a and 2b), confirming that the point mutation in *HT1* was the cause of the high O_3-sensitivity and stomatal malfunction in *suu*. Thus, *suu* was renamed *ht1-8D*, taking into account the nomenclature from Hashimoto-Sugimoto et al., 2016. We characterized the

stomatal responses of *ht1-8D*, Col-0 with ProHT1:HT1 A109V and *ht1-2*, where HT1 lacks 14 amino acids from the kinase domain (Hashimoto et al., 2006). Interestingly, while both *ht1-8D* and *ht1-2* completely lacked high CO_2-induced stomatal closure (Figure 12A) as well as low CO_2-induced stomatal opening (Figure 12B), they had opposite stomatal conductance – approximately twice as high and half as high as in Col-0 in *ht1-8D* and *ht1-2*, respectively. Recently, similar stomatal behaviour was documented in *ht1-2* and *ht1-3*, the latter harbouring another dominant mutation in *HT1* that leads to arginine-to-lysine substitution at position 102 of the HT1 protein (R102K) (Hashimoto-Sugimoto et al., 2016). Moreover, four new recessive HT1 lines that had mutations in the regions required for HT1 kinase activity, all had high leaf temperature at ambient and high CO_2 concentration, indicating low stomatal conductance (Hashimoto-Sugimoto et al., 2016). Both HT1 A109V and HT1 R102K retain kinase activity *in vitro* (III, Fig. 4a, Hashimoto-Sugimoto et al., 2016). Thus, it is conceivable that HT1 has a dual role in stomatal regulation – one executed via its kinase activity, which is impaired in the recessive *ht1* mutants, leading to constitutive activation of high CO_2 signalling, and another via structure or interaction that is hindered in plants expressing the HT1 A109V or HT1 R102K. The two dominant mutations in the *HT1* gene are located close to each other, indicating that the region harbouring R102 and A109 is important for HT1 function, e.g. via protein-protein interactions, and that the two dominant substitutions are likely to impair the role of HT1 in a similar manner.

Interestingly, efficient stomatal closure in response to ABA-treatment occurred in *ht1-8D* and *ht1-2* (III, Fig. 2c) as well as in *ht1-3* (Hashimoto-Sugimoto et al., 2016), further indicating that HT1 is a CO_2-specific regulator of stomatal signalling that is not required for ABA-responses. To date, very few other CO_2-specific stomatal regulators have been identified, including the carbonic anhydrases (Hu et al., 2010) and the more controversial RHC1 (Tian et al., 2015), whereas most identified proteins required for efficient CO_2-induced stomatal closure are also essential for ABA response. The latter include for example SLAC1 (Negi et al., 2008; Vahisalu et al., 2008), OST1 (Xue et al., 2011; Merilo et al., 2013), PP2Cs (Merilo et al., 2013) and ABA receptors (Merilo et al., 2013; Chater et al., 2015). Thus, full characterization of the role of the HT1 kinase as well as identification of its substrates and regulators is important to understand stomatal responses to CO_2 and the mutant versions of HT1 can be a useful tool in this process.

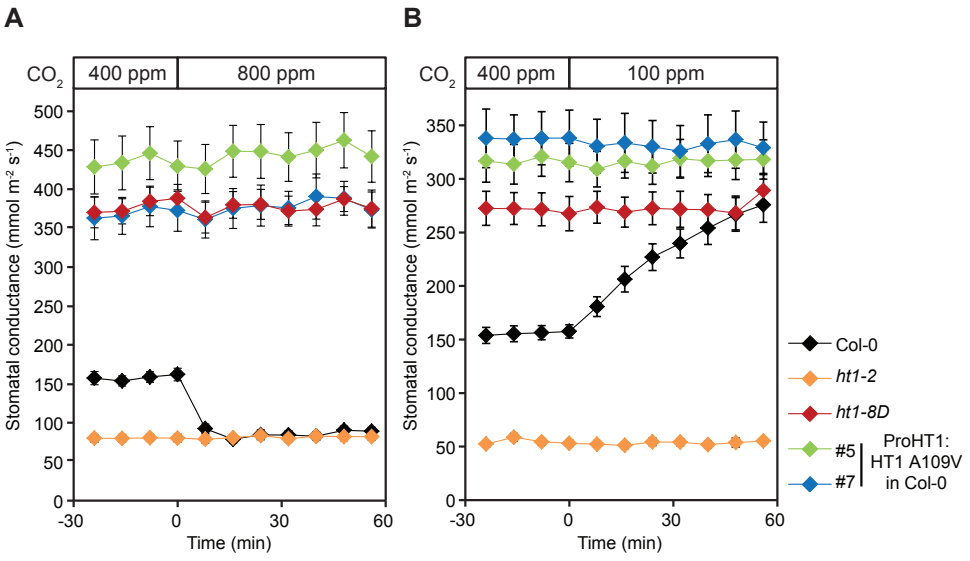

Figure 12. Stomatal responses to CO_2 in *ht1* mutants and wild-type plants expressing HT1 A109V. (A) Mean ± SEM stomatal conductance, CO_2 concentration was elevated from 400 ppm to 800 ppm at time point zero (n = 16-21). (B) Mean ± SEM stomatal conductance, CO_2 concentration was reduced from 400 ppm to 100 ppm at time point zero (n = 12-14). ProHT1::HT1 A109V in Col-0 denote plants where the expression of HT1 A109V is driven by the HT1 promoter. The figure is modified from III, Fig. 2a and 2b.

5.2.3. GHR1 is involved in CO_2-induced stomatal closure

The O_3-sensitive *rcd7* plant line that had been separated from the line with *suu* mutation (see above) was characterized and mapped (IV). The mutation caused moderate O_3-sensitvity (IV, Fig. 1a, 1b and 1c) and slightly increased stomatal conductance (IV, Fig. 1d) suggesting that stomatal function in this mutant could be studied further. The *rcd7* mutation was inherited in a recessive manner and was mapped to a region in the lower arm of chromosome 4 (IV, Fig. 1e). Ten candidate genes in this region were selected for further analysis based on the genome re-sequencing of the *rcd7* mutant. Homozygous insertion mutants for these genes were analysed for O_3-sensitivity and among them, a T-DNA insertion line in *GHR1* showed severe O_3-induced leaf damage (IV, Supplemental Fig. 1, line SALK_031493C). Three other plant lines harbouring an insertion in *GHR1* were also very O_3-sensitive (IV, Fig. 2a and 2b) and plants from the F1 cross between *rcd7* and a strong T-DNA allele (GK_760C07) showed *rcd7*-like O_3-sensitivity (IV, Fig. 2c and 2d), indicating that the mutation underlying the phenotypes of *rcd7* was caused by the alanine-to-threonine substitution (A618T) at position 618 in GHR1 – an LRR-RLK (leucine-rich repeat receptor-like kinase) involved in stomatal responses to ROS and ABA (Hua et al., 2012).

As a screen for O_3-sensitivity had been established as a good method for isolation of mutants with deficient stomatal regulation (Kangasjärvi et al., 2005;

Vahisalu et al., 2008, III, IV), a new screen was carried out to find additional regulators of stomatal closure as well as ROS-signalling. From this screen, three new alleles of *RCD7/GHR1* that caused O_3-sensitivity (IV, Fig. 3b), were identified (*rcd7-2*, *rcd7-3*, *rcd7-4*). All the newly identified mutations were located in the LRR-domains of RCD7/GHR1 (IV, Fig. 3a). Interestingly, two of these lines (*rcd7-3* and *rcd7-4*) had increased stomatal conductance similar to the T-DNA knock-out line of *GHR1* (GK_760C07), whereas the *rcd7-2* line had stomatal conductance similar to wild-type plants (IV, Fig. 3c). These results further support the notion that high stomatal conductance is not an essential prerequisite for O_3-sensitivity. Intriguingly, the response to a short-term O_3-pulse was impaired in *rcd7-2* that had normal stomatal conductance as well as in the other lines with high stomatal conductance (IV, Fig. 3c and 3d), suggesting that steady-state stomatal conductance and stomatal responsiveness to O_3 are both regulated by GHR1, but not via the same mechanism.

Thus, two independent O_3-sensitivity screens led to identification of GHR1 as a regulator of stomatal conductance and O_3-responses. GHR1, a receptor-like kinase that is required for stomatal responses to ABA and hydrogen peroxide, was shown to activate the SLAC1 anion channel in oocytes and phosphorylate the SLAC1 N-terminus in protoplast pull-down assays (Hua et al., 2012). Therefore, we assessed stomatal responses to various stimuli in an O_3-sensitive *GHR1* T-DNA insertion line *ghr1-3* (Figure 13A and 13B) in comparison with plants lacking the central SLAC1-activating kinase OST1 (*ost1-3*) to elaborate the role of GHR1 in guard cell signal transduction. Both *ghr1-3* and *ost1-3* completely lacked stomatal closure in response to treatment with O_3 and ABA (IV, Fig. 4a and 4c), which is in line with the previously described hydrogen peroxide and ABA-insensitivity of a *ghr1* mutant (Hua et al., 2012). However, GHR1 is not only involved in ABA- and ROS-signalling but has an important role in stomatal responsiveness to elevated CO_2 (Figure 13C) as well as darkness (IV, Fig. 4f). Indeed, *ghr1-3* plants were completely insensitive to increased CO_2-concentration and darkness, whereas *ost1-3* plants responded by steady, albeit slow, stomatal closure in both conditions. These data suggest that in the absence of OST1, GHR1 can compensate for its function in CO_2- and darkness-induced stomatal closure, whereas the opposite is not true. On the other hand, stomatal closure in response to a decrease in relative air humidity was absent in *ost1-3* but present in *ghr1-3* (IV, Fig. 4d and 4e), indicating that stomatal response to an increase in vapour pressure deficit is dependent on OST1, rather than GHR1. Importantly, GHR1 is not only required for efficient response to artificial day-time darkness, but also for normal circadian stomatal behaviour. The stomatal conductance of *ghr1-3* plants did not change markedly throughout the 24-hour period, whereas stomata in *ost1-3* opened even more than in wild-type plants in the morning and closed fully at night, although the closure was slower than in the wild-type plants (IV, Fig. 4g). These data indicate an important role for GHR1 in the control of plant WUE as lack of stomatal closure during night-time is an expensive deficiency leading to waste of water. Thus, GHR1 is a central regulator of stomatal responses that has a prominent role in CO_2 signalling as well as responses to both natural and artificial darkness.

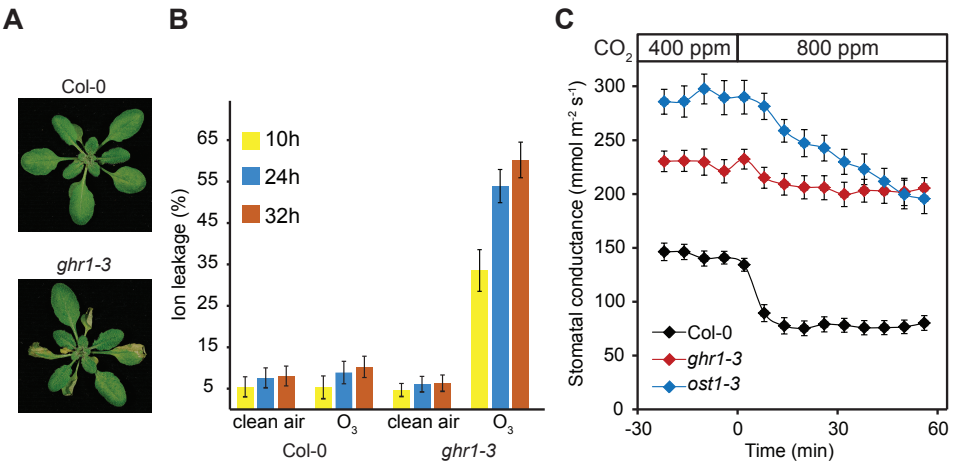

Figure 13. The O_3-sensitivity and stomatal response to elevated CO_2 of the *ghr1-3* mutant. (A) Leaf damage induced by 6 h of O_3 exposure (350 ppb). (B) Mean ± SD ion leakage from O_3-treated (350 ppb for 6 h) and control (clean air) plants 10, 24, and 32 h after the beginning of O_3 exposure (n = 4). O_3-treated *ghr1-3* plants were statistically significantly different from the wild type in all time points (One-way ANOVA with Tukey HSD *post hoc* test). (C) Mean ± SEM stomatal conductance; CO_2 concentration was elevated from 400 ppm to 800 ppm at time point zero (n = 9-17). The figure is modified from IV, Fig. 2a, 2b and 4b.

5.3. Guard cell CO_2 signal transduction (II, III, IV, unpublished data)

Analysis of the O_3-sensitivity of the Cvi-0 ecotype and characterization of a mutant line with impaired CO_2-induced stomatal closure led to the identification of MPK12 as a regulator of stomatal CO_2-responses (II). O_3-sensitivity screens led to the discovery of a dominant mutation in HT1 (III), a central CO_2-specific kinase, as well as several mutations in GHR1, a protein that contributes to stomatal responses to several factors, including elevated CO_2 (IV). Further research on the roles of these proteins in guard cell CO_2-sigalling led to the identification of a core regulatory pathway that is required for high CO_2-induced stomatal closure.

5.3.1. MPK12 and MPK4 interact with HT1 and regulate its activity

In order to find proteins that interact with HT1, we carried out a split-ubiquitin yeast two-hybrid (Y2H) screen. A mitogen-activated protein kinase, MPK4, was identified as an interactor of HT1. The silencing of MPK4 and MPK4-like in *N. tabacum* (NtMPK4 and NtMPK4L) resulted in increased stomatal aperture and O_3-sensitivity (Gomi et al., 2005; Yanagawa et al., 2016) and NtMPK4 was shown to participate in CO_2 signalling (Marten et al., 2008), thus MPK4 was a

promising candidate as a regulator of CO_2-responses in *A. thaliana* as well. The *mpk4* mutants are dwarfed, therefore we used *mpk4 NahG* plants (Petersen et al., 2000) for gas-exchange analysis as the expression of NahG enables the degradation of salicylic acid and thus rescues the size of *mpk4* plants. Stomatal responses of the plants expressing a constitutively active version of MPK4, MPK4 D198G/E202A (Berriri et al., 2012), were also analysed. However, both efficient high CO_2-induced stomatal closure and low CO_2-induced stomatal opening were present in *mpk4 NahG* as well as in plants with the constitutively active MPK4 (III, Supplemental Fig. 4). These results indicated that either MPK4 was not essential for CO_2 signalling in *A. thaliana* or the function of MPK4 could be compensated by other proteins.

Both MPK4 and MPK12 belong to the same subgroup of MPKs (Ichimura et al., 2002) and have high sequence similarity. Thus, a pair-wise split-ubiquitin Y2H assay was carried out to test if HT1 interacts with MPK12. Indeed, HT1 interacted with both MPK4 and MPK12 in yeast (II, Fig. 4a and 4b, III, Supplemental Fig. 5), whereas the interaction was impaired both in MPK12 G53R (II, Fig. 4a and 4b) and HT1 A109V (III, Supplemental Fig. 5), indicating that G53 in MPK12 identified in Cvi-0 and A109 in HT1 identified in *suu* are required for the interaction of the two proteins. In bimolecular fluorescence complementation assays, both HT1 and HT1 A109V interacted with MPK12 (III, Fig. 3b and 3c) and both MPK12 and MPK12 G53R interacted with HT1 (II, Fig. 4c and 4d). However, the interaction of the mutant versions of HT1 and MPK12 with the wild-type versions of MPK12 and HT1, respectively, was significantly weaker (II, Fig. 4c and 4d, III, Fig. 3b and 3c). Similarly, the interaction of wild-type HT1 with MPK4 was stronger than for HT1 A109V (III, Fig. 3c). These data indicate that both MPK12 and MPK4 interact with HT1 and thus likely regulate stomatal CO_2-responses via HT1. As the stomatal conductance of the *mpk12-4 ht1-2* double mutant was as low as in *ht1-2* (II, Fig. 4f), we concluded that *HT1* is epistatic to *MPK12* and thus MPK12 is expected to function upstream of HT1 in guard cell signal transduction. This is interesting as HT1 is known as a Raf-like MAPKKK (Ichimura et al., 2002; Hashimoto-Sugimoto et al., 2016) and in a classical MAP kinase signalling pathway, an MAPKKK regulates an MKK (MAP kinase kinase) that in turn regulates an MPK (Colcombet and Hirt, 2008). However, a recent analysis of the *A. thaliana* kinome suggests that the Raf-like MAPKKKs are mis-annotated and do not actually function in MAP kinase signalling cascades (Zulawski et al., 2014), indicating that the role of HT1 is expected to be different from classical MAPKKKs.

HT1 and HT1 A109V were active *in vitro*, exhibiting both autophosphorylation as well as phosphorylation of the artificial substrate casein (III, Fig. 4a). MPK12 displayed moderate autophosphorylation activity *in vitro* that was lost in MPK12 G53R (III, Fig. 5c), indicating that G53 is required for MPK12 kinase activity. However, neither MPK12 nor the hyperactive MPK12 Y122C could phosphorylate kinase-inactive HT1 K113M *in vitro* (III, Fig. 5c). We tested if MPK12 could affect HT1 activity by *in vitro* inhibition assays. Wild-type MPK12 inhibited HT1 autophosphorylation as well as phosphorylation of casein

by HT1, whereas inhibition of HT1 A109V by MPK12 (III, Fig. 4b, 4c and 4d; Figure 14A) and HT1 by MPK12 G53R (II; Fig. 5a and 5b; Figure 14B) was clearly less efficient. These data indicate that MPK12 acts as an inhibitor of the HT1 kinase in stomatal CO_2-signal transduction and A109 in HT1 as well as G53 in MPK12 are crucial for efficient inhibition of HT1 activity. Moreover, MPK4 also inhibited HT1 autophosphorylation (II, Fig. 6a, III, Fig. 6d), as well as phosphorylation of casein by HT1 (II, Fig. 6a), whereas the inhibition was clearly weaker for MPK4 G55R (II, Fig. 6a). These results indicate that in addition to MPK12, MPK4 also regulates HT1 activity and the conserved glycine residue at position 53 in MPK12 and 55 in MPK4 is required for the inhibition of HT1 activity by these MPKs. As the glycine-to-arginine substitution in this position causes a protrusion of the arginine side chain in the MPK structure (II, Fig. 6b and 6c), it is conceivable that the interaction of the MPKs with HT1 is hindered, leading to severely reduced inhibition of HT1 activity by the mutant versions of the MPKs.

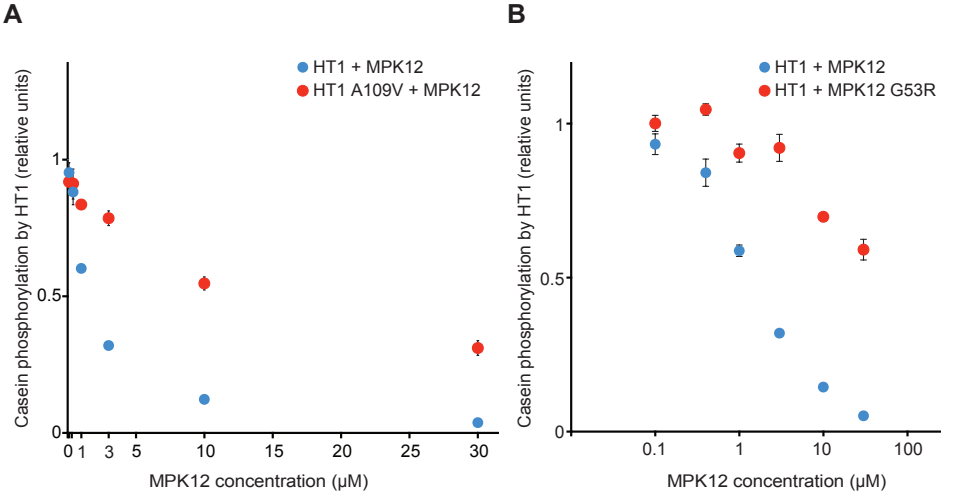

Figure 14. MPK12 inhibits HT1 activity and the inhibition is reduced by A109V substitution in HT1 or G53R substitution in MPK12. (A) Mean ± SEM relative casein phosphorylation by HT1 or HT1 A109V in the presence of different MPK12 concentrations (n = 3). (B) Mean ± SEM relative casein phosphorylation by HT1 in the presence of different concentrations of MPK12 or MPK12 G53R (n = 3). Modified from II, Fig. 5b and III, Fig. 4d.

5.3.2. The role of the HT1 kinase in CO_2 signal transduction

High CO_2-induced stomatal closure was completely absent in the *ht1-8D* and *ht1-2* mutants (Figure 12A) and slower than in wild-type plants in the *mpk12-4* mutants (Figure 10A). As the activation of SLAC1 anion channel is required for efficient CO_2-induced stomatal closure (Vahisalu et al., 2008), we tested if

HT1 affected SLAC1 activation in the *X. laevis* oocytes, a system that is often used to characterize the function of heterologous ion channels and their regulators. We assayed the effect of HT1 on SLAC1 activation by OST1 and GHR1 in oocytes, as OST1 is a key activator of SLAC1 (Geiger et al., 2009; Lee et al., 2009b; Vahisalu et al., 2010) and GHR1 is a central regulator of stomatal closure in response to elevated CO_2 concentration (Figure 13C). SLAC1 was activated by OST1 (III, Fig. 5a) and GHR1 (III, Fig. 5b) in *X. laevis* oocytes as described previously (Geiger et al., 2009; Hua et al., 2012). The activation of SLAC1 by both OST1 and GHR1 was inhibited by wild-type HT1, as well as HT1 A109V and the kinase-inactive HT1 K113W (III, Fig. 5a and 5b), suggesting that HT1 functions as an inhibitor of SLAC1-activation also in guard cells and neither the A109 nor HT1 kinase activity are required for the inhibition. HT1-induced inhibition of SLAC1 activation both by OST1 and GHR1 raises the question of the direct target of HT1. Whether the inhibition occurs at the level of SLAC1 or are both GHR1 and OST1 regulated by HT1, remains to be studied in the future. As several CPKs have also been shown to activate SLAC1 in oocytes (Geiger et al., 2010; Brandt et al., 2012; Scherzer et al., 2012; Brandt et al., 2015), the effect of HT1 on the activation of SLAC1 by CPKs could help to clarify this point.

Although the kinase activity of HT1 was not crucial for the inhibition of SLAC1 activation in oocytes (III, Fig 5a and 5b), it likely has a role in the function of HT1 *in planta*. The targets of HT1 have remained elusive to date, but as HT1 inhibited OST1- and GHR1-induced SLAC1 activation in oocytes, we tested if the HT1 kinase could phosphorylate SLAC1, GHR1 or OST1 *in vitro*. GHR1 and the N-terminus of SLAC1 were phosphorylated by HT1 *in vitro* (Figure 15). HT1 has been reported to phosphorylate OST1 before (Tian et al., 2015). However, in those assays a version of HT1 that lacked 45 N-terminal amino acids was used. In accordance with this, we found that the short version of HT1 weakly phosphorylated the kinase-inactive OST1 K50N (III, Supplemental Fig. 9b) and the full-length HT1 phosphorylated OST1 K50N either weakly (III, Supplemental Fig. 9a) or not at all (III, Fig. 6a). Thus, SLAC1 and GHR1, and potentially also OST1, are candidate targets of the HT1 kinase. Whether the phosphorylation of these proteins by HT1 occurs also *in planta*, remains to be studied in the future. As GHR1 is more important for CO_2-induced stomatal closure than OST1 (Figure 13C), it can be considered a likely substrate of the HT1 kinase. The phosphorylation of GHR1 by HT1, but not by HT1 A109V, was inhibited by both MPK12 and MPK4 *in vitro* (III, Fig. 6b and 6d), suggesting that both MPK12 and MPK4 may hinder the phosphorylation of GHR1 by HT1 also in *planta*.

The ability of the kinase-inactive HT1 K113W to inhibit SLAC1 activation by both OST1 and GHR1 in oocytes similar to the wild-type HT1 is puzzling. As HT1 can phosphorylate GHR1, SLAC1 and possibly OST1 *in vitro* (III, Fig. 6a and Supplemental Fig. 9a), it is appealing to hypothesize that the regulation of SLAC1 activation requires HT1 kinase activity. This would explain the phenotypes of the plant lines harbouring recessive mutations in *HT1* that disrupt the

gene or result in the loss of the kinase activity of the corresponding protein, e.g. as in *ht1-2* (Hashimoto et al., 2006). If HT1 kinase activity was required for the inhibition of SLAC1 activation, it would not be inhibited by the HT1 in *ht1-2* and other plant lines with kinase-inactive HT1. This would lead to constitutive activation of SLAC1 and result in low stomatal conductance, as observed in *ht1-2* (Hashimoto et al., 2006; Figure 12). Moreover, this would cause a loss of the responsiveness to changes in CO_2-concentration as the constitutive activation of SLAC1 could not be turned off by the HT1 kinase. Thus, the requirement of HT1 kinase activity for the regulation of SLAC1 activation would explain the phenotypes of *ht1-2* and other recessive *ht1* mutants (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016), but would contradict the data obtained from oocytes (III, Fig. 5). While the *X. laevis* oocyte system can be used to study various properties and regulators of ion channels, it is a heterologous as well as a robust system, which may not always be sufficient to identify quantitative differences. For example, it is conceivable that there is a difference in the efficiency of inhibition of SLAC1 activation by wild-type HT1 and the kinase-inactive HT1 K113W, but this is not of sufficient magnitude to be detected in the oocyte system. Importantly, in all the experiments conducted in oocytes, the interaction of SLAC1 with OST1 or GHR1 was enhanced by using fusion proteins with the N-terminal or C-terminal part of YFP, respectively. Potentially, this approach could hinder the discovery of differences in the strength of inhibition between different versions of the HT1 kinase. Thus, the role of HT1 kinase activity in the regulation of SLAC1 activation needs to be analysed further with other methods.

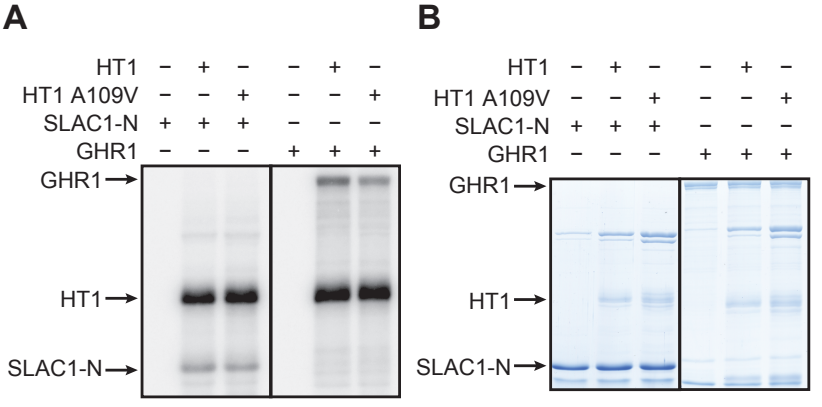

Figure 15. HT1 and HT1 A109V phosphorylate GHR1 and SLAC1 N-terminus. Autoradiograph (A) and Coomassie Brilliant Blue G-250 staining (B) are shown. Modified from III, Fig. 6a.

5.3.3. The regulation of CO_2-induced stomatal closure by MPKs and HT1

As MPK12 inhibited HT1 activity *in vitro* (II, Fig. 5a and 5b, III, Fig. 4b and 4d), we assayed if the addition of MPK12 to the oocytes would rescue the HT1-induced inhibition of SLAC1 activation by GHR1. Indeed, when MPK12 was added to GHR1, SLAC1 and HT1 in oocytes, SLAC1 activation was restored, whereas this was not true for the HT1 A109V (Figure 16). These data suggest that in guard cells, MPK12 acts as a positive regulator of CO_2-induced stomatal closure by inhibition of HT1, which is an inhibitor of SLAC1 activation.

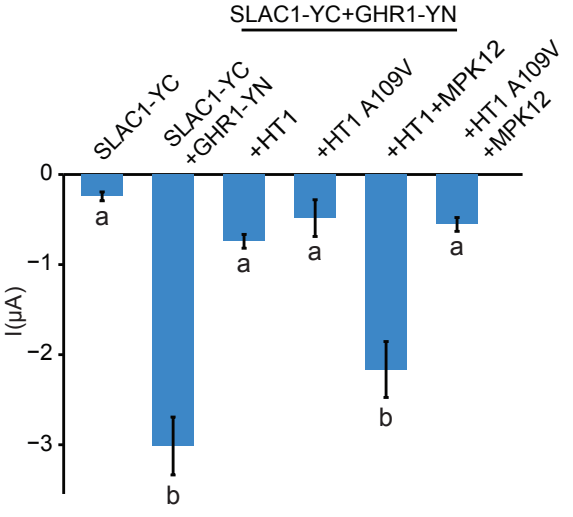

Figure 16. SLAC1 activation by GHR1 is inhibited by HT1 and HT1 A109V in oocytes. MPK12 restores SLAC1 activation in the presence of GHR1 and HT1, but not HT1 A109V. Mean ± SEM currents at -140 mV are shown. Statistically significantly different groups are denoted with different letters (One-way ANOVA + Tukey unequal N HSD *post hoc* test, n = 8-12.). Split-YFP (bimolecular fluorescence complementation) was used for SLAC1 and GHR1 coexpression and is indicated by -YN or -YC in the names of respective proteins. Modified from III, Fig. 5c.

These results led to a model that provides an explanation for the phenotypes found in the *ht1-8D* and *mpk12-4* mutants and the Col-S2 plant line. By this model, in wild-type plants at ambient CO_2 concentration, HT1 inhibits SLAC1 activation by OST1 and GHR1, leading to normal steady-state stomatal conductance. The increase of CO_2-concentration leads to activation or release of MPK12 by a yet unknown mechanism, subsequent inhibition of the activity of HT1 by MPK12, and stomatal closure, as SLAC1 can be activated by OST1 and GHR1. In the *ht1-8D* plants, MPK12 is not capable of inhibiting the HT1 A109V, leading to constitutive HT1-induced inhibition of SLAC1 activation that results in high steady-state stomatal conductance as well as loss of stomatal responsive-

ness to elevated CO_2-concentration (Figure 12A). The situation is similar in the *mpk12-4* plants, where MPK12 is not present and thus inhibition of HT1 activity cannot occur, resulting in constitutive inhibition of SLAC1 activation by HT1 and high stomatal conductance (II, Fig. 2a). The weakened inhibition of HT1 activity by MPK12 G53R also explains the similar phenotypes of *mpk12-4* and Col-S2 plants (II, Fig. 2a). However, stomatal closure in response to elevated CO_2 is completely absent in *ht1-8D* (Figure 12A), whereas it is merely slower in *mpk12-4* and Col-S2 (Figure 10A), suggesting that there are other inhibitors of HT1 that can rescue SLAC1 activation in response to increased CO_2-concentration in the absence of MPK12. MPK4, which is preferentially expressed in guard cells (Petersen et al., 2000; Rodriguez et al., 2010), a close relative of MPK12 (Ichimura et al., 2002) and capable of interaction with (III, Fig. 3c) as well as inhibition of HT1 (II, Fig. 6a, III, Fig. 6d), is the most likely candidate protein that could compensate for the loss of MPK12-induced inhibition of HT1 activity in *mpk12-4* and Col-S2 plants, leading to only partially impaired CO_2-induced stomatal closure. As another dominant mutation in *HT1*, leading to R102K substitution in the corresponding protein, caused similarly increased stomatal conductance and loss of CO_2-responsiveness as the A109V substitution (Hashimoto-Sugimoto et al., 2016; Figure 12) it is conceivable that the R102 is also required for efficient interaction with and/or inhibition of HT1 activity by MPK12. This hypothesis remains to be tested in the future.

We assayed the role of MPK4 in stomatal CO_2 signalling via generation of transgenic plant lines where *MPK4* expression was silenced in a guard-cell specific manner in the *mpk12-4* background, resulting in the lack of both MPK12 and MPK4 in the stomata of these plants. Several independent T1 transgenic plants all had completely lost high CO_2-induced stomatal closure (III, Fig. 7a and 7b) as well as low CO_2-induced stomatal opening (III, Supplemental Fig. 11). The expression of the silencing construct was driven by either MPK12 or HT1 promoters, as both of these genes have strong expression in the guard cells (Jammes et al., 2009; Hashimoto et al., 2006). Subsequent analysis of two and three T3 lines, where *MPK4* was silenced in the *mpk12-4* background using the MPK12 promoter or the HT1 promoter, respectively, was carried out. The lines were denoted *mpk4 GC* in *mpk12-4 (ProMPK12)* and *mpk4 GC* in *mpk12-4 (ProHT1)*, respectively. The growth phenotype of the plant lines indicated that the MPK12 promoter yields a more guard cell specific expression of the transgene, as the lines where the silencing of *MPK4* was driven by the HT1 promoter were dwarfed (Figure 17) similar to the *mpk4* mutants (Petersen et al., 2000), indicating that silencing of MPK4 outside of guard cells had occurred.

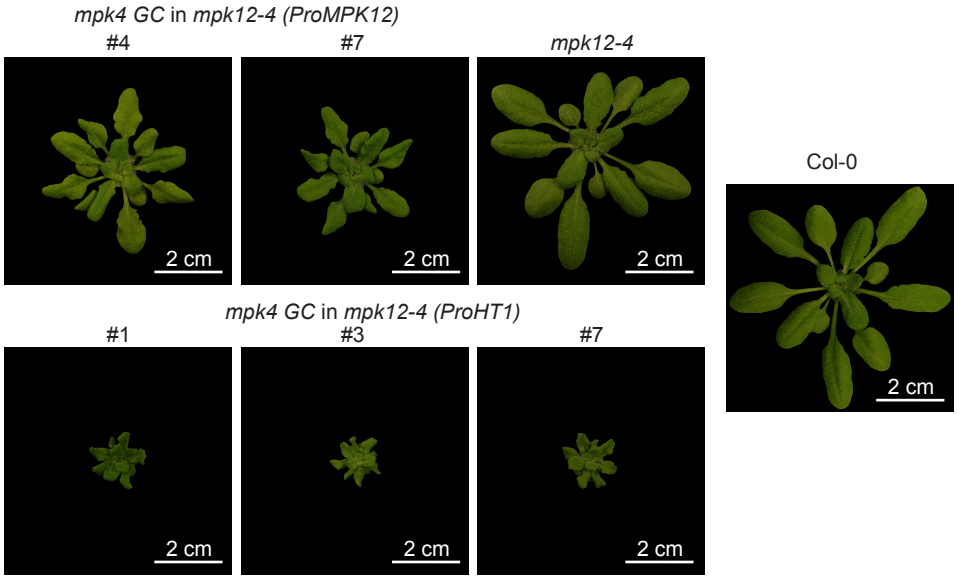

Figure 17. Growth phenotype of the T3 lines with guard cell specific silencing of *MPK4* expression in the *mpk12-4* background. Plant lines where *MPK4* silencing is driven by MPK12 and HT1 promoters are denoted by *mpk4 GC* in *mpk12-4 (ProMPK12)* and *mpk4 GC* in *mpk12-4 (ProHT1)*, respectively. Images of 25-30 days old plants are shown.

Gas-exchange analysis of the T3 lines confirmed the complete loss of CO_2-induced stomatal closure in plants that lack both MPK12 and MPK4 (Figure 18). The stomatal conductance was not as high as in the *mpk12-4* or *ht1-8D* in the T3 lines (Figure 12 and 18). This could be explained by partial silencing of MPK4 outside guard cells that may lead to excess SA-synthesis and stunted growth as in the *mpk4* mutants (Petersen et al., 2000). As SA has been shown to induce stomatal closure (Miura and Tada, 2014) and plants with higher SA levels, such as C24, have lower stomatal conductance (Figure 8A; Okuma et al., 2014), increased SA levels could explain the decreased stomatal conductance in the T3 plants with silenced MPK4 expression.

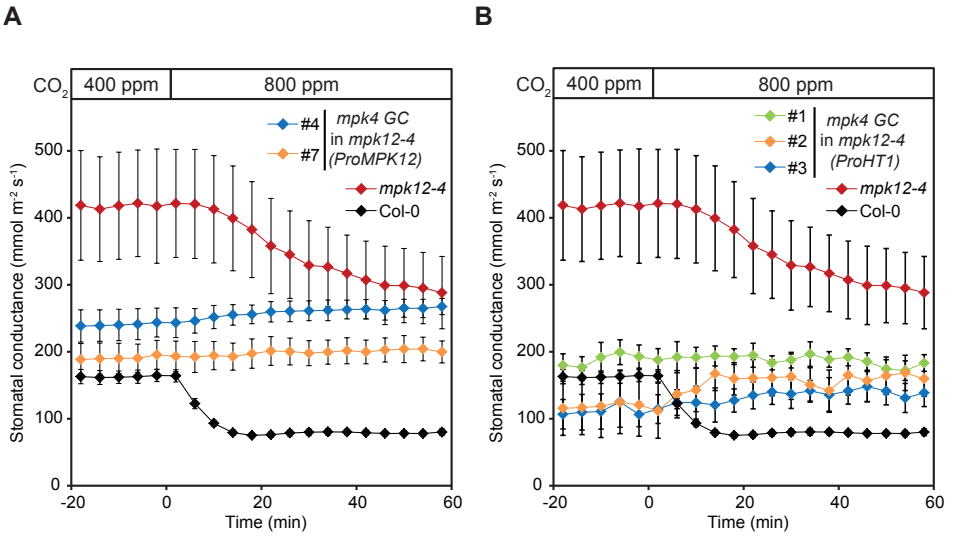

Figure 18. Response to elevated CO_2 in transgenic T3 lines with silenced *MPK4* expression in the guard cells of *mpk12-4*. (A) Plant lines where MPK4 silencing is driven by MPK12 promoter. (B) Plant lines where MPK4 silencing is driven by HT1 promoter. In (A) and (B), mean ± SEM stomatal conductance is shown; CO_2 concentration was elevated from 400 ppm to 800 ppm at time point zero (n = 3-4). For Col-0 and *mpk12-4*, same data is shown in (A) and (B).

As lack of both MPK12 and MPK4 resulted in complete abolition of stomatal closure in response to elevated CO_2 (Figure 18) and both MPK12 and MPK4 were capable of interaction with and inhibition of HT1 activity *in vitro* (II, Fig. 5, III, Fig. 4 and 6), it is likely that both of these MPKs have an important role in the regulation of guard cell CO_2 signalling. Based on the defective stomatal responsiveness to elevated CO_2 in *mpk12* (II), *ht1* (III), *ghr1* mutants (IV) and interaction studies, *in vitro* kinase assays and oocyte experiments, a model for a part of guard cell CO_2-specific signal transduction in response to increased CO_2 concentration is proposed (Figure 19). The reasons for impairment of CO_2-induced stomatal closure in *ht1-2* and other recessive *ht1* mutants, *ht1-8D* (and possibly *ht1-3*), *mpk12-4* and Col-S2 can be explained in the frames of this model and have been shown in Figure 19. In this model, increase in CO_2 concentration results in higher bicarbonate levels, which are perceived by a yet to be described mechanism. This leads to activation of MPK12 and MPK4, whereas it remains to be characterized, whether this occurs via phosphorylation, change in the location of the MAP kinases, or release from inhibition by upstream regulators. Thereafter, MPK12 and MPK4 inhibit HT1 kinase activity, thus suppressing the negative regulation of SLAC1 and leading to the activation of anion fluxes through SLAC1 by the GHR1 and OST1 kinases. In the *ht1-8D* and *mpk12* mutants, the inhibition of HT1 activity by MPK12 and MPK4, or only MPK12, respectively, is impaired. This leads to constitutive inhibition of SLAC1 activation by HT1 and high stomatal

conductance as well as loss or impairment of CO_2-induced stomatal closure in *ht1-8D* and *mpk12*, respectively. In the recessive *ht1* mutants such as *ht1-2*, HT1 is incapable of inhibiting SLAC1 activation by GHR1 and OST1, resulting in constitutive SLAC1 activation manifested in low stomatal conductance and loss of CO_2-induced stomatal closure.

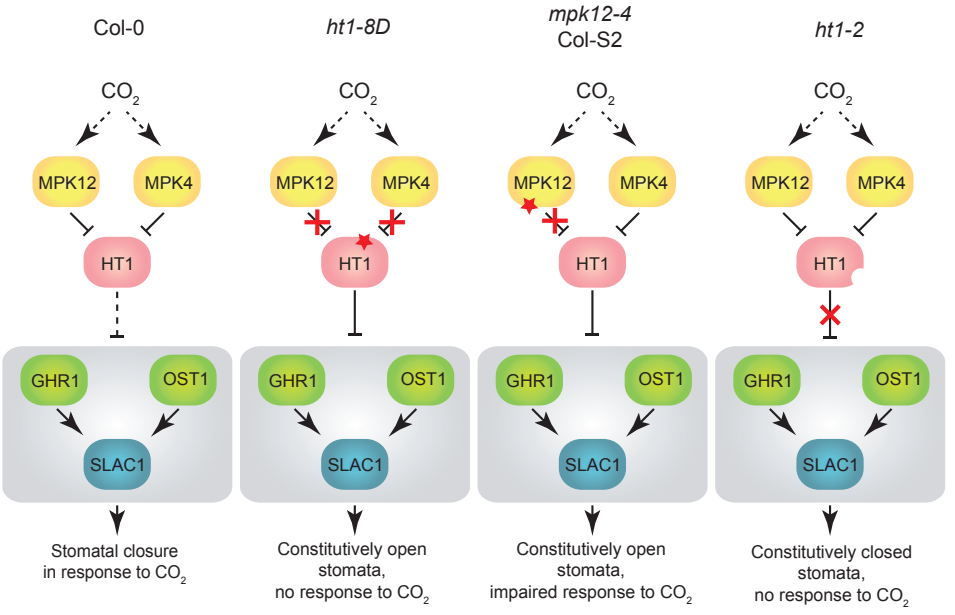

Figure 19. The core CO_2-signalling module and its failure in the *ht1-8D*, *mpk12-4*, *ht1-2* mutants and the NIL Col-S2. Elevation of CO_2 concentration results in the activation of MPK12 and MPK4 by yet unknown mechanisms. The MPKs inhibit the activity of HT1, leading to suppression of negative regulation of SLAC1 and to the concomitant activation of anion fluxes through SLAC1 by the GHR1 and OST1 kinases. In *ht1-8D*, *mpk12-4* and Col-S2 the inhibition of HT1 activity by MPK12 and MPK4, or only MPK12, respectively, is impaired. This leads to constitutive inhibition of SLAC1 activation by HT1 and high stomatal conductance as well as loss or impairment of CO_2-induced stomatal closure in *ht1-8D* and *mpk12* or Col-S2, respectively. In *ht1-2*, HT1 is incapable of inhibiting SLAC1 activation by GHR1 and OST1, resulting in constitutive SLAC1 activation manifested in low stomatal conductance and loss of CO_2-induced stomatal closure.

Importantly, low CO_2-induced stomatal opening was also absent in *ht1-2*, *ht1-8D* (Figure 12B) and *mpk12-4* plants with guard cell specific silencing of MPK4 expression (III, Supplemental Fig. 11) and impaired in *mpk12* mutants (Figure 10B). The molecular mechanisms and regulatory pathways controlling stomatal opening in response to a decrease in CO_2 concentration are largely unknown. PATROL, a protein that controls the subcellular localization of the major guard cell H^+-ATPase OST2 (Merlot et al., 2007) is a regulator of this process as *patrol* plants lack stomatal opening response to reduction of CO_2-concentration

(Hashimoto-Sugimoto et al., 2013). The double mutant of carbonic anhydrases CA1 and CA4 also shows impaired response to reduced CO_2 (Hu et al., 2010).

On one hand, the responsiveness to low CO_2 can be viewed as a reverse of the CO_2-induced stomatal closure. Reduction of CO_2 concentration would lead to a decrease in bicarbonate levels, resulting in reduced activation of MPK12 and MPK4; that in turn would lead to weaker inhibition of HT1 activity by MPKs, leading to increased inhibition of SLAC1 activation by GHR1 and OST1 and thus less anion efflux, resulting in more open stomata. In the *ht1-8D* and *mpk12* mutants, HT1 cannot be inhibited by MPKs and thus the degree of activation of the MPKs would not affect the stomatal aperture, resulting in no low CO_2-induced stomatal opening. However, if the loss of low CO_2-induced stomatal opening in plants with defects in the MPK12-HT1 signalling unit was solely due to inhibition of anion efflux through SLAC1, the stomatal conductance and response to low CO_2 concentration in the *ht1-8D* and *mpk12-4* would be expected to be similar to the *slac1* mutants. This is not true, as the stomatal conductance of *slac1-3* plants was lower than in *ht1-8D* (Figure 20A) and whereas the response to a decrease in CO_2 concentration was completely absent in *ht1-8D* and very weak in *mpk12-4*, the stomata of *slac1-3* opened in response to low CO_2 (Figure 20B). These data indicate that MPKs and HT1 are also required for active stomatal opening response to low CO_2 concentration, possibly via the regulation of PATROL and/or the activation of OST2. The high stomatal conductance of *ht1-8D* and *mpk12-4* suggests that OST2 may be hyperactivated in these plants. Further analysis of H^+-ATPase activation in these plant lines as well as interaction studies with the MPKs, HT1, PATROL and OST2 could help to understand the role of MPKs and HT1 in the regulation of low CO_2-induced stomatal opening.

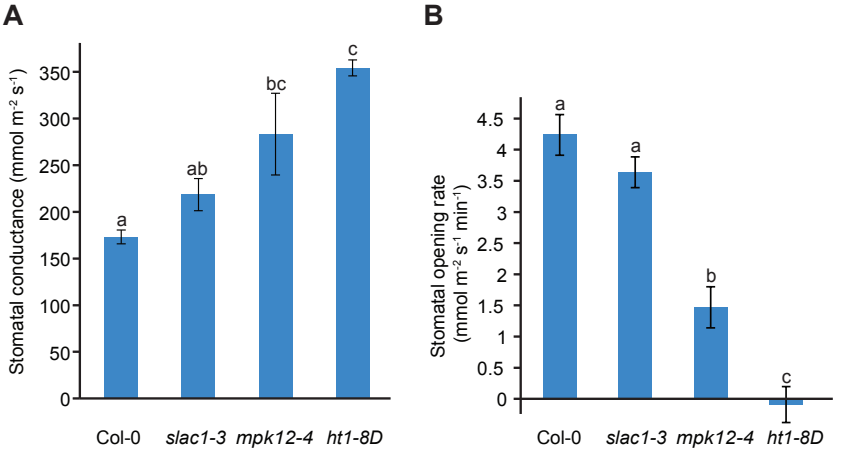

Figure 20. Stomatal conductance and opening rate in response to a reduction in CO_2 concentration. (A) Mean ± SEM stomatal conductance. (B) Mean ± SEM stomatal opening rate in response to reduction of CO_2 concentration from 400 ppm to 100 ppm. In (A) and (B), different letters mark statistically significantly different groups (One-way ANOVA, Tukey unequal N *post hoc* test, n = 4-6).

5.3.4 Guard cell signalling in response to changes in CO_2 concentration

In order to assess the importance of the MPK-HT1 signalling unit in CO_2 signal transduction in comparison with the mutants of key anion channels and their regulators, high CO_2-induced stomatal closure and low CO_2-induced stomatal opening were characterized in a range of mutants. These included plants deficient in the S-type and R-type anion channels SLAC1 (*slac1-3,* Negi et al., 2008; Vahisalu et al., 2008) and QUAC1/ALMT12 (*almt12-1,* Meyer et al., 2010; Sasaki et al., 2010), respectively; *ost1-3* (Yoshida et al., 2002), *ghr1-3* (IV) and *cpk3 cpk5 cpk6 cpk11* (Brandt et al., 2015) as plants deficient in key regulators of SLAC1 activators; *ca1 ca4* (Hu et al., 2010) that lacks efficient bicarbonate production and *ht1-2* (Hashimoto et al., 2006), *ht1-8D* (III) and *mpk12-4* (II) as plants deficient in the MPK-HT1 signalling module.

In response to elevated CO_2, stomatal closure was impaired in all the studied mutants, except for *almt12-1* and the quadruple *cpk* mutant (Figure 21 and 23A). This suggests that anion efflux through SLAC1, but not ALMT12, is of major importance in CO_2-induced stomatal closure, as shown before (Raschke et al., 2003). Furthermore, the CPKs 3, 5, 6 and 11 either are not required for efficient CO_2-induced stomatal closure or there are other CPKs, such as CPK21 and CPK23 (Geiger et al., 2010) that could compensate for their loss in the studied quadruple mutant. Whereas stomatal closure was slowed down in plants deficient in SLAC1 or its regulator OST1, this impairment was not as severe as in the *ht1-2*, *ht1-8D* or *ghr1-3* mutants (Figure 21 and 23A). These data further corroborate the central role of GHR1 in CO_2-induced stomatal closure and highlight the importance of proper regulation of HT1 in guard cells.

Stomatal opening in response to low CO_2 occurred in the *cpk* quadruple mutant, *almt12-1*, *ghr1-3* and *slac1-3*, whereas it was partially or completely impaired in other mutants (Figure 22 and 23B). These data indicate that reduced anion channel activation *per se* does not block stomatal opening in response to reduced CO_2 concentration. The complete loss or severe impairment of low CO_2-induced stomatal opening in *ht1-2*, *ht1-8D* and *mpk12-4* mutants (Figure 22 and 23B) indicates that the respective proteins contribute to the regulation of stomatal opening, in addition to the regulation of SLAC1 activation described above. Further analysis of the interaction of the components of e.g. light-induced stomatal opening pathway with MPK12, MPK4 and HT1 could give valuable insight into the molecular mechanisms of stomatal opening in response to reduced CO_2 concentration. Stomatal opening was also mildly impaired in *ost1-3* mutants (Figure 22 and 23B), which could be explained by the high stomatal conductance of these plants, resulting in a lower opening potential for the stomata.

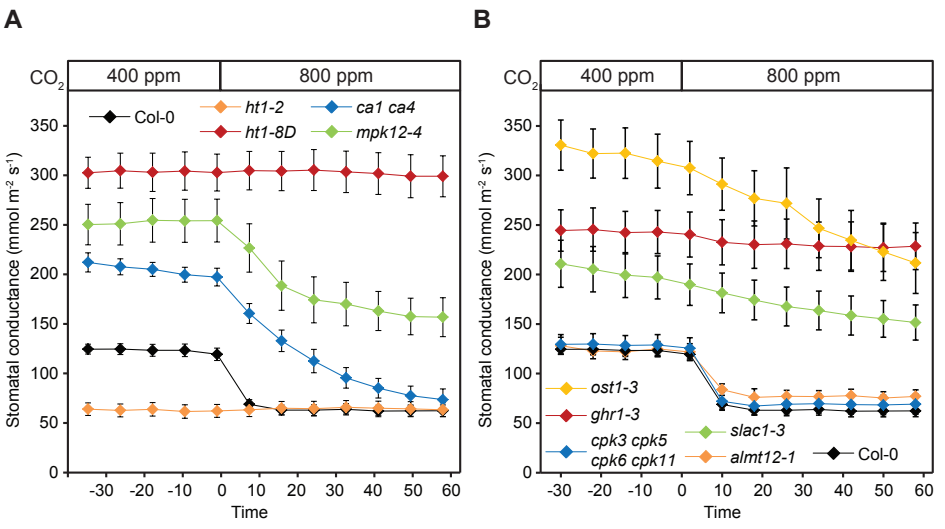

Figure 21. Stomatal closure in response to elevated CO_2 in various mutants of stomatal signalling. (A) Stomatal conductance in mutants related with HT1-MPK12 signalling module and bicarbonate production. (B) Stomatal conductance of mutants related with anion channel activation. In (A) and (B), mean ± SEM (n = 4-6) stomatal conductance is shown; CO_2 concentration was elevated from 400 ppm to 800 ppm at time point zero. The data for Col-0 is the same in (A) and (B).

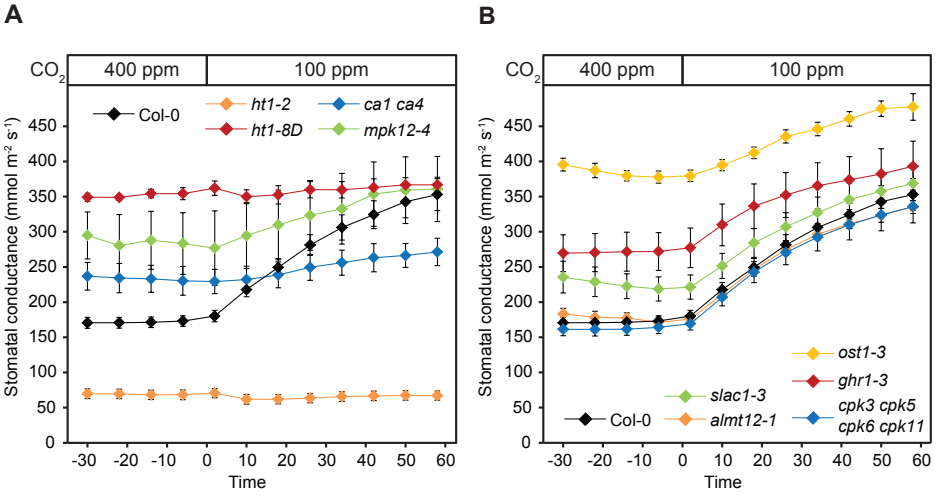

Figure 22. Stomatal opening in response to reduced CO_2 in various mutants of stomatal signalling. (A) Stomatal conductance in mutants related with HT1-MPK12 signalling module and bicarbonate production. (B) Stomatal conductance of mutants related with anion channel activation. In (A) and (B), mean ± SEM (n = 4-6) stomatal conductance is shown; CO_2 concentration was reduced from 400 ppm to 100 ppm at time point zero. The data for Col-0 is the same in (A) and (B).

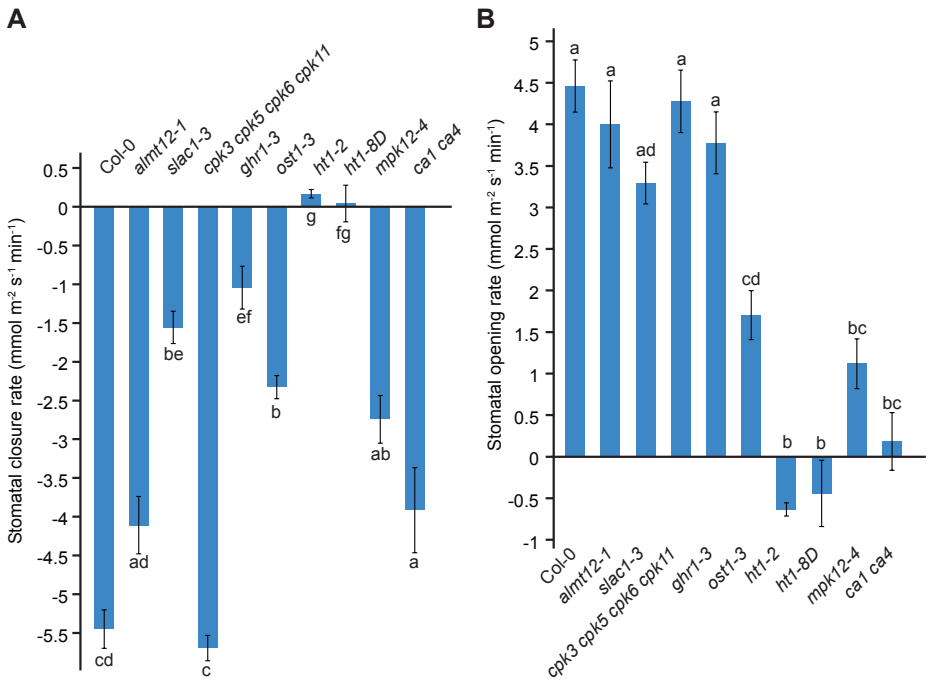

Figure 23. Stomatal closure and opening rates of various mutants of stomatal signalling. (A) Mean ± SEM stomatal closure rate. (B) Mean ± SEM stomatal opening rate. The data in (A) and (B) has been calculated based on the experiments presented in Figure 21 and 22, respectively. Different letters mark statistically significantly different groups (One-way ANOVA, Tukey unequal N *post hoc* test, n = 4-6).

The *ca1 ca4* double mutants had interesting CO_2-response patterns. Albeit the initial stomatal closure in the *ca1 ca4* double mutant was slower than in wild type plants in response to elevated CO_2, the magnitude of the response was greater than in any other studied plant line (Figure 21and 23A). As the conversion of CO_2 to bicarbonate occurs also spontaneously and the efficiency of this process should be higher at 800 ppm of CO_2, compared to 400 ppm, the strong closure response of the *ca1 ca4* mutants could be expected. However, low CO_2-induced stomatal opening was severely impaired in the *ca1 ca4* plants (Figure 22 and 23B). Intuitively, the lack of the two carbonic anhydrases should lead to lower levels of bicarbonate, which would be expected to promote, not suppress, stomatal opening. Possibly, the carbonic anhydrases have a role in the activation of stomatal opening other than via the regulation of bicarbonate levels, e.g. via interaction with other involved proteins. As the behaviour of *ca1 ca4* double mutant was similar to *mpk12-4* (Figures 21-23), the potential interaction of these proteins merits further study. As CA4 is localized in the plasma membrane and CA1 in the chloroplast (Hu et al., 2015), it is conceivable that the former preferentially catalyses the conversion of CO_2 to bicarbonate whereas the latter the conversion of bicarbonate

to CO_2, similar to the carbonic anhydrases CAH8 and CAH6 that function in the mechanism that enables concentrating CO_2 for photosynthesis in *Chlamydomonas reinhardtii* (Moroney et al., 2011). This would provide one possible explanation for the increased stomatal conductance in the *ca1 ca4* plants as the amount of CO_2 for photosynthesis would be decreased, requiring more open stomata for efficient CO_2 uptake. Similar explanation has been proposed for the increased stomatal conductance and decreased CO_2-responsiveness of rice plants that lack the chloroplastic βCA1 (Chen et al., 2017). Alternatively, lower levels of bicarbonate, which has been shown to enhance SLAC1 activation (Xue et al., 2011; Tian et al., 2015; Wang et al., 2016), could lead to reduced SLAC1 activation, manifested in increased stomatal conductance.

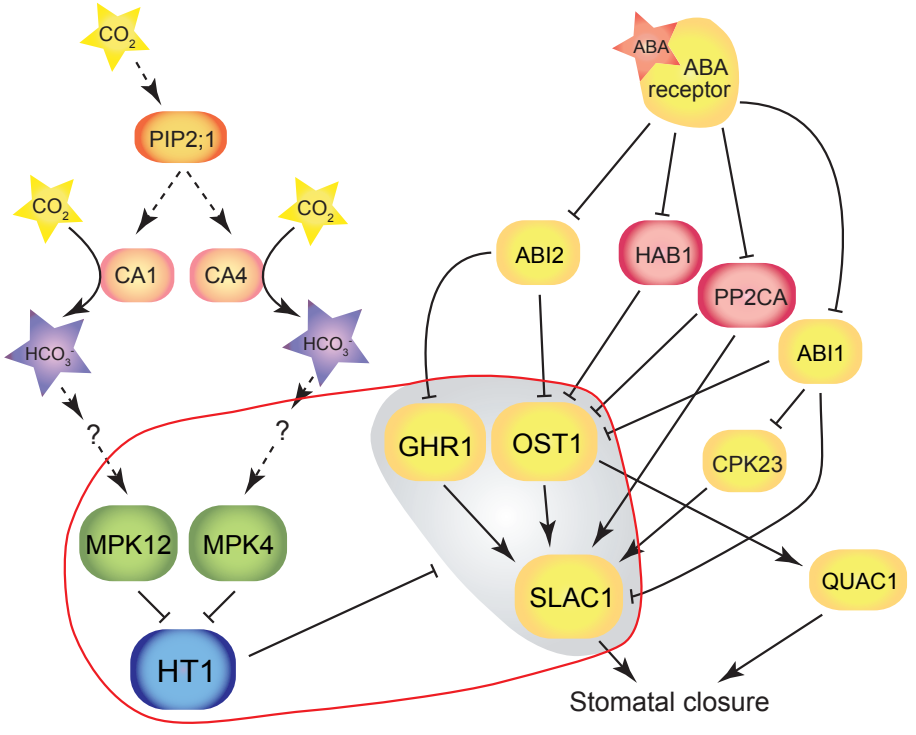

Figure 24. Guard cell ABA and CO_2 signalling. The components of ABA signalling pathway that have been shown to be important for CO_2 signal transduction as well are shown in yellow. The part of the model that is based on the research described in this thesis is marked by the red line. The aquaporin PIP2;1 has been proposed to facilitate CO_2 uptake. The carbonic anhydrases CA1 and 4 catalyse the conversion of CO_2 to bicarbonate that activates MPK12 and 4 via yet to be characterized mechanisms. The MPKs inhibit the activity of the HT1 kinase, which acts as an inhibitor of SLAC1 activation by GHR1 and OST1, thus suppressing stomatal closure in response to CO_2. Inhibition of HT1 activity by MPK12 and 4 in response to elevated CO_2 concentration enables the activation of SLAC1 by GHR1 and OST1, leading to CO_2-induced stomatal closure.

Altogether, HT1 and MPK12 are essential for normal stomatal responsiveness to both high and low CO_2 concentration. Deficiency in these proteins results in complete impairment in stomatal CO_2 signal transduction, whereas the lack of components common to the CO_2 and ABA signalling pathway mostly results in the weakening, but not complete loss of CO_2-responses (Figure 21-23 and Merilo et al., 2013). The combined stomatal ABA and CO_2 signal transduction pathway is shown in Figure 24. Identification of the mechanisms of CO_2 and/or bicarbonate perception and upstream regulators of MPK12 and MPK4, as well as thorough structural and biochemical analyses together with *in planta* experiments are required to better understand the regulation of stomatal responses to CO_2.

5.4. Stomatal responsiveness to ABA and CO_2 in ferns (V)

Understanding the evolution of stomatal responses to ABA and CO_2 would help to predict the behaviour of various plant species in the conditions of drought stress or changing levels of atmospheric CO_2 and thus benefit to the modelling of the effects of the changing environment on various ecosystems. Comparative studies of stomatal behaviour in plants with different evolutionary age, such as angiosperms *versus* older land plants, can contribute to this goal. The presence of active stomatal responses to ABA and elevated CO_2 in ferns has remained controversial to date. Furthermore, growth conditions and species have often not been taken into account when assessing and discussing the stomatal responsiveness of ferns.

Thus, we studied the effect of growth conditions on stomatal responses to ABA and CO_2 in three temperate fern species (V). The ferns *Athyrium filix-femina*, *Dryopteris filix-mas* and *Dryopteris carthusiana* were collected from nature and grown in two different growth environments in the laboratory. These were termed "growth cabinet" (constant 70% relative air humidity, 12/12 h light/dark cycle) and "growth room" (~30-40% relative air humidity [RH] during day, 40-65% at night, 16/8 h light/dark cycle). Stomatal responses to ABA and changes in CO_2 concentration were studied. Stomatal closure in response to ABA was present in *A. filix-femina* and *D. filix-mas* grown in the growth cabinet, but not in the growth room, whereas *D. carthusiana* lacked ABA-response in both conditions (V, Fig. 1c). Thus, both species and growth conditions affected stomatal responsiveness to ABA in ferns, whereas the angiosperm *A. thaliana* had efficient ABA-induced stomatal closure irrespective of growth conditions (V, Fig. 1b). Possibly, growth at the lower RH in the growth room induced ABA biosynthesis in ferns as has been shown before for other species (Bauer et al., 2013; McAdam and Brodribb, 2015), leading to stomatal closure and no further stomatal response to ABA-treatment. ABA levels did not increase in ferns after short-term exposure to low RH (McAdam and Brodribb, 2015), but response of fern ABA levels to prolonged exposure to low RH remains to be characterized. The modest magnitude of ABA-responses in ferns (V, Fig. 1c) could explain why no differences in stomatal conductance between ferns grown

in the growth room and in the growth cabinet, were detected. This could also be one reason why ferns have been previously reported to lack stomatal ABA-responsiveness (Brodribb and McAdam, 2011). Alternatively, the ABA supplied to the transpiration stream as in Brodribb and McAdam, 2011, could be incapable of inducing stomatal closure, whereas ABA applied by leaf spraying (V) or to epidermal strips (Ruszala et al., 2011; Cai et al., 2017) may reach guard cells directly and be more efficient in promoting stomatal closure. Although ABA-induced stomatal closure was present in two studied fern species, the response was clearly weaker than in the angiosperm *A. thaliana* (V, Fig 1b and 1c), indicating that even though stomatal ABA-responses are present in some fern species, the mechanisms of these responses are at least partly different between angiosperms and ferns. Further analysis of ABA-responsiveness in ferns across the evolutionary tree will help to clarify the importance and mechanisms of ABA-induced stomatal closure in ferns.

Stomatal responses to a reduction of CO_2 concentration from 400 ppm to 100 ppm and elevation of the CO_2 level back to 400 ppm were present in all ferns irrespective of growth conditions (V, Fig. 2c and 2d). Stomatal closure in response to elevated CO_2 concentration also occurred in all fern species, with the exception of the *D. filix-mas* grown in the growth room (V, Fig. 2c and 2d). The stomatal conductance of the *A. filix-femina* after the change of CO_2 concentration from 100 ppm to 400 ppm was statistically significantly higher than the pre-treatment value at 400 ppm when grown in the growth room (V, Fig. 2c and 2d). Thus, *D. filix-mas* and *A. filix-femina* displayed a trend of reduced stomatal responsiveness when grown in the lower RH of the growth room, similar to the ABA experiments (V, Fig. 1c). This is in accordance with the previously documented increased stomatal response to CO_2 elevation in angiosperms grown at high RH (Talbott et al., 2003). The dampening of stomatal responses to ABA and CO_2 in *D. filix-mas* and *A. filix-femina* grown in the growth room suggests that the underlying mechanisms responsible for the effect of growth conditions may be similar and potentially related with ABA levels.

The stomatal opening in response to a reduction of CO_2 concentration from 400 ppm to 100 ppm was fast in ferns, whereas stomatal closure in response to above-ambient CO_2 level was slow and steady (V, Fig. 2c), unlike the fast response in *A. thaliana* (V, Fig. 2a). This difference could explain the previously documented presence and absence of stomatal response to sub-ambient and above-ambient CO_2 in ferns, respectively (Brodribb et al., 2009; Brodribb and McAdam, 2013), since the slow response to above-ambient CO_2 in ferns could remain undetected in shorter experiments. The slow stomatal closure in response to above-ambient CO_2 and modest decrease in stomatal conductance in response to ABA are in accordance with the previously proposed slow dynamics of stomatal responsiveness in ferns (Hetherington and Woodward, 2003). The reasons for the slow stomatal closure in ferns could be related with the specific morphology of fern stomata that lack subsidiary cells and have less lateral movement and reduced shuttling of osmotica between guard cells and epidermal cells (Franks and Farquhar, 2007). On the other hand, as the fern stomata are not in direct

contact with the epidermal cells (Franks and Farquhar, 2007), they should be capable of fast opening, which indeed occurs in response to a reduction of CO_2 concentration (V, Fig. 2c). Thus, the kinetics of fern stomatal responses may largely be controlled by the morphological features of the stomata.

Fern stomatal responses were proposed to be regulated by purely hydropassive mechanisms (Brodribb and McAdam, 2011). We tested this hypothesis by analysis of stomatal responsiveness to reduced air humidity at low (50 ppm) and ambient (400 ppm) CO_2 levels in *A. filix-femina*. As low CO_2 induced stomatal opening in ferns (V, Fig. 2c and 2d), the stomatal response to a sharp reduction in air humidity should have been faster at low than at ambient CO_2 levels, due to higher transpiration that would lead to greater water loss and should induce faster closure by the hydropassive mechanism. However, the response to a reduction in air humidity was slower, not faster at low CO_2 irrespective of growth conditions (V, Fig. 3), indicating that active control of stomatal aperture is present at least in *A. filix-femina*.

Thus, our analysis of stomatal responses in three temperate fern species indicated that active regulation of stomatal responses is present in ferns, but fern stomatal responses to ABA and CO_2 depend on growth conditions as well as species. Thus, large-scale analysis of stomatal behaviour of ferns with different evolutionary age and growth habitat would be necessary to better understand the evolution of stomatal responsiveness in land plants.

6. CONCLUSIONS

The main conclusions of the current thesis can be summarized as follows:

- The O_3-tolerance and O_3-sensitivity of the Arabidopsis C24 and Te accessions, respectively, have both stomata-related and -unrelated causes. Different stomatal conductance and kinetics of stomatal response to prolonged O_3-treatment were found in C24 and Te, indicating an important role for stomata in determining O_3-sensitivity. Differences in gene expression and SA levels between C24 and Te pointed at stomata-unrelated mechanisms that also contribute to the O_3-tolerance and -sensitivity of the respective accessions.
- The O_3-sensitivity of the Arabidopsis Cvi-0 accession is partly caused by increased stomatal conductance due to a G53R substitution in MPK12 and partly by a presently unknown locus on chromosome 3.
- Several mutations in *GHR1* that encodes an LRR-RLK, cause increased O_3-sensitivity. GHR1 has a central role in stomatal regulation as it is required for efficient stomatal responses to changes in CO_2 concentration, darkness, ABA and O_3, as well as for adequate circadian stomatal behaviour.
- The G53R substitution in MPK12 or complete lack of the protein result in increased stomatal conductance, reduced WUE and impaired stomatal responses to changes in CO_2 concentration. The G53R in MPK12 is important for efficient interaction with HT1 and inhibition of HT1 activity.
- A dominant mutation in *HT1* that leads to A109V substitution in the HT1 protein causes O_3-sensitivity, increased stomatal conductance and complete lack of stomatal responses to changes in CO_2 concentration. The A109V in HT1 is important for efficient interaction with MPK12 and inhibition of HT1 activity by MPK12. HT1 inhibits both OST1- and GHR1-induced activation of SLAC1 in oocytes whereas MPK12 suppresses the effect of HT1. Thus, MPK12 acts as an inhibitor of HT1.
- Both MPK4 and MPK12 are involved in CO_2 signal transduction as stomatal responses to changes in CO_2 concentration are completely absent in *mpk12-4* plants with guard cell specific silencing of *MPK4* expression. MPK4 also interacts with HT1 and inhibits its activity.
- The MAP kinases 4 and 12 act as positive regulators of CO_2-induced stomatal closure by inhibiting the activity of the negative regulator HT1, which inhibits SLAC1 activation by OST1 and GHR1. Plants with deficiencies in the MPK-HT1 signalling unit have severely impaired stomatal CO_2-responses, whereas the CO_2-responses are merely moderately impaired in plants with mutations in key anion channels and their activators. Thus, MPK4, MPK12 and HT1 are extremely important for efficient stomatal responses to changes in CO_2 concentration.
- Fern stomatal responses to CO_2 and ABA depend on species and growth conditions, which should be taken into account when drawing major conclusions on the evolution of the mechanisms of stomatal regulation. Stomatal

response to reduced air humidity requires active control and cannot be explained by purely hydropassive mechanisms.

Altogether, the studies introduced in this thesis led to the identification of key components of guard cell CO_2-signalling pathway via analysis of different O_3-sensitive plant lines. The importance of GHR1, MPK12 and MPK4 in stomatal CO_2-responses was discovered and further evidence for the role of HT1 was acquired. Studies on the molecular mechanisms of action of these proteins led to the identification of MPK-HT1 signalling unit that is one of the very few CO_2-specific regulatory nodes in guard cell signal transduction networks discovered to date. Thus, the results presented in this thesis bring important insight into the molecular mechanisms of plant stomatal CO_2-responses and provide useful information for further research on this topic. The obtained knowledge could also be useful for applications in developing crops with higher water use efficiency.

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SUMMARY IN ESTONIAN

Õhulõhede CO_2 signaaliülekande regulaatorvalkude tuvastamine läbi osoonitundlikkuse

Taimed on elu aluseks Maal: nad valmistavad süsihappegaasist (CO_2) ja veest fotosünteesi käigus orgaanilisi ühendeid ning vabastavad hapnikku, pakkudes nõnda teistele organismidele toitu ja võimaldades aeroobset ainevahetust. Lisaks olulisele tähtsusele toidu tootmises on taimed lähtematerjaliks näiteks ravimite, rõivaste, paberi ja paljude teiste toodete valmistamisel. Seega on äärmiselt oluline mõista, kuidas taimed toimivad, et oleks võimalik aretada taimi, kes on kõrgema saagikuse, kasulikuma biokeemilise koostise või suurenenud stressitaluvusega.

Taime lopsakas kasv sõltub suurel määral gaasivahetusest keskkonnaga – sellest, kui edukalt ta omastab õhust süsihappegaasi ning kui palju läheb seejuures kaduma väärtuslikku vett. Gaasivahetus toimub läbi lehepinnal paiknevate õhulõhede, mille kaudu siseneb taime süsihappegaas ning lahkuvad veeaur ja hapnik. Õhulõhe moodustub kahest sulgrakust, mille veesisaldus määrab rakkude vahele jääva avause ehk õhupilu suuruse. Kärme ja täpne õhupilu avatuse regulatsioon vastavalt keskkonnas toimuvatele muutustele on taimede ellujäämiseks ja kasvuks hädavajalik. Õhulõhed avanevad kui ioonid ja vesi sisenevad sulgrakkudesse, mistõttu rakud paisuvad ning kumerduvad ja õhupilu laieneb. Õhulõhede sulgumine toimub vastupidiselt: ioonid ja vesi lahkuvad sulgrakkudest, rakkude ruumala väheb ning õhupilu kitseneb. Taime tasandil toob õhulõhede avanemine ja sulgumine vastavalt kaasa õhulõhede juhtivuse kasvu või kahanemise. Õhulõhed avanevad valguse, madala CO_2 kontsentratsiooni ja kõrge õhuniiskuse mõjul. Õhulõhed sulguvad vastusena pimedusele, kõrgele CO_2 kontsentratsioonile ja madalale õhuniiskusele. Ka saasteained, näiteks osoon, ning patogeenid põhjustavad õhulõhede sulgumist. Taimesiseste signaalidena reguleerivad õhulõhede sulgumist taimehormoon abstsiishape (ABA), lämmastikmonooksiid (NO), reaktiivsed hapniku ühendid (ROS) ja Ca^{2+} ioonid. Õhulõhede regulatsiooni mehhanismide mõistmine on eelduseks kõrgema veekasutuse efektiivsusega taimesortide aretamisel, kes oleksid edukamad muutuvates ja keerulistes keskkonnatingimuses.

Käesolevas doktoritöös uuriti erinevate osoonitundlike hariliku müürlooga (*Arabidopsis thaliana*) taimeliinide osoonitundlikkuse põhjusi ning seeläbi avastati süsihappegaasi mõjul õhulõhede sulgumist reguleeriva signaaliülekande ahela olulised komponendid. Töös uuriti ka sõnajalgade õhulõhede reaktsioone süsihappegaasile ja ABAle, et selgitada, kuidas on õhulõhede regulatsiooni mehhanismid erinevates taimerühmades evolutsioneerunud.

Esmalt uuriti kahe erinevates tingimustes evolutsioneerunud hariliku müürlooga ökotüübi, C24 ja Te, osoonitaluvuse ja -tundlikkuse põhjusi. Leiti, et C24 ökotüübi osoonitaluvusse panustavad nii madal õhulõhede juhtivus, millest on tingitud madalam omastatud osooni kogus, kui ka kõrgem salitsüülhappe tase, mis peegeldab pidevalt aktiveeritud kaitsemehhanisme ning arvatavasti aitab

kaasa osoonist tingitud kahjustuste vähendamisele. Te ökotüübi osoonitundlikkus oli arvatavasti vähemalt osaliselt põhjustatud kõrgemast õhulõhede juhtivusest ning sellest tingitud suuremast omastatud osooni kogusest. Samas olid C24 ja Te ökotüüpide geenikspressiooni profiilid erinevad, mis viitas, et lisaks õhulõhede erinevast käitumisest tingitud põhjustele on nende taimeliinide erineva osoonitundlikkusega seotud ka erinev geenikspressiooni regulatsioon.

Järgnevalt uuriti veel ühe hariliku müürlooga ökotüübi, Cvi-0, tugeva osoonitundlikkuse põhjusi ning leiti, et sellega oli seotud kaks erinevat genoomi piirkonda, millest üks oli seotud ka kõrgema õhulõhede juhtivuse ning CO_2 kontsentratsiooni muutusest tingitud õhulõhede avanemise ja sulgumise häirumisega. Geneetiline kaardistamine näitas, et mainitud fenotüüpide põhjuseks oli punktmutatsioon mitogeen-aktiveeritud proteiinkinaasi (MPK) kodeerivas geenis *MPK12*, mis viis G53R asenduseni vastavas valgus. Lisaks leiti mutantne taimeliin, kus *MPK12* geen puudus, õhulõhede juhtivus oli kõrge ning reaktsioonid CO_2 kontsentratsiooni muutustele häiritud. Selgus, et MPK12 valgul on tähtis roll nii õhulõhede avanemisel kui sulgumisel vastavalt madala või kõrge CO_2 kontsentratsiooni mõjul.

Käesolevas töös uuriti ka hariliku müürlooga mutante, kes leiti sõeluuringute käigus, kus otsiti osoonitundlikke taimeliine. Sel viisil avastati dominantne mutatsioon geenis *HT1*, mis kodeerib õhulõhede CO_2-vastuses keskse rolliga valku. Mutatsioon põhjustas A109V asenduse HT1 valgus, mis tõi kaasa tugeva osoonitundlikkuse, kõrge õhulõhede juhtivuse ning muutis õhulõhed täiesti tundetuks CO_2 kontsentratsiooni muutustele. Lisaks leiti mitmeid mutatsioone geenis *GHR1* ning näidati, et GHR1 funktsioon on oluline õhulõhede reageerimisel pimedusele, CO_2 kontsentratsiooni tõusule, ABAle ja osoonile ning samuti vajalik õhulõhede normaalseks käitumiseks ööpäeva lõikes.

Täiendav MPK12 ja HT1 valkude toimemehhanismide uurimine viis kõrge CO_2 kontsentratsiooni poolt põhjustatud õhulõhede sulgumiseks olulise signaaliülekande ahela avastamiseni. Leiti, et MPK12 interakteerus HT1 valguga ning takistas *in vitro* katsetes HT1 valgu autofosforüülumist ning ka HT1-vahendatud kaseiini fosforüülimist. Kui kasutati G53R asendusega MPK12 koos metsiktüüpi HT1 valguga või A109V asendusega HT1 koos metsiktüüpi MPK12 valguga, siis oli valkudevaheline interaktsioon ning MPK12-vahendatud HT1 aktiivsuse inhibitsioon nõrgem. HT1 takistas kinaaside OST1 ja GHR1 põhjustatud SLAC1 anioonkanali aktivatsiooni suure kannuskonna (*Xenopus laevis*) ootsüütides ning MPK12 lisamisel SLAC1 aktivatsioon taastus. Lisaks leiti, et MPK12 valgule väga sarnane MPK4 interakteerus samuti HT1 valguga ning vähendas *in vitro* katsetes HT1 aktiivsust. Taimed, kelles puudus *MPK12* ning *MPK4* ekspressioon oli sulgrakuspetsiifiliselt vaigistatud, olid täiesti tundetud CO_2 kontsentratsiooni muutustele, just nagu taimed, kelle HT1 valgus oli A109V asendus. Mainitud tulemuste põhjal pakuti välja mudel MPK12, MPK4 ja HT1 rolli kohta õhulõhede vastuses kõrgele CO_2 kontsentratsioonile. Mudeli kohaselt surub HT1 kinaas alla OST1- ja GHR1-sõltuva SLAC1 anioonkanali aktivatsiooni, takistades nõnda õhulõhede sulgumist. Kõrge CO_2 kontsentratsioon põhjustab MPK12 ja MPK4 aktiveerumise, mis omakorda inhibeerivad HT1 aktiivsust. See võimal-

dab SLAC1 aktivatsiooni OST1 ja GHR1 kaudu ning toob kaasa õhulõhede sulgumise kõrge CO_2 kontsentratsiooni mõjul. Kuna CO_2 tajumise ja järgneva signaaliülekande mehhanismide kohta õhulõhedes oli seni veel üsna vähe teada, aitavad siin kirjeldatud tulemused oluliselt kaasa CO_2 kontsentratsiooni muutustest põhjustatud õhulõhede reaktsioonide mõistmisele.

Viimasel ajal on taimebioloogide hulgas kirgi kütnud küsimus õhulõhede regulatsiooni mehhanismide evolutsioonist. Nimelt on erinevates uurimisrühmades mõõdetud sõnajalgade õhulõhede reaktsioone ABAle ja CO_2-le ning saadud vastuolulisi tulemusi. Ühelt poolt on leitud, et sõnajalgade õhulõhed ei reageeri ABAle ja kõrgele CO_2 kontsentratsioonile ja välja on pakutud hüpotees, mille kohaselt sõnajalgades puuduvad aktiivsed õhulõhede sulgumise kontrolli mehhanismid ning õhulõhede reaktsioonid keskkonnatingimustele toimuvad hüdropassiivselt ehk sõltuvalt lehe veesisaldusest ja veepotentsiaali muutustest. Teiselt poolt on leitud, et sõnajalgades on olemas kõik õhulõhede regulatsioonis osalevad ABA signaali ülekandeks vajalikud komponendid ning sõnajalgade õhulõhed reageerivad nii ABAle kui kõrgele CO_2 kontsentratsioonile. Käesolevas töös uuriti kolme Eestis kasvava sõnajalaliigi õhulõhede reaktsioone ABAle ja CO_2 kontsentratsiooni muutustele ning leiti, et sõnajalgade õhulõhede käitumine sõltub taimeliigist ning kasvutingimustest. Samuti leiti, et ainuüksi hüdropassiivne mehhanism ei ole piisav, et selgitada naistesõnajala (*Athyrium filix-femina*) õhulõhede käitumist vastusena madalale õhuniiskusele. Saadud tulemused viitavad, et sõnajalgadega tehtud uurimustes tuleks kindlasti arvesse võtta taimede liiki ning kasvutingimusi ning selleks, et teha kaugeleulatuvaid järeldusi õhulõhede regulatsiooni mehhanismide evolutsiooni kohta, on tarvis laiaskaalalist analüüsi, mis kaasab erineva evolutsioonilise vanuse ning kasvukeskkonnaga sõnajalaliike.

Käesolevas töös avastati osoonitundlike taimeliinide analüüsi kaudu mitmed õhulõhede CO_2-vastuses olulised valgud ning pakuti välja mudel nende rolli kohta kõrge CO_2 kontsentratsiooni mõjul toimuvas õhulõhede sulgumises. Lisaks leiti, et sõnajalgade õhulõhede reaktsioonid ABAle ja CO_2-le on liigispetsiifilised ja sõltuvad kasvutingimustest. Saadud tulemused aitavad senisest paremini mõista õhulõhede käitumist muutuva CO_2 kontsentratsiooni tingimustes ning on sisendiks rakendusteadusele, mille eesmärgiks on kõrgema veekasutuse efektiivsusega taimede aretamine, kes oleksid edukamad muutuvates ja keerulistes keskkonnatingimuses.

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