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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which are referred to in the text by the corresponding Roman numerals:

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V. Zhang, Jingbo; De-oliveira-Ceciliato, Paulo; Takahashi, Yohei; Schulze, Sebastian; Dubeaux, Guillaume; Hauser, Felix; Azoulay-Shemer, Tamar; **Tõldsepp, Kadri**; Kollist, Hannes; Rappel, Wouter-Jan; Schroeder, Julian I. (2018). Insights into the Molecular Mechanisms of CO_2-Mediated Regulation of Stomatal Movements. Current Biology, 28 (23), R1356−R1363.10.1016/j.cub.2018.10.015.

Author's contribution

I. I performed some of the experiments, analysed the data, visualized the results and wrote the manuscript.
II. I performed *in vitro* kinase assays and immunoblot analyses, participated in visualizing the data, and writing and editing the manuscript.
III. I performed *in vitro* kinase assays and immunoblot analyses, participated in visualizing the data, and writing and editing the manuscript.
IV. I performed some of the *in vitro* kinase assays and participated in visualizing the data and editing the manuscript.
V. I wrote a part of the manuscript.

ABBREVIATIONS

ABA	abscisic acid, a plant hormone
ABA2	*ABA DEFICIENT 2*, a gene that encodes a cytosolic short-chain dehydrogenase/reductase which is involved in the conversion of xanthoxin to ABA-aldehyde during ABA biosynthesis
ABI1 and ABI2	ABA INSENSITIVE 1 and 2, PP2Cs
ADP	adenosine diphosphate
AHA1	H^+-ATPASE 1, a plasma membrane proton pump which mediates proton efflux from guard cells
AHG1	ABA-HYPERSENSITIVE GERMINATION 1, a PP2C
AHG3/PP2CA	ABA-HYPERSENSITIVE GERMINATION 3/PROTEIN PHOSPHATASE 2CA, a PP2C
Ala (A)	alanine
ALMT12/QUAC1	ALUMINUM-ACTIVATED MALATE TRANSPORTER 12/QUICK-ACTIVATING ANION CHANNEL 1, a rapid (R-type) anion channel which mediates malate and sulfate efflux from guard cells
ANOVA	analysis of variance, a collection of statistical models and their associated estimation procedures used to analyse the differences among group means in a sample
ANPs	ARABIDOPSIS NUCLEUS- AND PHRAGMOPLAST-LOCALIZED KINASE 1-RELATED PROTEIN KINASEs, MAPKKKs
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartate
AtABCB14	*Arabidopsis thaliana* ATP-BINDING CASSETTE B14, a transporter which mediates malate uptake into guard cells
AtNRT1.1/CHL1	*Arabidopsis thaliana* NITRATE TRANSPORTER 1.1/CHLORINA 1, a dual-affinity nitrate transporter which mediates nitrate uptake into guard cells
ATP	adenosine triphosphate
AtRBOHD and AtRBOHF	*Arabidopsis thaliana* RESPIRATORY BURST OXIDASE D and F, nicotinamide adenine dinucleotide phosphate oxidase catalytic subunits which function in ABA-induced ROS production in Arabidopsis guard cells

BiFC	bimolecular fluorescence complementation, a technique used in the investigation and visualization of protein-protein interactions
BIG	a calossin-like protein, a protein which is involved in elevated CO_2-induced stomatal closure
$[Ca^{2+}]_{cyt}$	cytosolic free calcium concentration
βCA1 and βCA4	β-carbonic anhydrases 1 and 4, enzymes that catalyse the interconversion between CO_2 and bicarbonate
CBC1 and CBC2	CONVERGENCE OF BLUE LIGHT AND CO_2 1 and 2, kinases which have been proposed to function as negative regulators of CO_2-induced stomatal closure
CFP	CYAN FLUORESCENT PROTEIN, used as a reporter for protein localization in cells
Col-0	an *Arabidopsis thaliana* accession from Germany
CPK	CALCIUM-DEPENDENT PROTEIN KINASE
Cvi-0	an *Arabidopsis thaliana* accession from Cape Verde islands
Cys (C)	cysteine
GCA2	*GROWTH CONTROLLED BY ABSCISIC ACID 2*, a gene that encodes a protein that might function downstream of, or at convergence point of CO_2 and ABA signalling
GFP	GREEN FLUORESCENT PROTEIN, used as a reporter for protein localization in cells
GHR1	GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1, a plasma membrane leucine-rich repeat receptor-like kinase which mediates SLAC1 activation
$GHR1^{ID}$	GHR1 cytoplasmic domain (amino acid residues 653-1053)
$GHR1^{KD}$	GHR1 kinase domain (amino acid residues 764-1053)
Glu (E)	glutamate
Gly (G)	glycine
GORK	GATED OUTWARDLY-RECTIFYING K^+ CHANNEL, an outwardly-rectifying potassium channel which mediates potassium efflux from guard cells
GST-tag	glutathione S-transferase-tag, a tag which is used for affinity purification of recombinant proteins
HAB1 and HAB2	HYPERSENSITIVE TO ABA 1 and 2, PP2Cs
H^+-ATPase	proton-ATPase, a plasma membrane proton pump which pumps protons out of the cell, thereby

	creating electric potential differences across the plasma membrane
6xHis-tag	hexahistidine-tag, a tag used for affinity purification of recombinant proteins
HT1	HIGH LEAF TEMPERATURE 1, a kinase which is a central regulator of CO_2-induced stomatal movements
KAT1 and KAT2	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 and 2, voltage-dependent inward-rectifying potassium channels which mediate potassium uptake into guard cells
K_d	dissociation constant
KIN7	KINASE 7, a leucine-rich repeat protein kinase family protein which is responsible for the phosphorylation of TPK1 vacuolar K^+ channel
KUP6 and KUP8	K^+ UPTAKE PERMEASE 6 and 8, potassium transporters which mediate K^+ efflux during ABA-induced stomatal closure
Lys (K)	lysine
MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
MAPKK	MITOGEN-ACTIVATED PROTEIN KINASE KINASE
MAPKKK	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE
MBP	Myelin basic protein, an artificial substrate for protein kinases
MeJA	methyl jasmonate, a plant hormone
MST	microscale thermophoresis, a technology for biophysical analysis of interactions between biomolecules
NCED3 and *NCED5*	*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* and *5,* genes that encode 9-cis-epoxycarotenoid dioxygenase which is a key enzyme in the biosynthesis of abscisic acid
NIL	near-isogenic line
OST1	OPEN STOMATA 1, an SnRK2 which activates SLAC1 via phosphorylation
PATROL1	PROTON ATPASE TRANSLOCATION CONTROL 1, a protein which controls the location of H^+-ATPase in CO_2 signalling
PIP2;1	PLASMA MEMBRANE INTRINSIC PROTEIN 2;1, an aquaporin
PP2Cs	PROTEIN PHOSPHATASE 2Cs, negative regulators of ABA signalling

PYR/PYL/RCARs	PYRABACTIN RESISTANCE 1 (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR), ABA receptors
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RHC1	RESISTANT TO HIGH CARBON DIOXIDE 1, a MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE)-type transporter which may function as a bicarbonate sensing component in the CO_2 signalling pathway
ROS	reactive oxygen species
SA	salicylic acid, a plant hormone
SEM	standard error of the mean
Ser (S)	serine
SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1, a slow (S-type) anion channel which mediates nitrate and chloride efflux from guard cells
SLAH3	SLAC1 HOMOLOGUE 3, a slow (S-type) anion channel which mediates nitrate and chloride efflux from guard cells
SnRK2s	SUCROSE NON-FERMENTING 1 (SNF1)-RELATED KINASE 2s, positive regulators of ABA signalling
Strep-tag	an affinity tag used for purification of recombinant proteins, consists of eight amino acid residues
T-DNA	transfer DNA
Thr (T)	threonine
TILLING	Targeting Induced Local Lesions in Genomes
TPK1	TWO PORE K^+ CHANNEL 1, a voltage-independent vacuolar potassium channel which mediates potassium efflux from guard cell vacuoles
Tyr (Y)	tyrosine
Val (V)	valine
WUE	water use efficiency, ratio of the carbon assimilation to transpiration, reflects the water loss for the production of a unit of biomass
Y2H	yeast two-hybrid screening, a molecular biology technique used to discover protein-protein interactions
YFP	YELLOW FLUORESCENT PROTEIN, used as a reporter for protein localization in cells

INTRODUCTION

Plants are essential to life on Earth – in the process of photosynthesis, they use solar energy to convert carbon dioxide (CO_2) and water into organic compounds, whereas oxygen is released as a by-product. Organic compounds derived from CO_2 assimilation are used as a source of energy by nearly all living organisms. The breakdown of these organic compounds occurs via aerobic respiration which requires oxygen released in the photosynthetic process. The fixation of CO_2 and its release during the breakdown of organic molecules form the global carbon cycle.

Stomatal pores, that are on the surfaces of the majority of aerial parts of plants, facilitate gas exchange between plants and the external atmosphere. Each stomatal pore is surrounded by two highly specialized guard cells which sense various endogenous and environmental stimuli, such as CO_2, light, temperature, hormones and pathogens and adjust the stomatal pore size to balance CO_2 uptake for photosynthesis and loss of water vapour through transpiration. The opening and closing of stomatal pores are regulated by changes in guard cell volume. Rapid modulations in the guard cell volume are mediated by complex signalling networks and ion transport in guard cells.

Stomatal opening promotes plant growth by enhancing CO_2 uptake for photosynthesis and transpirational water loss which is essential for the uptake of nutrients from the soil. However, stomatal opening also causes undesirable water loss from plants under drought stress. Therefore, appropriate control of stomatal apertures is essential for the optimization of plant growth under multiple stress conditions in nature.

In the light, photosynthesis causes a reduction in the $[CO_2]$ in the intercellular space of leaves (C_i), which induces stomatal opening and influx of CO_2 for further assimilation. At the leaf level, the ratio of CO_2 uptake to water loss determines plant water use efficiency (WUE) which is a parameter of crop quality and performance under water deficit and is an important selection trait. In darkness, there is no photosynthesis, C_i rises and triggers stomatal closure. A continuing rise in atmospheric $[CO_2]$ alter rates of transpirational water loss and CO_2 uptake. The $[CO_2]$ inside the leaves further increases and causes a reduction in stomatal apertures globally, even during light periods. CO_2-induced stomatal closure reduces water loss and hence, it may improve plant water use efficiency.

Since water availability is a major constraint of crop yield and is the single most important factor limiting food production, optimization of CO_2-controlled stomatal movements may enable breeding of crops that conserve water while maximizing photosynthesis. While stomatal closure induced by elevated $[CO_2]$ may improve water use efficiency and yield of crop plants in water-limiting environments, development of plants which stomata respond less strongly to CO_2 and thus enable enhanced CO_2 uptake by leaves may increase crop yield in the regions where water resources are not limiting.

Presently, the molecular mechanisms by which plants sense CO_2 concentration and transduce the CO_2 signal to regulate water loss, are not fully understood. This thesis contributes to the understanding of the mechanism of how the CO_2 signal is transduced in the guard cells and proposes a new model for stomatal CO_2 signalling.

1. REVIEW OF LITERATURE

1.1. Ion channels and transporters in stomatal opening and closing

1.1.1. Ion channels and transporters in stomatal opening

During stomatal opening, which is induced by light, low [CO_2] or high humidity, an increase in guard cell volume is driven by accumulation of ions and/or solutes, mainly K^+, Cl^-, malate and sugars, which increases guard cell water potential and creates a driving force for water uptake from apoplast into guard cells (Schroeder *et al.*, 2001) (Figure 1). Increases in guard cell volume and hence turgor pressure widen the stomatal pore. Since mature guard cells lack plasmodesmata, solute uptake and efflux occur via plasma membrane ion channels and transporters (Wille and Lucas, 1984). The solute transported across the plasma membrane also pass across the vacuolar membrane (tonoplast) (Jezek and Blatt, 2017).

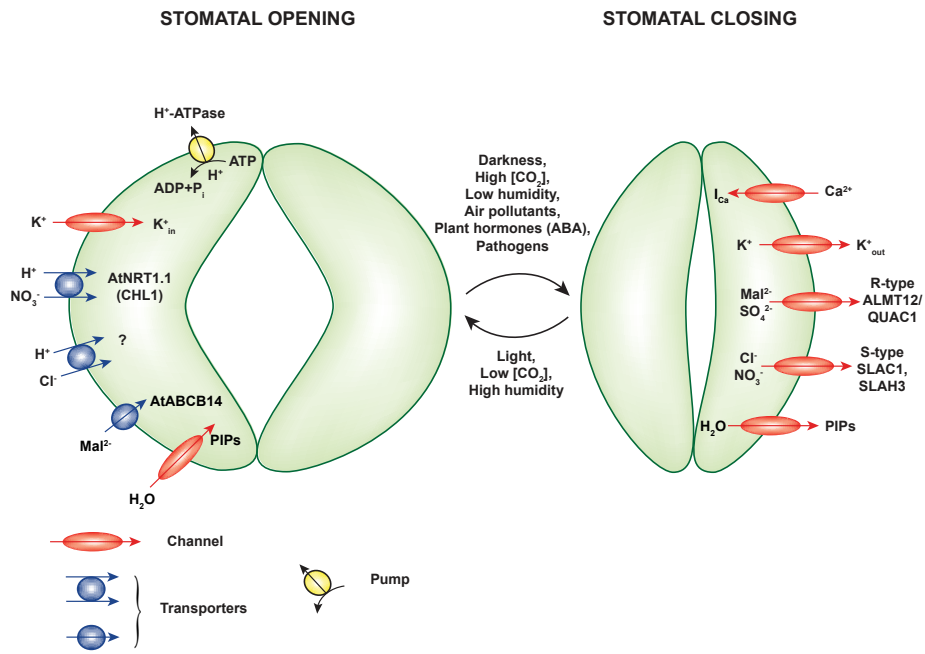

Figure 1. Guard cells respond to various stimuli causing stomata to open or close. Efflux of protons and influx of cations, anions and water leads to stomatal opening (left), whereas influx of Ca^{2+} and release of cations, anions and water leads to stomatal closure (right).

Stomatal opening requires activation of plasma membrane H^+-ATPases via phosphorylation of sites mainly clustered in the C-terminal domain (Kinoshita and Shimazaki, 1999, Rudashevskaya *et al.*, 2012). Phosphorylation of the penultimate Thr residue at the C-terminus by a yet unidentified kinase has been suggested to be involved in transducing the response to blue light (Svennelid *et al.*, 1999, Takemiya *et al.*, 2013, Haruta *et al.*, 2015). Activation of plasma membrane H^+-ATPases via phosphorylation drives H^+ efflux from the cytosol and hyperpolarization of the guard cell plasma membrane (Kinoshita and Shimazaki, 1999) (Figure 1).

Membrane hyperpolarization leads to activation of inward-rectifying K^+ channels including KAT1 (Anderson *et al.*, 1992, Schachtman *et al.*, 1992, Nakamura *et al.*, 1995, Ichida *et al.*, 1997) and KAT2 (Pilot *et al.*, 2001), and K^+ influx into guard cells (Schroeder *et al.*, 1987) (Figure 1). While the activity of the inward-rectifying K^+ channels is increased by extracellular acidification (Blatt, 1992), the channels are inhibited by elevated cytosolic free Ca^{2+} concentration (Schroeder and Hagiwara, 1990). The activity of the inward-rectifying K^+ channels can also be modulated by phosphorylation. For example, it has been proposed that phosphorylation of KAT1 and KAT2 by a Ca^{2+}-dependent protein kinase (CPK13) inhibits the channels and reduces stomatal opening (Ronzier *et al.*, 2014).

K^+ uptake during stomatal opening is balanced by uptake of counter ions Cl^-, NO_3^- and malate (Pandey *et al.*, 2007) (Figure 1). The molecular basis of the mechanism of Cl^- uptake is unknown. It is hypothesized that the influx of Cl^- occurs via H^+/anion symporters or OH^-/anion antiporters (Pandey *et al.*, 2007) (Figure 1). Uptake of NO_3^- has been reported to be mediated by a nitrate transporter encoded by *AtNRT1.1/CHL1* (Tsay *et al.*, 1993, Guo *et al.*, 2003) and malate has been shown to be transported into guard cells by a transporter AtABCB14 (Lee *et al.*, 2008) (Figure 1).

Accumulation of K^+ and anions followed by uptake of water leads to guard cell swelling and stomatal opening (Schroeder *et al.*, 1987) (Figure 1).

1.1.2. Ion channels in stomatal closing

During stomatal closure, which is induced by darkness, high [CO_2], low humidity, air pollutants (ozone, sulfur dioxide) pathogen elicitors or plant hormones (abscisic acid (ABA), methyl jasmonate (MeJA)), guard cells release ions/solutes and water which leads to a reduction in guard cell volume and turgor pressure and narrowing of the stomatal pore (Schroeder *et al.*, 2001) (Figure 1).

Stomatal closing requires extrusion of anions from guard cells through plasma membrane anion channels (Schroeder and Keller, 1992) (Figure 1). There are two types of anion channels in the guard cell plasma membrane which activation leads to anion efflux: rapid (R-type) and slow (S-type) anion channels (Schroeder and Keller, 1992) (Figure 1). Both types of anion channel currents are enhanced by increases in cytosolic Ca^{2+} concentration (Hedrich *et*

al., 1990, Schroeder and Hagiwara, 1990), and both are activated by depolarization (Keller *et al.*, 1989, Schroeder and Keller, 1992). The R-type channels exhibit rapid activation kinetics over a narrow voltage range, whereas the S-type channels activate over a much broader range of voltages and exhibit slow activation kinetics (Linder and Raschke, 1992, Schroeder and Keller, 1992). It has been reported that activation of the rapid and slow channels occurs within 5–50 ms and 5–50 s, respectively. (Linder and Raschke, 1992, Schroeder and Keller, 1992). The R-type channels show rapid deactivation at hyperpolarized potentials and are inactivated during prolonged stimulation (Linder and Raschke, 1992, Schroeder and Keller, 1992). In contrast, the S-type channels show extremely slow voltage-dependent deactivation (Linder and Raschke, 1992, Schroeder and Keller, 1992). The rapid channels are permeable to Cl^-, NO_3^-, SO_4^- and malate (Keller *et al.*, 1989, Hedrich and Marten, 1993, Roberts, 2006), and the slow channels exhibit permeability to NO_3^-, Cl^- and malate (Schmidt and Schroeder, 1994) (Figure 1). While the R-type channels provide a limited contribution to long-term anion efflux, the S-type channels provide a central molecular mechanism for controlling anion efflux and the resulting stomatal closure (Schroeder and Keller, 1992).

An R-type channel ALMT12/QUAC1 mediates anion efflux during stomatal closure (Meyer *et al.*, 2010) (Figure 1). ALMT12/QUAC1 is highly permeable to sulfate and malate and its activity depends on extracellular malate (Hedrich and Marten, 1993, Meyer *et al.*, 2010). The cytosolic C-terminus of ALMT12/QUAC1 has been proposed to be involved in the voltage-dependent deactivation mechanism of ALMT12/QUAC1 (Mumm *et al.*, 2013).

An S-type channel SLAC1 has been proposed to have an essential role in stomatal closure (Negi *et al.*, 2008, Vahisalu *et al.*, 2008). Besides *SLAC1*, its homologue *SLAH3* is also expressed in guard cells (Geiger *et al.*, 2011) (Figure 1). However, the channels have different biophysical properties. SLAH3 shows 20 times higher permeability to NO_3^- than to Cl^- (Geiger *et al.*, 2011), whereas SLAC1 is eight times more permeable to NO_3^- than to Cl^- (Geiger *et al.*, 2009). In addition to higher preference for nitrate, SLAH3 requires an increase in extracellular NO_3^- to induce its activity (Geiger *et al.*, 2011). SLAC1 has been shown to be impermeable to HCO_3^- and it exhibits negligible permeability to malate (Geiger *et al.*, 2009). Since SLAC1 and SLAH3 co-localize in the plasma membrane of guard cells, they may release Cl^- and NO_3^- in a concerted action during stomatal closure (Negi *et al.*, 2008, Vahisalu *et al.*, 2008, Geiger *et al.*, 2011). Both SLAC1 and SLAH3 are activated via phosphorylation (Geiger *et al.*, 2009, Geiger *et al.*, 2010, Geiger *et al.*, 2011). Following phosphorylation, the channels open likely by a conformational change.

Efflux of anions leads to depolarization of the plasma membrane (Linder and Raschke, 1992, Schroeder and Keller, 1992) which in turn deactivates inward-rectifying K^+ channels and activates outward-rectifying K^+ channels, resulting in K^+ efflux from guard cells (Schroeder *et al.*, 1987) (Figure 1). In contrast to the inward-rectifiers, the outward-rectifying channels are insensitive to cytosolic free Ca^{2+} concentration (Schroeder and Hagiwara, 1990) and their activity

is enhanced by cytosolic alkalinization (Blatt, 1992). It has been proposed that the activity of outward-rectifying K^+ channel GORK can be specifically enhanced by a Ca^{2+}-dependent protein kinase (CPK33) which results in faster stomatal closure (Ache *et al.*, 2000, Hosy *et al.*, 2003, Corratgé-Faillie *et al.*, 2017).

Release of anions and K^+ leads to a decrease in osmotic potential and creates a driving force for water efflux. Efflux of ions and water results in shrinkage of guard cells and stomatal closing (Schroeder *et al.*, 2001) (Figure 1).

During stomatal closure, an elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) arises from Ca^{2+} influx through plasma membrane Ca^{2+}-permeable channels (I_{Ca}) (Figure 1) and Ca^{2+} release from internal stores, such as endoplasmic reticulum, vacuole and plastids (McAinsh and Pittman, 2009). $[Ca^{2+}]_{cyt}$ has been observed to oscillate (Webb *et al.*, 1996b). Oscillations of $[Ca^{2+}]_{cyt}$ are likely generated by changes in the rate at which Ca^{2+} enters and exits the cytosol (Evans *et al.*, 2001). It has been suggested that stimulus-induced oscillations in $[Ca^{2+}]_{cyt}$ encode information that is used to specify the outcome of signalling pathways (Evans *et al.*, 2001, McAinsh and Pittman, 2009).

1.2. ABA signalling in guard cells

1.2.1. Abscisic acid

Abscisic acid (ABA) ($C_{15}H_{20}O_4$) is a sesquiterpenoid which belongs to a class of organic compounds known as isoprenoids or terpenoids (Nambara and Marion-Poll, 2005). ABA is an important phytohormone that controls plant growth and development and mediates plant protective responses against abiotic stresses, such as drought, cold and salinity (Cutler *et al.*, 2010). ABA is *de novo* biosynthesized from C_{40} carotenoids which originate from isopentenyl pyrophosphate synthesized in plastids through the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Nambara and Marion-Poll, 2005). Vascular tissues are probably the main sites of ABA biosynthesis from where ABA is transported to target cells. In addition, ABA could also be synthesized in guard cells and trigger in concert with transported ABA a downstream signalling cascade leading to stomatal closure (Nambara and Marion-Poll, 2005). The endogenous ABA level is modulated by the balance between biosynthesis and catabolism, as well as by the rate of transport to its sites of action. ABA is biologically inactivated either through hydroxylation and subsequent catabolic pathways or by conjugation with glucose (Nambara and Marion-Poll, 2005).

1.2.2. Core ABA signalling pathway

The earliest events of ABA signal transduction occur via a central signalling module comprised of proteins belonging to three protein classes: PYRABACTIN RESISTANCE 1 (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) (PYR/PYL/RCARs), which play a central role in ABA perception (Ma *et al.*, 2009, Park *et al.*, 2009), PROTEIN PHOSPHATASE 2Cs (PP2Cs), such as ABI1 and ABI2 (Gosti *et al.*, 1999, Merlot *et al.*, 2001), HAB1 (Leonhardt *et al.*, 2004) and PP2CA (Kuhn *et al.*, 2006), which act as negative regulators of ABA signalling, and SUCROSE NON-FERMENTING 1 (SNF1)-RELATED KINASE 2s (SnRK2s), such as OST1, which are positive regulators (Mustilli *et al.*, 2002, Yoshida *et al.*, 2006b) (Figure 2).

In the presence of ABA, PYR/PYL/RCARs form a complex with PP2Cs which leads to inhibition of the PP2C activity and relieves repression of positive factors, such as SnRK2s, which, in turn, target membrane proteins, ion channels and transcription factors (Danquah *et al.*, 2014, Munemasa *et al.*, 2015) (Figure 2). In guard cells, ABA triggers combined activation of S- and R-type anion channels which leads to transient depolarization of the plasma membrane (Roelfsema *et al.*, 2004). ABA can also cause $[Ca^{2+}]_{cyt}$ elevation in guard cells, and enhance the ability of $[Ca^{2+}]_{cyt}$ to activate the S-type anion channels (Hubbard *et al.*, 2012). Anion efflux through the activated anion channels, SLAC1 (Negi *et al.*, 2008, Vahisalu *et al.*, 2008) and QUAC1 (Meyer *et al.*, 2010, Sasaki *et al.*, 2010), and depolarization of the plasma membrane leads to K^+ efflux through outward-rectifying K^+ channels, such as GORK (Ache *et al.*, 2000, Hosy *et al.*, 2003) (Figure 2). Release of anions and K^+ leads to a decrease in osmotic potential and creates a driving force for water efflux through PIP2;1 aquaporins (Grondin *et al.*, 2015), which results in shrinkage of guard cells and stomatal closing (Figure 2).

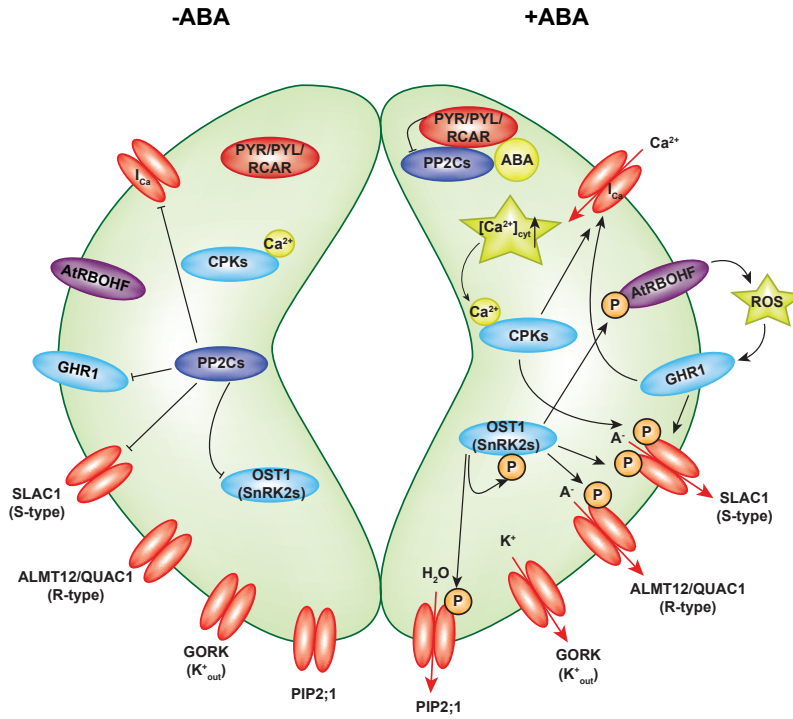

Figure 2. ABA signal transduction in guard cells (modified from Munemasa *et al*., 2015). (Left) In the absence of ABA, PP2Cs dephosphorylate SnRK2s and an S-type anion channel SLAC1 (Geiger *et al.*, 2009, Lee *et al.*, 2009b, Brandt *et al.*, 2012, Brandt *et al.*, 2015). CPKs are not deactivated by PP2Cs but PP2Cs prevent $[Ca^{2+}]_{cyt}$ activation of the S-type anion channels by direct dephosphorylation (Brandt *et al*., 2015). A PP2C, ABI2, also prevents SLAC1 activation by a plasma membrane leucine-rich repeat receptor-like kinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) (Hua *et al*., 2012).
(Right) In the presence of ABA, PYR/PYL/RCAR ABA receptors bind to and inhibit PP2Cs (Melcher *et al.*, 2009, Miyazono *et al.*, 2009, Nishimura *et al.*, 2009, Santiago *et al.*, 2009a, Yin *et al.*, 2009, Li *et al.*, 2015) which leads to activation of Ca^{2+}-independent protein kinases (SnRK2s) (Belin *et al.*, 2006, Ng *et al.*, 2011, Xie *et al.*, 2012). SnRK2s, in turn, activate S- and R-type anion channels (Geiger *et al.*, 2009, Lee *et al.*, 2009b, Vahisalu *et al.*, 2010, Imes *et al.*, 2013). Ca^{2+} influx through Ca^{2+}-permeable (I_{Ca}) channels results in $[Ca^{2+}]_{cyt}$ increases that activate CPKs which mediate Ca^{2+}-dependent regulation of the S-type anion channels (Kim *et al.*, 2010, Zhang *et al.*, 2014, Munemasa *et al.*, 2015). CPKs are also required for activation of the I_{Ca} channels (Munemasa *et al*., 2015). Activation of the S- and R-type anion channels causes plasma membrane depolarization, which drives K^+ efflux through voltage-dependent outward-rectifying K^+ channels, such as GORK (Ache *et al.*, 2000, Hosy *et al.*, 2003). The loss of K^+ and anions leads to a decrease in osmotic potential and creates a driving force for water efflux through PIP2;1 aquaporins (Grondin *et al.*, 2015), resulting in guard cell turgor decrease and stomatal closure. ROS, produced by a NADPH-oxidase (AtRBOHF) (Kwak *et al.*, 2003), could activate GHR1 that mediates ABA-induced elevation in $[Ca^{2+}]_{cyt}$ and activation of the S-type anion channels (Hua *et al*., 2012).

1.2.2.1. ABA receptors (PYR/PYL/RCARs)

There are 14 soluble PYR/PYL/RCAR proteins, highly conserved at the amino acid sequence level, in Arabidopsis (PYR1, PYL1-PYL13) (Ma *et al.*, 2009, Park *et al.*, 2009, Santiago *et al.*, 2009b). Some of them are dimeric (PYR1, PYL1, and PYL2), whereas others are monomeric (PYL4-PYL6 and PYL8-PYL10) or exist in monomer-dimer equilibrium (PYL3) in solution (Nishimura *et al.*, 2009, Santiago *et al.*, 2009a, Yin *et al.*, 2009, Dupeux *et al.*, 2011, Hao *et al.*, 2011, Nakagawa *et al.*, 2014, Li *et al.*, 2015). The monomeric receptors have generally higher binding affinities for ABA (K_d ~1 μM) than the dimeric receptors (K_d > 50 μM) in the absence of PP2Cs (Dupeux *et al.*, 2011, Hao *et al.*, 2011). However, in the presence of PP2Cs, both groups of receptors bind ABA with high affinity (K_d in the nanomolar range), indicating formation of a stable ternary complex (Ma *et al.*, 2009, Miyazono *et al.*, 2009, Santiago *et al.*, 2009b, Yin *et al.*, 2009). A number of monomeric receptors (PYL5-6, PYL8-10) are able to inhibit PP2Cs *in vitro* in the absence of ABA, whereas dimeric receptors (PYR1, PYL1-3) cannot (Dupeux *et al.*, 2011, Hao *et al.*, 2011). However, the inhibitory effect of the monomeric receptors is greatly reduced compared with their action in the presence of ABA, indicating that ABA enhances the affinity of the receptors for PP2Cs (Ma *et al.*, 2009, Santiago *et al.*, 2009b, Dupeux *et al.*, 2011, Hao *et al.*, 2011).

Structural studies have revealed a mechanism for PP2C inhibition by ABA receptors (Melcher *et al.*, 2009, Miyazono *et al.*, 2009, Nishimura *et al.*, 2009, Santiago *et al.*, 2009a, Yin *et al.*, 2009, Li *et al.*, 2015). Binding of ABA into the receptor binding pocket induces conformational changes resulting in the exposure of a hydrophobic surface on the ABA receptors that associates with the active site of PP2Cs. Interaction of PP2Cs with the hydrophobic surface of ABA-bound receptors inhibits PP2C phosphatase activity (Melcher *et al.*, 2009, Miyazono *et al.*, 2009, Nishimura *et al.*, 2009, Santiago *et al.*, 2009a, Yin *et al.*, 2009, Li *et al.*, 2015).

It has been suggested that the *PYR/PYL/RCAR* genes function redundantly in ABA signalling (Park *et al.*, 2009). Guard cells of *pyr1 pyl1 pyl2 pyl4* quadruple mutants showed impaired ABA-induced stomatal closure (Nishimura *et al.*, 2010), whereas guard cells of duodecuple *pyr1 pyl1 pyl2 pyl3 pyl4 pyl5 pyl7 pyl8 pyl9 pyl10 pyl11 pyl12* mutants exhibited an extreme insensitivity to ABA (Zhao *et al.*, 2018). Progressive inactivation of the *PYR/PYL/RCAR* genes also led to gradually higher steady-state stomatal conductance and had an additive effect on stomatal aperture (Gonzalez-Guzman *et al.*, 2012).

1.2.2.2. Protein phosphatase 2Cs (PP2Cs)

PP2Cs represent a major phosphatase family in plants. PP2Cs are monomeric serine/threonine phosphatases, which require Mn^{2+} or Mg^{2+} for their activity (Schweighofer *et al.*, 2004). Arabidopsis PP2Cs have been clustered into 10 groups (A-J) (Schweighofer *et al.*, 2004). At least six (ABI1, ABI2, HAB1/ P2C-HA, HAB2, AHG1, AHG3/PP2CA) of the nine PP2Cs from group A act as negative regulators of the ABA signalling pathway (Gosti *et al.*, 1999, Merlot *et al.*, 2001, Leonhardt *et al.*, 2004, Saez *et al.*, 2004, Kuhn *et al.*, 2006, Yoshida *et al.*, 2006b, Nishimura *et al.*, 2007, Rubio *et al.*, 2009) and have been shown to dephosphorylate certain serine/threonine protein kinases in subfamily 2 of SUCROSE NON-FERMENTING 1 (SNF1)-related kinases (SnRK2s) (Lee *et al.*, 2009b, Umezawa *et al.*, 2009, Vlad *et al.*, 2009).

Mutations in genes that encode PP2Cs (HAB1, AHG3) caused ABA hypersensitive phenotypes (Leonhardt *et al.*, 2004, Saez *et al.*, 2004, Kuhn *et al.*, 2006), whereas overexpression led to reduced ABA sensitivity (Saez *et al.*, 2004, Kuhn *et al.*, 2006). Plants carrying dominant mutations *abi1-1* or *abi2-1* exhibited ABA insensitivity (Koornneef *et al.*, 1984, Leung *et al.*, 1994, Meyer *et al.*, 1994, Leung *et al.*, 1997, Rodriguez *et al.*, 1998), whereas revertants of *abi1-1* were hypersensitive to ABA and revertants of *abi2-1* displayed wild-type ABA sensitivity (Gosti *et al.*, 1999, Merlot *et al.*, 2001).

1.2.2.3. SNF1-related kinases (SnRK2s)

In Arabidopsis, there are 10 SnRK2s, designated as SRK2A-SRK2J (Yoshida *et al.*, 2002) or SnRK2.1-SnRK2.10 (Hrabak *et al.*, 2003, Kobayashi *et al.*, 2004). OPEN STOMATA 1 (OST1)/SnRK2.6/SRK2E has been shown to play a key role in stomatal responses to ABA (Merlot *et al.*, 2002, Mustilli *et al.*, 2002, Yoshida *et al.*, 2002, Xie *et al.*, 2006). Recessive *ost1* mutants (*ost1-1*, *ost1-2*, *ost1-4*) displayed impaired ABA-induced stomatal closure as well as inhibition of light-induced stomatal opening (Merlot *et al.*, 2002, Mustilli *et al.*, 2002, Xie *et al.*, 2006). In contrast, stomatal regulation by darkness, light or CO_2-free air was not affected (Mustilli *et al.*, 2002). OST1 shares a role with SnRK2.2/ SRK2D and SnRK2.3/SRK2I in seed germination and seedling development, whereas SnRK2.2 and SnRK2.3 contribute to stomatal regulation. *snrk2.2/ 2.3/2.6* triple-mutants showed an extreme insensitivity to ABA in seed germination and seedling development and had completely disrupted stomatal responses to ABA (Fujii and Zhu, 2009, Fujita *et al.*, 2009, Nakashima *et al.*, 2009).

Structural studies have provided insights into the mechanisms of activation and inhibition of SnRK2s (Ng *et al.*, 2011, Soon *et al.*, 2012, Xie *et al.*, 2012, Zhou *et al.*, 2012). It has been proposed that in the presence of ABA, SnRK2s are activated via a two-step mechanism: 1) ABA-mediated partial activation through the release of PP2C-mediated inhibition, 2) full activation via autophosphorylation of S175 (Belin *et al.*, 2006, Ng *et al.*, 2011, Xie *et al.*, 2012).

In the absence of ABA, SnRK2s interact with PP2Cs (Yoshida *et al.*, 2006a, Lee *et al.*, 2009b, Umezawa *et al.*, 2009, Vlad *et al.*, 2009) which directly regulate the activity of SnRK2s (Umezawa *et al.*, 2009). Similarly to the activation, SnRK2s are inhibited via a two-step mechanism: 1) the kinase activity is partially reduced via dephosphorylation of the critical pS175 residue, and 2) the kinase is fully inhibited by forming a stable complex, in which the catalytic sites of the PP2C and the SnRK2 directly interact with each other (Umezawa *et al.*, 2009, Vlad *et al.*, 2009, Soon *et al.*, 2012, Xie *et al.*, 2012, Zhou *et al.*, 2012).

1.2.2.4. Targets of the ABA-activated OST1 kinase

Several proteins involved in controlling the stomatal aperture have been identified as targets of the OST1 kinase (Figure 2): a NADPH oxidase catalytic subunit AtRBOHF (Sirichandra *et al.*, 2009), anion channels SLAC1(Geiger *et al.*, 2009, Lee *et al.*, 2009b, Vahisalu *et al.*, 2010, Brandt *et al.*, 2012) and ALMT12/QUAC1 (Imes *et al.*, 2013), an inward-rectifying K^+ channel KAT1 (Sato *et al.*, 2009), a K^+ uptake transporter KUP6 (Osakabe *et al.*, 2013) and PIP2;1 aquaporins (Grondin *et al.*, 2015).

Phosphorylation of Ser13 and Ser174 in the N-terminal cytosolic domain of AtRBOHF by OST1 was proposed to regulate the activity of AtRBOHF in response to ABA (Sirichandra *et al.*, 2009). ABA induces production of ROS by AtRBOHF and AtRBOHD in Arabidopsis guard cells (Kwak *et al.*, 2003) (Figure 2). ROS, in turn, activates plasma membrane Ca^{2+}-permeable channels (I_{Ca}) and elevates $[Ca^{2+}]_{cyt}$ (Allen *et al.*, 2000, McAinsh *et al.*, 2000, Pei *et al.*, 2000, Murata *et al.*, 2001, Wang *et al.*, 2013) (Figure 2). Elevated $[Ca^{2+}]_{cyt}$ is linked to downstream signalling events via several Ca^{2+} sensors, including Ca^{2+}-dependent protein kinases (CPKs), which mediate Ca^{2+}-dependent regulation of S-type anion channels (Kim *et al.*, 2010, Zhang *et al.*, 2014, Munemasa *et al.*, 2015) (Figure 2). In addition to the Ca^{2+}-permeable channels, ROS could activate a plasma membrane leucine-rich repeat receptor-like kinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1 (GHR1) that mediates ABA-induced elevation in $[Ca^{2+}]_{cyt}$ and activation of the S-type anion channels (Hua *et al.*, 2012) (Figure 2). ROS may also activate MAPK cascades in guard cell signalling to promote stomatal closure (Danquah *et al.*, 2014, Liu and He, 2017).

SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) has been reported as the most likely S-type anion channel required for the plasma membrane depolarization and stomatal closure (Negi *et al.*, 2008, Vahisalu *et al.*, 2008) (Figure 2). Mutations in *SLAC1* resulted in a strong impairment of the S-type anion channel activity and over-accumulation of osmoregulatory anions in guard cell protoplasts (Negi *et al.*, 2008, Vahisalu *et al.*, 2008). Stomata of *SLAC1* mutants (*slac1-1* and *slac1-2*) exhibited a strong insensitivity to ABA and were defective in regulation of transpiration in response to drought stress

(Negi *et al.*, 2008, Vahisalu *et al.*, 2008). Phosphorylation of Ser59 and Ser120 in the N-terminal domain of SLAC1 has been suggested to be required for ABA-induced stomatal closure (Vahisalu *et al.*, 2010, Brandt *et al.*, 2015). The activation of SLAC1 could be abolished by PP2Cs which could directly dephosphorylate SLAC1 or inhibit the activity of OST1 and thus prevent the phosphorylation of SLAC1 (Geiger *et al.*, 2009, Lee *et al.*, 2009b, Brandt *et al.*, 2012, Brandt *et al.*, 2015) (Figure 2).

Besides the S-type channel SLAC1, an R-type channel ALMT12/QUAC1 also mediates anion efflux during ABA-induced stomatal closure (Meyer *et al.*, 2010, Imes *et al.*, 2013) (Figure 2). OST1 interacted with ALMT12/QUAC1 and enhanced its activity in *X. laevis* oocytes (Imes *et al.*, 2013). Plants carrying mutations in *ALMT12/QUAC1* (*almt12-1* and *almt12-2*) exhibited partially impaired ABA-induced stomatal closure (Meyer *et al.*, 2010).

A K^+ transporter KUP6 has also been reported to be one of the targets of the ABA-activated OST1 kinase (Osakabe *et al.*, 2013). OST1 interacted with KUP6 *in planta* and phosphorylated its C-terminal region (Osakabe *et al.*, 2013). The guard cells of *kup6 kup8 gork* triple-mutant showed strongly impaired ABA-sensitivity, indicating that KUP6, together with KUP8 and GORK, could mediate K^+ efflux during ABA-induced stomatal closure (Hosy *et al.*, 2003, Osakabe *et al.*, 2013).

ABA-dependent water permeability of guard cells has been suggested to be mediated by PIP2;1 aquaporins (Grondin *et al.*, 2015). OST1 phosphorylated Ser121 in the cytosolic peptide of the plasma membrane intrinsic protein 2;1 (PIP2;1 aquaporin) (Figure 2) *in vitro* and enhanced the PIP2;1 water transport activity in *X. laevis* oocytes (Grondin *et al.*, 2015). Expression of a phosphomimetic form (Ser121Asp) of PIP2;1 in *pip2;1* plants constitutively enhanced water permeability of guard cells and restored ABA-dependent stomatal closure (Grondin *et al.*, 2015).

ABA not only induces stomatal closure by triggering efflux of anions and potassium via plasma membrane ion channels but also inhibits ion uptake which is required for stomatal opening (Schroeder *et al.*, 2001). Phosphorylation of Thr306 in the C-terminal region of an inward-rectifying K^+ channel KAT1 by OST1 has been suggested to be required for ABA-induced inhibition of inward K^+ currents which prevents stomatal opening (Sato *et al.*, 2009, Acharya *et al.*, 2013). Since ABA-activated OST1 promoted ROS production in guard cells, the inhibition of inward K^+ channels by OST1 might also occur indirectly (Acharya *et al.*, 2013). Namely, ABA-mediated ROS production in guard cells has been shown to inhibit H^+-ATPase activity which leads to plasma membrane depolarization (Zhang *et al.*, 2004). Depolarization, in turn, prevents voltage-dependent activation of the inward K^+ channels and stomatal opening (Acharya *et al.*, 2013).

1.3. CO_2 signalling in guard cells

Leaf stomata close in response to higher than ambient CO_2 concentrations and conversely, low [CO_2] triggers opening of stomata. The CO_2 concentration in leaves changes as a result of photosynthesis and respiration (Engineer *et al.*, 2016).

Both mesophyll and guard cells could contribute to CO_2 sensing (Lawson, 2009, Mott, 2009, Lawson *et al.*, 2014, Engineer *et al.*, 2016). Experiments have shown that mesophyll tissue enhances the stomatal response to high and low [CO_2] (Mott *et al.*, 2008, Mott, 2009, Fujita *et al.*, 2013). However, the precise nature of the mesophyll to stomata signal is unknown and it remains to be determined whether the level of mesophyll-derived amplifying factor is itself regulated by CO_2 concentration changes or is it constitutively present and amplifies CO_2 sensing mechanisms in guard cells (Mott, 2009, Lawson *et al.*, 2014, Engineer *et al.*, 2016). The ability of stomata to respond to [CO_2] changes in isolated epidermis suggests that the components for CO_2 sensing and signal transduction reside inside the guard cells (Mott, 2009). However, the primary CO_2/HCO_3^- sensors remain unknown. Several proteins have been reported to control stomatal aperture in response to CO_2 concentration changes, including carbonic anhydrases (Hu *et al.*, 2010), a MATE-type transporter RHC1 (Tian *et al.*, 2015), protein kinases HT1 (Hashimoto *et al.*, 2006) and OST1 (Xue *et al.*, 2011), anion channels SLAC1 (Negi *et al.*, 2008, Vahisalu *et al.*, 2008) and ALMT12/QUAC1 (Meyer *et al.*, 2010), and a Munc13-like protein PATROL1 (Hashimoto-Sugimoto *et al.*, 2013) (Figure 3) but it remains to be clarified how these components interact and are integrated into the signalling network.

Low [CO_2]- and light-induced stomatal opening pathways have been suggested to interact with one another. However, the degree of interaction needs to be elucidated (Lawson, 2009, Mott, 2009, Lawson *et al.*, 2014). Also, it is a subject of debate whether stomatal responses to CO_2 and light are linked to mesophyll photosynthesis via the mesophyll signal or whether guard cell photosynthesis itself provides a metabolite signal which induces stomatal movements (Lawson, 2009, Mott, 2009, Lawson *et al.*, 2014).

This thesis elucidates a mechanism of how the CO_2 signal is transduced in the guard cells to promote stomatal closure.

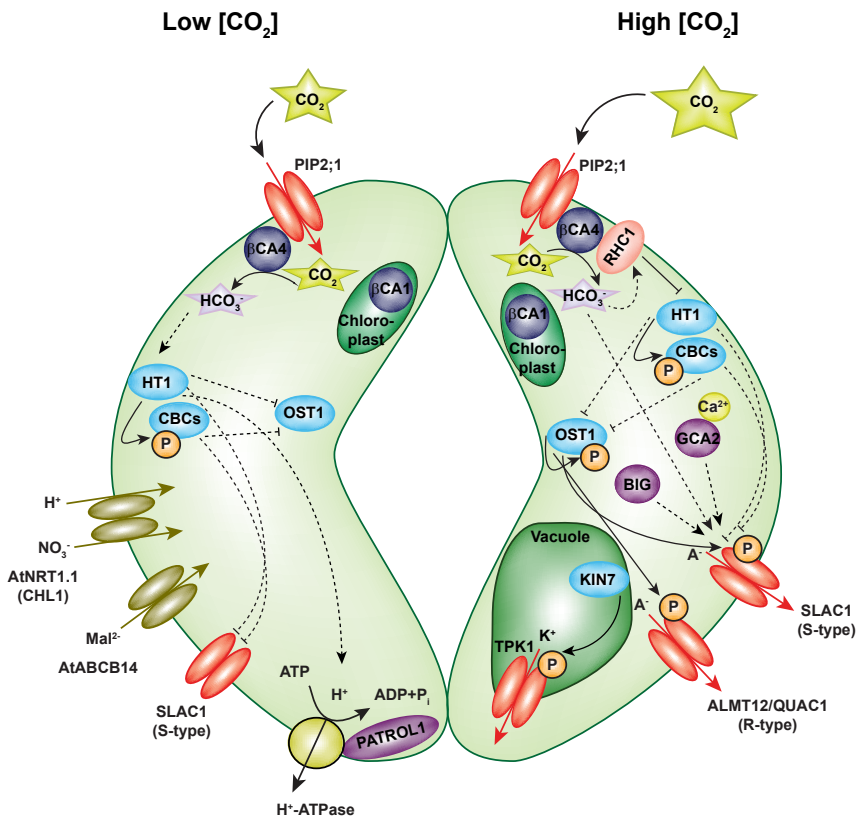

Figure 3. CO_2 signal transduction in guard cells.
(Left) Activation of H^+-ATPases and stomatal opening in response to low [CO_2] remain to be elucidated. Protein kinases (HT1, CBCs) (Hashimoto *et al.*, 2006, Hiyama *et al.*, 2017), ion transporters (AtNRT1.1/CHL1 and AtABCB14) (Guo *et al.*, 2003, Lee *et al.*, 2008), and a Munc13-like protein PATROL1 (Hashimoto-Sugimoto *et al.*, 2013) have been proposed to have a role in low [CO_2]-induced stomatal opening.
(Right) The elevated CO_2 concentration in leaves leads to an enhanced influx of CO_2 into guard cells through PIP2;1 aquaporins (Wang *et al.*, 2016b). The PIP2;1 aquaporins interact with a βCA4 carbonic anhydrase (Wang *et al.*, 2016b), which together with a βCA1 accelerate the formation of bicarbonate that acts as an intracellular signalling molecule in guard cells (Hu *et al.*, 2010, Xue *et al.*, 2011, Tian *et al.*, 2015, Wang *et al.*, 2016b). Protein kinases (HT1, OST1, CBCs, KIN7) (Hashimoto *et al.*, 2006, Xue *et al.*, 2011, Hiyama *et al.*, 2017, Isner *et al.*, 2018), intracellular calcium ions (Young *et al.*, 2006) and ion channels (SLAC1, ALMT12/QUAC1, TPK1) (Negi *et al.*, 2008, Vahisalu *et al.*, 2008, Meyer *et al.*, 2010, Xue *et al.*, 2011, Isner *et al.*, 2018) mediate CO_2-induced stomatal closure. However, it remains to be elucidated how these components interact and are integrated into the signalling network. Arrows and blocks represent positive and negative regulation, respectively. Regulation pathways are predicted to be direct (lines) or are unknown and remain to be further investigated (dashed lines).

1.3.1. Roles for aquaporins and carbonic anhydrases in guard cell CO_2 signalling

Most of the plant aquaporins facilitate transmembrane water transport, but they can also transport small neutral molecules such as gases (CO_2, ammonia), reactive oxygen species (hydrogen peroxide), and metalloids (boric acid, silicic acid, antimonite, arsenite) (Maurel *et al.*, 2016). Plasma membrane intrinsic proteins (PIPs), which constitute one of the subfamilies of aquaporins (Groszmann *et al.*, 2016), can transport CO_2 through the membranes of guard cells (Heckwolf *et al.*, 2011, Uehlein *et al.*, 2012, Heinen *et al.*, 2014, Groszmann *et al.*, 2016, Wang *et al.*, 2016b). However, their role in stomatal regulation remains to be clarified, since CO_2- and ABA-induced stomatal closure was not significantly impaired in *pip2;1* mutants (Wang *et al.*, 2016b).

PIP2;1 aquaporins interact with a beta-carbonic anhydrase (βCA4) (Wang *et al.*, 2016b) which accelerates the conversion of CO_2 into HCO_3^- (Figure 3) (Hu *et al.*, 2010). It has been suggested that HCO_3^- acts as an intracellular signalling molecule that mediates CO_2 signal transduction in guard cells (Hu *et al.*, 2010, Xue *et al.*, 2011, Tian *et al.*, 2015, Wang *et al.*, 2016b). βCAs likely do not function as noncatalytic CO_2 receptors, since the activity of carbonic anhydrases was required for CO_2-mediated stomatal regulation (Hu *et al.*, 2010, Hu *et al.*, 2015).

Two of six carbonic anhydrases (βCAs) in Arabidopsis, βCA1 and βCA4, are highly expressed in guard cells (Hu *et al.*, 2010). The plasma membrane-localized βCA4 and the βCA1, which is localized in chloroplast, have been shown to function in early CO_2 signal transduction cascade (Hu *et al.*, 2010, Hu *et al.*, 2015) (Figure 3). *ca1 ca4* double-mutant plants displayed greatly impaired low- and high [CO_2]-induced stomatal responses, whereas ABA-induced stomatal closing and blue light-induced opening remained functional (Hu *et al.*, 2010). βCA-mediated CO_2-triggered stomatal responses were proposed not to be directly linked to photosynthesis (Hu *et al.*, 2010).

1.3.2. Transporters in guard cell CO_2 signalling

A carbonic anhydrase βCA4 interacts with a plasma membrane-localized MULTIDRUG AND TOXIC COMPOUND EXTRUSION (MATE)-type transporter, RESISTANT TO HIGH CARBON DIOXIDE 1 (RHC1) (Tian *et al.*, 2015) (Figure 3). It has been suggested that RHC1 may function as a bicarbonate sensing component in the CO_2 signalling pathway (Tian *et al.*, 2015). Recently, it was also reported that intracellular bicarbonate ions could directly interact with an S-type anion channel SLAC1 and upregulate its activity (Zhang *et al.*, 2018) (Figure 3). Thus, SLAC1 might function as a secondary HCO_3^- sensor in guard cells (Zhang *et al.*, 2018). However, the precise mechanism by which intracellular bicarbonate directly affects the activity of SLAC1 remains to be elucidated (Zhang *et al.*, 2018).

A plasma membrane-localized ABC transporter ATP-BINDING CASSETTE B14 (AtABCB14) and a nitrate transporter NITRATE TRANSPORTER 1.1/CHLORINA 1 (NRT1.1/CHL1) have also been suggested to have a role in CO_2-induced stomatal movements (Guo *et al.*, 2003, Lee *et al.*, 2008) (Figure 3). The transporter AtABCB14, which mediates malate uptake into guard cells, might function as a negative regulator of CO_2-induced stomatal closure (Lee *et al.*, 2008). Besides mediating nitrate accumulation into guard cells during the light-induced stomatal opening, it is likely that the NRT1.1/CHL1 also functions in low [CO_2]-induced stomatal opening pathway (Guo *et al.*, 2003).

1.3.3. Protein kinases in guard cell CO_2 signalling

HIGH LEAF TEMPERATURE1 (*HT1*) is mainly expressed in guard cells and encodes a Ca^{2+}-independent Ser/Thr kinase which requires Mg^{2+} for its activity (Hashimoto *et al.*, 2006, Hashimoto-Sugimoto *et al.*, 2016). The plasma membrane-associated HT1 kinase is predicted to be a Raf-like Group C MAPKKK which has been shown to have a CO_2-specific role in guard cells (Ichimura *et al.*, 2002, Hashimoto *et al.*, 2006, Hashimoto-Sugimoto *et al.*, 2016) (Figure 3).

Recessive *ht1-2* and dominant *ht1-3* mutants exhibited completely disrupted stomatal responses to low and high [CO_2] but retained normal response to ABA (Hashimoto *et al.*, 2006, Hashimoto-Sugimoto *et al.*, 2016). The *ht1-2* mutants carried a deletion of highly conserved amino acids at the kinase domain, which led to a disruption of the HT1 kinase activity and resulted in plants which stomata showed a constitutive high [CO_2] response (Hashimoto *et al.*, 2006, Hashimoto-Sugimoto *et al.*, 2016). In contrast, the *ht1-3* mutants, which had widely open stomata, harboured a mutation at position 102 (R102K) in a non-conserved region, which did not significantly affect the kinase activity but was proposed to affect HT1 interactions with its targets (Hashimoto-Sugimoto *et al.*, 2016).

HT1 has been indicated to be epistatic to *βCA1* and *βCA4* since stomata of *ca1 ca4 ht1-2* triple-mutant plants exhibited a constitutive high [CO_2] phenotype similar to the *ht1-2* mutants (Hu et al., 2010). The constitutive high [CO_2] response was also observed in *rhc1 ht1-2* and *ost1-3 ht1-2* double-mutants, indicating that *HT1* was also epistatic to *RHC1* and *OST1* (Matrosova *et al.*, 2015, Tian *et al.*, 2015).

In addition to CO_2-induced stomatal movements, HT1 might function in the light-induced stomatal opening pathway (Hashimoto *et al.*, 2006, Matrosova *et al.*, 2015). The *ht1-2* mutants showed an impaired red light-induced stomatal opening, whereas the response to blue light was functional (Hashimoto *et al.*, 2006, Matrosova *et al.*, 2015).

CONVERGENCE OF BLUE LIGHT AND CO_2 1 (*CBC1*) and *CBC2* encode Ser/Thr protein kinases which belong to a Group C of MAPKKKs (Ichimura *et al.*, 2002, Hiyama *et al.*, 2017). CBCs interacted with the HT1 kinase and were

proposed to be the substrates of HT1 (Hiyama *et al.*, 2017) (Figure 3). *cbc1 cbc2* double-mutant plants showed constitutively closed stomata that did not respond to [CO_2] concentration changes and blue light but exhibited a normal response to ABA (Hiyama *et al.*, 2017). CBCs were suggested to integrate the signals from blue light and low [CO_2] and induce stomatal opening by inhibiting S-type anion channels either directly via phosphorylation or indirectly via other kinases and phosphatases (Hiyama *et al.*, 2017).

Besides playing a central role in ABA signalling cascade, OST1 mediates CO_2 signal transduction in guard cells (Xue *et al.*, 2011) (Figure 3). *ost1* mutants (*ost1-1*, *ost1-2*, *ost1-3*) exhibited impaired CO_2-induced stomatal closure (Xue *et al.*, 2011, Merilo *et al.*, 2013) and HCO_3^-/CO_2-induced activation of S-type anion currents (Xue *et al.*, 2011). OST1 was suggested to be phosphorylated by HT1, which was proposed to inhibit the OST1 kinase activity and prevent the activation of SLAC1 by OST1 (Tian *et al.*, 2015).

1.3.4. Ion channels in guard cell CO_2 signalling

Elevated intracellular [HCO_3^-] mediates the activation of S-type anion currents in guard cells (Xue *et al.*, 2011, Wang *et al.*, 2016b) (Figure 3). An S-type anion channel SLAC1 was demonstrated to be a central positive mediator of CO_2-induced stomatal closure (Negi *et al.*, 2008, Vahisalu *et al.*, 2008, Xue *et al.*, 2011) (Figure 3). *slac1* mutants (*slac1-1*, *slac1-3*) exhibited impaired elevated [CO_2]-induced stomatal closure and bicarbonate-induced activation of S-type anion currents (Vahisalu *et al.*, 2008, Xue *et al.*, 2011).

Compared with ABA-triggered activation, SLAC1 may require phosphorylation at different sites to activate in response to CO_2 (Yamamoto *et al.*, 2016). Since CO_2- but not ABA-sensitive phenotype was restored in *slac1-4* plants expressing a truncated SLAC1 protein (SLAC1 ΔNC), it is likely that phosphorylation of the N- and C-terminal regions of SLAC1 are not essential for CO_2-induced stomatal closure (Yamamoto *et al.*, 2016). SLAC1 was proposed to activate in response to CO_2 via phosphorylation of Tyr243 located on the cytoplasmic side of the membrane since *slac1-2* mutants expressing a SLAC1 Y243F protein showed impaired stomatal response to CO_2 but intact response to ABA (Yamamoto *et al.*, 2016).

Recently, a putative bicarbonate interacting residue (Arg256) within the cytosolic side of SLAC1 anion channel was identified (Zhang *et al.*, 2018). A mutation in Arg256 impaired CO_2/HCO_3^- but not ABA-induced activation of SLAC1, indicating that Arg256 might be required for CO_2- but not ABA-induced stomatal closure (Zhang *et al.*, 2018). Thus, SLAC1 may function not only in anion transport but also as a secondary HCO_3^- sensor in guard cells (Zhang *et al.*, 2018).

Besides the S-type anion channel SLAC1, a guard cell R-type anion channel ALMT12/QUAC1 contributes to CO_2-induced stomatal closing (Meyer *et al.*,

2010) (Figure 3). Plants lacking ALMT12/QUAC1 showed partially impaired high [CO_2]-induced stomatal response (Meyer *et al.*, 2010).

Stomatal closing also requires K^+ efflux from guard cell vacuoles via vacuolar K^+ channels (Isner *et al.*, 2018) (Figure 3). A vacuolar K^+ channel TPK1 and a receptor-like protein kinase KIN7, which is responsible for phosphorylation of TPK1, have been suggested to be involved in guard cell CO_2 and ABA signalling (Isner *et al.*, 2018) (Figure 3). *tpk1* mutants showed reduced ABA-induced stomatal closure, whereas the guard cells of *kin7* mutants displayed delayed ABA response (Isner *et al.*, 2018). However, both *tpk1* and *kin7* mutants were markedly unresponsive to elevated [CO_2] (Isner *et al.*, 2018).

1.3.5. Other proteins identified to function in guard cell CO_2 signalling

A Munc13-like protein PROTON ATPASE TRANSLOCATION CONTROL 1 (PATROL1) has been indicated to be essential for stomatal opening in response to low [CO_2] (Hashimoto-Sugimoto *et al.*, 2013) (Figure 3). *patrol1* mutants showed impaired CO_2-dependent leaf temperature change and stomatal opening in response to low [CO_2] (Hashimoto-Sugimoto *et al.*, 2013). The *patrol1* mutation also disturbed the plasma membrane targeting of a H^+-ATPase AHA1, indicating that PATROL1 functioned in targeting the AHA1 to the plasma membrane (Hashimoto-Sugimoto *et al.*, 2013) (Figure 3). However, it remains to be clarified, how the H^+-ATPase is regulated by CO_2.

Recently, a novel allele of the Arabidopsis *BIG* locus was identified (He *et al.*, 2018) (Figure 3). The *BIG* gene was suggested to have a specific role in high [CO_2]-induced stomatal closure (He *et al.*, 2018). *BIG* mutants were compromised in elevated [CO_2]-induced stomatal closure and bicarbonate activation of S-type anion currents but showed intact response to ABA (He *et al.*, 2018). In addition to controlling stomatal aperture, *BIG* was also suggested to be required for the control of stomatal development by elevated [CO_2] (He *et al.*, 2018).

Similarly to ABA, high [CO_2] induces an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) (Webb *et al.*, 1996a), which is required for CO_2-induced activation of S-type anion channels (Xue *et al.*, 2011). CO_2 was demonstrated to modulate $[Ca^{2+}]_{cyt}$ transient patterns and prime Ca^{2+} sensors which could mediate specificity in Ca^{2+} signalling in Arabidopsis guard cells (Young *et al.*, 2006). An ABA-insensitive mutant, *gca2* (*growth controlled by abscisic acid*), showed similar $[Ca^{2+}]_{cyt}$ transient rate at low and high [CO_2], and exhibited impaired stomatal closure in response to high [CO_2] (Young *et al.*, 2006). Since the *gca2* mutant also showed an altered ABA-induced $[Ca^{2+}]_{cyt}$ pattern in guard cells (Allen *et al.*, 2001), GCA2 might function downstream of, or at the convergence point of CO_2 and ABA signalling (Young *et al.*, 2006).

1.3.6. Convergence of CO_2 and ABA signalling

The molecular, biochemical and cellular mechanisms underlying interactions between CO_2 and ABA signalling, as well as the precise signalling convergence point remain to be resolved (Engineer *et al.*, 2016). It has been proposed that the convergence point of CO_2 and ABA signalling may locate downstream of ABA synthesis and ABA receptors but upstream of, or at the level of OST1 activation. Alternatively, elevated [CO_2] may increase ABA concentration or ABA signal transduction in guard cells to mediate stomatal closing (Xue *et al.*, 2011, Merilo *et al.*, 2013, Chater *et al.*, 2015, Tian *et al.*, 2015).

Recently, new insights into the interaction between CO_2 and ABA signalling were provided (Hsu *et al.*, 2018). It was proposed that CO_2 and ABA signal transduction converge downstream of OST1 activation and that basal ABA signalling and OST1 activity enhance stomatal closure in response to elevated [CO_2] (Hsu *et al.*, 2018) (Figure 4). It was reported that stomata of ABA biosynthesis mutants (*nced3 nced5* and *aba2-1*) remained responsive to [CO_2] elevation, whereas stomata of ABA receptor quadruple (*pyr1 pyl1 pyl2 pyl4*) and hextuple (*pyr1 pyl1 pyl2 pyl4 pyl5 pyl8*) mutants displayed delayed response to high [CO_2] (Hsu *et al.*, 2018). Also, elevated [CO_2] did not trigger an increase in ABA concentration in guard cells and did not enhance the kinase activity of OST1 (Hsu *et al.*, 2018).

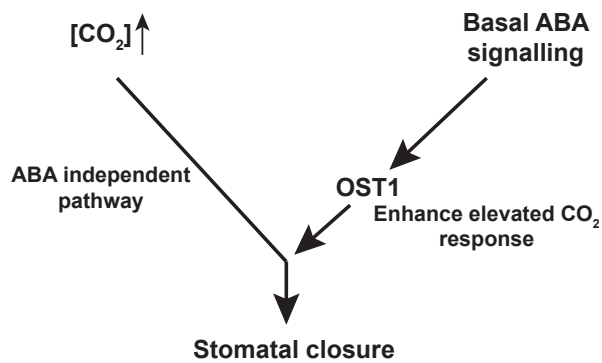

Figure 4. Interaction between elevated [CO_2] and ABA signal transduction pathways (from Hsu *et al.*, 2018).
Elevated [CO_2] and ABA signal transduction pathways converge downstream of OST1 activation. The elevated [CO_2] signal transduction pathway is ABA-independent, but basal ABA-signal transduction and OST1 kinase activity are required for amplifying and accelerating the CO_2-induced stomatal closure.

1.4. Plant mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinase (MAPK) cascades mediate intracellular transmission and amplification of extracellular stimuli, resulting in the induction of appropriate cellular responses (Ichimura *et al.*, 2002, Suarez-Rodriguez *et al.*, 2010). In plants, MAPK cascades are involved in various physiological (Rasmussen *et al.*, 2012, Meng and Zhang, 2013), developmental (Xu and Zhang, 2015) and hormonal (Jagodzik *et al.*, 2018) responses, and are also included in guard cell signalling networks which are involved in regulation of stomatal apertures in response to endogenous and environmental stimuli (Zhang *et al.*, 2014, Lee *et al.*, 2016).

MAPK cascades are composed of at least three types of reversibly phosphorylated kinases, MAPK kinase kinases (MAP3Ks, MAPKKKs or MKKKs), MAPK kinases (MAP2Ks, MAPKKs, MKKs or MEKs) and MAPKs (MPKs), which are highly conserved enzymes in all eukaryotes (Ichimura *et al.*, 2002, Colcombet and Hirt, 2008, Suarez-Rodriguez *et al.*, 2010). Often signals received by receptor proteins activate MAP3Ks, which are Ser/Thr kinases and phosphorylate two amino acid residues of downstream MAP2Ks at a conserved S/T-X_{3-5}-S/T motif (X denotes any amino acid residue) of the MAP2K activation loop (Ichimura *et al.*, 2002, Colcombet and Hirt, 2008, Suarez-Rodriguez *et al.*, 2010). Activated MAP2Ks, in turn, phosphorylate downstream MAPKs on Thr and/or Tyr residues at a conserved T-X-Y motif (Ichimura *et al.*, 2002, Colcombet and Hirt, 2008, Suarez-Rodriguez *et al.*, 2010). Phosphorylated MAPKs then transduce the signals to various effector proteins in the cytoplasm or nucleus, which include other kinases, enzymes or transcription factors, regulating their functional activity, turnover and localization (Ichimura *et al.*, 2002, Colcombet and Hirt, 2008, Suarez-Rodriguez *et al.*, 2010). The signalling specificity of MAPK cascades can be maintained by spaciotemporal constraints and dynamic protein-protein interactions, and by mechanisms that include cross-inhibition, feedback control, and scaffolding (Suarez-Rodriguez *et al.*, 2010, Bigeard and Hirt, 2018). MAPK specificity can also be modulated via protein phosphatases (Luan, 2003, Suarez-Rodriguez *et al.*, 2010, Bigeard and Hirt, 2018).

In Arabidopsis genome, approximately 110 genes encode MAPK cascade components, 80 MAP3Ks, 10 MAP2Ks and 20 MAPKs (Ichimura *et al.*, 2002, Colcombet and Hirt, 2008, Suarez-Rodriguez *et al.*, 2010). Based on sequence similarities, Arabidopsis MAPKs and MAP2Ks can be divided into four groups (A-D) (Ichimura *et al.*, 2002, Colcombet and Hirt, 2008, Suarez-Rodriguez *et al.*, 2010). MAP3Ks are divided into two large subfamilies: the MEKK (MAPK/ERK kinase kinase)-like MAP3Ks (Group A), for which there is functional evidence that they act as MAP3Ks *in planta*, and RAF-like (Groups B and C), for which the only functional evidence comes from non-plant systems (Ichimura *et al.*, 2002, Colcombet and Hirt, 2008, Suarez-Rodriguez *et al.*, 2010).

1.4.1. MPK4 and MPK12 in guard cell signalling

MPK4 is expressed in various plant tissues and it is also highly expressed in guard cells (Petersen *et al.*, 2000). Initially, the role of MPK4 in negatively regulating plant defences against pathogens was identified (Petersen *et al.*, 2000). Loss of MPK4 activity resulted in constitutive defence responses, including elevated salicylic acid (SA) levels, increased resistance to virulent pathogens and constitutive pathogenesis-related (*PR*) gene expression (Petersen *et al.*, 2000, Brodersen *et al.*, 2006). The role of Arabidopsis MPK4 in stomatal-based defences has also been examined (Berriri *et al.*, 2012). However, the function of MPK4 in plant immunity was probably not related to the regulation of stomatal closure since *mpk4-2* lines expressing constitutively active MPK4 had wild-type-like stomatal responses to pathogens (Berriri *et al.*, 2012).

NtMPK4, a close orthologue of Arabidopsis MPK4 and MPK12 in *Nicotiana* species, and a homologue of NtMPK4 (NtMPK4L) have been suggested to be involved in the regulation of stomatal movements (Gomi *et al.*, 2005, Marten *et al.*, 2008, Yanagawa *et al.*, 2016). Silencing of *NtMPK4* or *NtMPK4L* in *N. tabacum* plants led to an increased guard cell size and greater stomatal aperture (Gomi *et al.*, 2005, Marten *et al.*, 2008, Yanagawa *et al.*, 2016). *NtMPK4*- and *NtMPK4L*-silenced plants exhibited reduced stomatal closure in response to ozone (Gomi *et al.*, 2005, Yanagawa *et al.*, 2016). In addition, silencing of *NtMPK4* caused an impaired stomatal response to elevated [CO_2], whereas ABA sensitivity was not altered (Gomi *et al.*, 2005, Marten *et al.*, 2008). Since darkness did not activate plasma membrane S-type anion channels in *NtMPK4*-silenced guard cells, NtMPK4 was also required for darkness-induced stomatal closure (Marten *et al.*, 2008).

Similarly to NtMPK4, MPK4 in another tobacco species *N. attenuata* (NaMPK4), functions in guard cell signalling (Hettenhausen *et al.*, 2012). Silencing of *NaMPK4* resulted in enlarged stomatal size and greater stomatal apertures (Hettenhausen *et al.*, 2012). Stomata of *NaMPK4*-silenced plants showed strongly impaired response to ABA, indicating that in contrast to NtMPK4, NaMPK4 was required for ABA-induced stomatal closure (Hettenhausen *et al.*, 2012). NaMPK4 was also suggested to have a role in darkness-induced stomatal closure and in guard cell-mediated defence responses (Hettenhausen *et al.*, 2012).

Two Arabidopsis MAPK genes, *MPK9* and *MPK12*, were shown to be highly and preferentially expressed in guard cells (Jammes *et al.*, 2009). Thus, these MPKs could have a role in the regulation of stomatal movements. Single *mpk9-1* and *mpk12-1* homozygous TILLING mutants did not show any altered stomatal movements in response to ABA (Jammes *et al.*, 2009). However, mutations in both *MPK9* and *MPK12* caused enhanced transpirational water loss and significantly impaired ABA-induced inhibition of stomatal opening (Jammes *et al.*, 2009). An *A. thaliana* natural accession from Cape Verde Islands (Cvi-0), in which conserved glycine residue in MPK12 is substituted with arginine at position 53, showed higher stomatal conductance due larger

guard cells and stomata, and reduced water use efficiency (Des Marais *et al.*, 2014). The G53R mutation in MPK12 partially impaired ABA-mediated inhibition of stomatal opening but not ABA-mediated stomatal closure (Des Marais *et al.*, 2014).

1.4.2. MAP kinase cascades involving MPK4 and MPK12

While cascades involving MPK4 have been characterized, no cascades involving MPK12 have been identified. It also remains to be clarified whether classical MAPK cascades including MPK4 and MPK12 function in stomatal signalling and which MAP3Ks and MAP2Ks may act upstream of MPK4 and MPK12 in guard cells.

Several studies have revealed the involvement MEKK1-MKK1/MKK2-MPK4 pathway in the repression of immune responses (Petersen *et al.*, 2000, Andreasson *et al.*, 2005, Brader *et al.*, 2007, Suarez-Rodriguez *et al.*, 2007, Gao *et al.*, 2008, Qiu *et al.*, 2008). Flagellin peptide (flg22) treatment activated MPK4 in wild-type plants but not in *mekk1* and *mkk1 mkk2* mutants, indicating that the activation of MPK4 by flg22 was controlled by MEKK1 through MKK1 and MKK2 (Ichimura *et al.*, 2006, Suarez-Rodriguez *et al.*, 2007, Gao *et al.*, 2008, Qiu *et al.*, 2008, Kong *et al.*, 2012). *mekk1* and *mpk4* single-mutants and *mkk1 mkk2* double-mutants were severely dwarfed, exhibited increased salicylic acid (SA) levels, constitutively expressed high levels of *Pathogenesis-Related* (*PR*) and SA-dependent defence genes, and showed enhanced resistance against pathogens (Petersen *et al.*, 2000, Ichimura *et al.*, 2006, Suarez-Rodriguez *et al.*, 2007, Gao *et al.*, 2008, Qiu *et al.*, 2008). The dwarf morphology and autoimmune phenotypes of *mekk1*, *mkk1 mkk2* and *mpk4* mutants could be suppressed by loss-of-function mutations in a nucleotide binding and leucine-rich repeat receptor (NLR) SUMM2 (Zhang *et al.*, 2012). SUMM2 have been suggested to sense the disruption of the MEKK1-MKK1/MKK2-MPK4 cascade and mediate the activation of defence responses (Zhang *et al.*, 2017b).

MEKK1-MKK1/MKK2-MPK4 cascade has also been shown to regulate ROS homeostasis and cell death (Ichimura *et al.*, 2006, Nakagami *et al.*, 2006, Gao *et al.*, 2008, Pitzschke *et al.*, 2009). *mekk1*, *mkk1 mkk2* and *mpk4* mutants accumulated high levels of H_2O_2, a known inducer of cell death in plants, and a number of genes related to redox control were misregulated in these mutants (Ichimura *et al.*, 2006, Nakagami *et al.*, 2006, Gao *et al.*, 2008). ROS enhanced MEKK1 kinase activity which, in turn, led to the activation of MPK4 in Arabidopsis protoplasts (Nakagami *et al.*, 2006). However, MPK4 was not activated by ROS in *mekk1* mutants, suggesting that MEKK1 was a positive regulator of MPK4 (Nakagami *et al.*, 2006).

MKK2-MPK4 module was suggested to mediate cold and salt stress tolerance in Arabidopsis (Teige *et al.*, 2004). *mkk2* mutants were impaired in MPK4 activation and were hypersensitive to cold and salt stress, whereas MKK2 overexpression lines exhibited constitutive activation of MPK4, consti-

tutively upregulated expression of stress-induced marker genes, and increased freezing and salt tolerance (Teige *et al.*, 2004).

MAP kinase cascades including ANPs-MKK6-MPK4 have also been studied extensively. ANPs-MKK6-MPK4 cascades have been suggested to regulate cytokinesis (Kosetsu *et al.*, 2010, Takahashi *et al.*, 2010, Zeng *et al.*, 2011) and might also have a role in the regulation of plant immune responses (Lian *et al.*, 2018).

To date, cascades which include MPK12 have not been identified. Although MAP2Ks responsible for phosphorylation of MPK12 *in vivo* remain unknown, MKK1, MKK6, MKK7 and MKK9 were able to phosphorylate MPK12 *in vitro* (Lee *et al.*, 2009a) and MKK6 interacted with MPK12 in yeast (Takahashi *et al.*, 2010).

1.4.3. Other MAP kinases in guard cell signalling

MPK3 has been indicated to be involved in ABA-induced inhibition of stomatal opening (Gudesblat *et al.*, 2006). Guard cell-specific antisense inhibition of *MPK3* expression in Arabidopsis led to a partial reduction of ABA-induced inhibition of stomatal opening but did not affect the promotion of stomatal closure by ABA (Gudesblat *et al.*, 2006). Since ABA-induced H_2O_2 synthesis was not significantly affected in *MPK3*-silenced lines, MPK3 probably acted downstream of H_2O_2 in ABA signalling (Gudesblat *et al.*, 2006).

MKK1-MPK6 module might have a role in an ABA-dependent signalling cascade causing H_2O_2 production (Xing *et al.*, 2008). ABA did not trigger H_2O_2 production in guard cells of *mkk1* and *mpk6* mutants, whereas H_2O_2 production was observed in *MKK1* overexpressing plants (Xing *et al.*, 2008). The activity of MPK6 was enhanced by ABA treatment in wild-type plants but not in the *mkk1* mutants, suggesting that MKK1 was required for ABA-triggered activation of MPK6 (Xing *et al.*, 2008).

Another study suggested that MKK1-MPK6 module might be involved in darkness-induced stomatal closure via functioning downstream of H_2O_2 (Zhang *et al.*, 2017a). *mkk1* and *mpk6* mutants were completely impaired in darkness-induced stomatal closure but darkness induced H_2O_2 production in guard cells of these mutants (Zhang *et al.*, 2017a). Darkness enhanced the activity of MPK6 in wild-type plants but not in the *mkk1* mutants, suggesting that MKK1 mediated the darkness-induced activation of MPK6 (Zhang *et al.*, 2017a).

MPK3 and MPK6, as well as their upstream MAPK kinases MKK4 and MKK5, were shown to play a role in stomatal immunity (Su *et al.*, 2017). *mpk3 mpk6* and *mkk4 mkk5* double mutants exhibited impaired stomatal closure in response to flg22 (Su *et al.*, 2017). flg22-triggered activation of MPK3 and MPK6 in wild-type plants but not in the *mkk4 mkk5* double mutants, indicating that MKK4 and MKK5 acted upstream of MPK3 and MPK6 (Su *et al.*, 2017).

A MAPKK kinase, MKKK20, was suggested to be involved in ABA-induced stomatal closure (Li *et al.*, 2017). Mutations in a gene encoding

MKKK20 caused an impaired stomatal response to ABA (Li *et al.*, 2017). ABA treatment did not enhance the kinase activity of MPK6 in *mkkk20*, *mkk5*, and *mkkk20 mkk5* mutants but enhanced the activity of MPK6 in *MKKK20* over-expression lines (Li *et al.*, 2017). A PP2C ABI1 was able to decrease the activity of MKKK20 *in vitro*, suggesting that the MKKK20 protein kinase activity could be regulated by ABI1 (Li *et al.*, 2017).

Another MAPKK kinase, MKKK18, might also play a role in guard cell ABA signalling (Mitula *et al.*, 2015). *mkkk18* mutants exhibited an impaired stomatal response to ABA, whereas *MKKK18* overexpression lines were hyper-sensitive to ABA-induced stomatal closure (Mitula *et al.*, 2015). The kinase activity of MKKK18 was decreased by ABI1 *in vitro*, suggesting that similarly to MKKK20, MKKK18 could be regulated by ABI1 (Mitula *et al.*, 2015).

2. AIM OF THE STUDY

The aim of this thesis was to study CO_2 signal transduction in guard cells and elucidate the molecular mechanisms of stomatal regulation. The specific aim was to characterize the role of MAP kinases in CO_2-induced stomatal movements.

To meet these aims, the following questions were investigated:

- could MAP kinases (MPK4, MPK9 and MPK12) have a CO_2-specific role in guard cells
- could MAP kinases (MPK4 and MPK12) function as CO_2 sensors in plants
- could MAP kinases (MPK4 and MPK12) interact with any identified components of CO_2 signalling pathway
- could MAP kinases (MPK4 and MPK12) modulate the kinase activity of their interaction partners
- which proteins could be the substrates for the interaction partners of MPK4 and MPK12, and could these proteins also function in CO_2-induced stomatal movements

Based on the results obtained from the investigation of the abovementioned questions, a new model for guard cell CO_2 signal transduction was proposed.

3. MATERIALS AND METHODS

The details of experimental procedures have been described in the Materials and Methods sections of the publications I–IV. Methods used to obtain the unpublished data are described below.

Microscale thermophoresis (MST)

The coding sequence of HT1 was cloned into a pET28a vector (Novagen) as described in the publication II. The sequences of MPK4, MPK11, MPK12 and MPK12 G53R were cloned into a pET52b (Novagen) vector using primers:
SmaI-MPK4-F (5'-GGGAATTCCCCGGGATGTCGGCGGAGAGTTG -3'),
SalI-MPK4-R (5'- CCCTTAAGGTCGACTCACACTGAGTCTTGAG -3'),
SmaI-MPK11-F (5'- GGGAATTCCCCGGGATGTCAATAGAGAAACCATTC -3'),
SalI-MPK11-R (5'-CCCTTAAGGTCGACTTAAGGGTTAAACTTGACTG -3'),
SmaI-MPK12-F (5'-GGGAATTCCCCGGGATGTCTGGAGAATCAAGCTC -3'),
SalI-MPK12-R (5'- CCCTTAAGGTCGACTCAGTGGTCAGGATTGAATTTG -3')
6xHis-tagged HT1 and Strep-tagged MPK4, MPK11, MPK12 and MPK12 G53R were expressed in *E. coli* BL21(DE3) cells. The cultures were grown in TB medium at 37 °C to OD_{600} ~0.6. Recombinant protein expression was induced with 0.3 mM IPTG at 18 °C for 20 h. 6xHis-HT1 was purified as described in publication II. Strep-tagged proteins were purified using Strep-Tactin® Superflow® (IBA) resin according to the manufacturer's protocol. Then, the samples (6xHis-HT1 and Strep-tagged MPKs) were run on a Superdex 75 10/300 GL size exclusion column (GE Healthcare) with a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 0.1% NP-40 at a flow rate of 0.5 mL/min on an ÄKTAmicro FPLC system (GE Healthcare). Purified proteins were concentrated using Amicon Ultra-2 Centrifugal Filter Units (NMWL 3000) (Millipore) and stored at -80 °C until use.

6xHis-tagged HT1 was fluorescently labelled using a kit for labelling of His-tagged proteins (Monolith NT™ His-Tag Labeling Kit RED-tris-NTA kit, Nanotemper Technologies) according to manufacturer's protocol. Unlabeled MPKs were titrated into a fixed concentration (10 nM) of labelled 6xHis-HT1. Binding reactions were carried out in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 0.1% NP-40. Samples were loaded in Monolith NT.115 premium coated capillaries (Nanotemper Technologies) and measured in the Monolith NT.115 apparatus (Nanotemper Technologies). The data were recorded at 25 °C using the red LED at 100% and infrared laser power at 40%. Data analyses were performed with the MO Affinity Analysis software (Nanotemper Technologies).

***In vitro* kinase assays with tomato HT1 and MPK12**

The coding sequences of tomato (*Solanum lycopersicum*) HT1 (tHT1), MPK12 (tMPK12) and MPK12 G52R (tMPK12 G52R) were cloned into a pET28a vector (Novagen) using primers:
BamHI-tHT1-2-for (5'- GCGGATCCATGGCTACTTCTTGTTTC -3')
XhoI-tHT1-2-rev (5'- GCCTCGAGTCAAACATTTACAGGTATAG -3')
BamHI-tMPK12-2-for (5'- GCGGATCCATGGAGGCAAGTACAGG -3')
XhoI-tMPK12-2-rev (5'- GCCTCGAGTCAGTGAGTTGGATCTGG -3')

A point mutation corresponding to the G52R mutation in tMPK12 was created with two-step PCR using primers:
1st PCR1:
BamHI-tMPK12-2-for (5'- GCGGATCCATGGAGGCAAGTACAGG -3')
tMPK12-2(G52R)-rev (5'- CACAAACGATGCGATAAGCTCC -3')
1st PCR2:
tMPK12-2(G52R)-for (5'- GGAGCTTATCGCATCGTTTGTG -3')
XhoI-tMPK12-2-rev (5'- GCCTCGAGTCAGTGAGTTGGATCTGG -3')
2nd PCR:
BamHI-tMPK12-2-for (5'- GCGGATCCATGGAGGCAAGTACAGG -3')
XhoI-tMPK12-2-rev (5'- GCCTCGAGTCAGTGAGTTGGATCTGG -3')

6xHis-tagged proteins (tHT1, tMPK12 and tMPK12 G52R) were expressed in *E. coli* BL21(DE3) cells. The cultures were grown in TB medium at 37 °C to OD_{600} ~0.6. Recombinant protein expression was induced with 0.3 mM IPTG at 18 °C for 20 h. The proteins were purified as described in the publication II.

In vitro kinase assays with tomato HT1 and MPK12 were performed as described in the publication II. The final concentrations of tMPK12 in the HT1 inhibition assays were 0 to 30 μM.

4. RESULTS AND DISCUSSION

4.1. MPK4 and MPK12 are involved in CO_2-induced stomatal movements

To study the role of MPK4 and MPK12 in stomatal regulation, we generated stable homozygous *mpk12 mpk4* double-mutant lines (T4 generation) (I). Since the Arabidopsis *mpk4* mutants are severely dwarfed due to inappropriate activation of defence responses (Zhang *et al.*, 2017b), it is not feasible to measure accurate stomatal conductance in these plants. Therefore, we suppressed *MPK4* expression specifically in guard cells in *mpk12-4* line, which completely lacked *MPK12* (II, Supplemental Fig. 3), and used two independent homozygous *mpk12-4 mpk4GC* lines (referred to as *mpk12 mpk4GC #1* and *mpk12 mpk4GC #2*) for physiological response and signalling analyses (I).

To explore whether MPK4 and MPK12 function in stomatal CO_2 signalling, we measured stomatal conductance in intact leaves as well as in full rosettes in intact plants in response to CO_2 concentration shifts. Stomatal conductance of leaves of wild-type (Col-0) plants decreased when [CO_2] was changed from 400 p.p.m. to 800 p.p.m. and increased followed by a shift from 800 p.p.m. to 150 p.p.m. (I, Fig. 1a). In contrast, stomatal responses to changes in CO_2 concentration were completely abolished in the leaves of *mpk12 mpk4GC #1* plants (I, Fig. 1a). Similarly, experiments with full rosettes of intact plants showed that stomatal responsiveness to both high- and low [CO_2] was fully abolished in independent *mpk12 mpk4GC* lines, whereas wild-type plants responded to [CO_2] shifts normally (Figure 5a,b). Compared to *mpk12 mpk4GC* double-mutants, *mpk12-4* (referred to as *mpk12*) single-mutants displayed only partially impaired CO_2 responses (Figure 5a,b). Thus, silencing of *MPK4* in the *MPK12*-deficient background clearly led to more severely impaired CO_2 responses than in the *mpk12* single-mutant (Figure 5a,b), demonstrating that both MPK4 and MPK12 contributed to CO_2-induced stomatal movements.

We also analysed stomatal density and stomatal index in the *mpk12 mpk4GC* lines, since the stomatal conductance of these lines was consistently higher than in the wild-type plants throughout the experiments (I). The average stomatal index in the leaves of *mpk12 mpk4GC* mutants was slightly lower than in the wild-type and *mpk12* plants, whereas there was no significant effect on stomatal density (I, Fig. 2a,b). This suggested that the enhanced stomatal conductance in the *mpk12 mpk4GC* double-mutants was due to more open stomata (I). Therefore, we also analysed the stomatal size of *mpk12 mpk4GC* lines by measuring stomatal heights and quantifying stomatal apertures (I). Both *mpk12 mpk4GC* lines did not have significantly larger stomatal heights but had significantly larger stomatal apertures compared with wild-type plants (I, Fig. 2c,d). Thus, the function of MPK4 and MPK12 is related to the regulation of stomatal aperture.

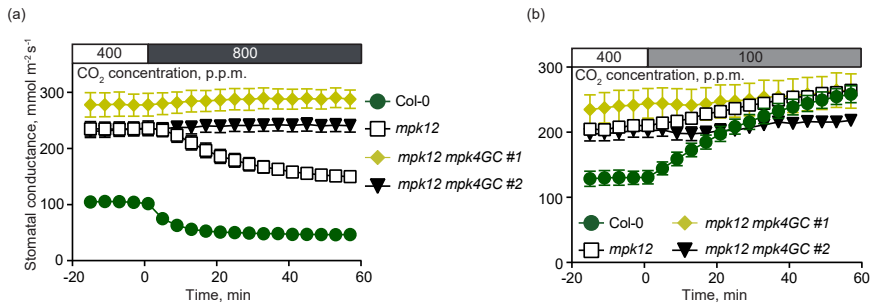

Figure 5. *mpk12 mpk4GC* double mutant plants show dramatically impaired stomatal CO_2 response.
(a–b) Time-resolved patterns of stomatal conductance in whole rosettes during changing CO_2 concentration from 400 to 800 p.p.m. (a) or from 400 to 100 p.p.m. (b) of Col-0 wild type, *mpk12* and *mpk12 mpk4GC* double mutant alleles. Error bars indicate ± SEM, n = 6–7. (Modified from the publication I, Fig. 1b,c)

To explore the role of MPK4 separately from MPK12 in stomatal CO_2 signalling, we generated *mpk4GC* lines in the wild-type (Col-0) background in which *MPK4* expression was silenced specifically in guard cells (T2 generation) (I). The suppression of *MPK4* expression in guard cells of two independent *mpk4GC* lines (referred to as *mpk4GC #1* and *mpk4GC #2*) was confirmed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (I, Supplemental Fig. 2). In contrast to *mpk4* mutants which displayed severe growth impairment, the *mpk4GC* lines (referred to as *mpk4GC #1* and *mpk4GC #2*) grew normally and were only slightly smaller than wild-type plants (Figure 6a). We measured stomatal conductance in response to low and high [CO_2] in full rosettes in intact *mpk4GC* mutant plants. Both *mpk4GC* lines exhibited wild-type-like stomatal responses to [CO_2] shifts (Figure 6b,c). However, compared to wild-type plants, stomatal conductance of *mpk4GC* lines was higher throughout experiments (Figure 6b,c).

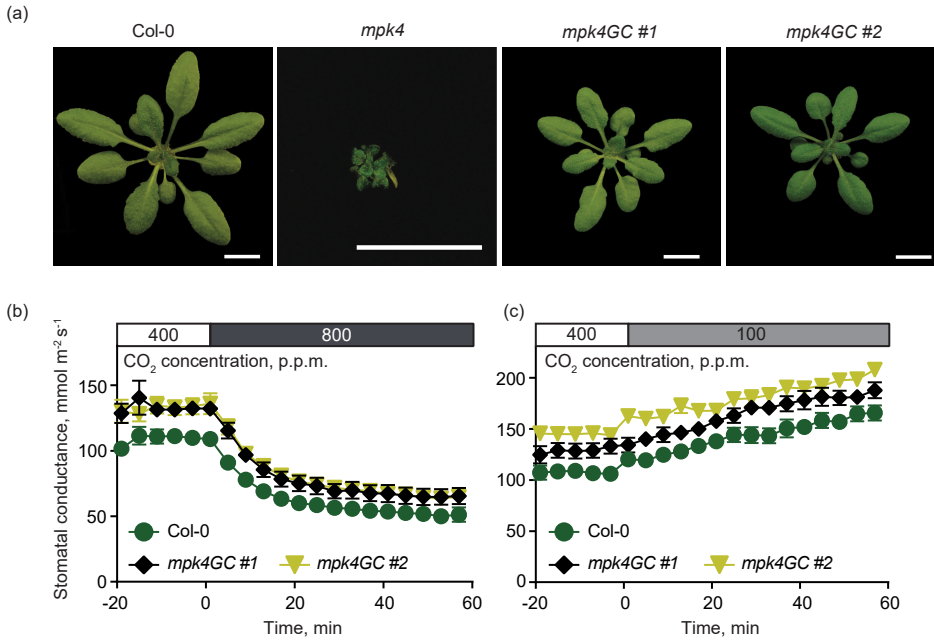

Figure 6. The function of MPK4 in stomatal signalling.
(a) Photos of wild-type (Col-0), *mpk4* and *mpk4GC* plants. Scale bars 1 cm.
(b-c) Time-course of stomatal conductance in whole rosettes during changing CO_2 concentration from 400 to 800 p.p.m. (b) or from 400 to 100 p.p.m. (c) of Col-0 wild type and *mpk4GC* lines. Error bars indicate ± SEM, n = 10-14. (Modified from the publication I, Fig. 3a–d)

To elucidate whether MPK4 and MPK12 could also function in guard cell ABA signalling, we measured stomatal conductance in intact leaves and in full rosettes in intact plants after ABA treatment (I). Spraying a 5 µM ABA solution on full rosettes resulted in declined stomatal conductance in wild-type (Col-0) plants as well as in *mpk12* and *mpk12 mpk4GC* lines (Figure 7). Similarly, applying 2 µM ABA in intact leaves to the transpiration stream via the petiole caused a strong ABA-induced reduction in the stomatal conductance in wild-type and *mpk12 mpk4GC #1* leaves (I, Fig. 1e,f). Silencing of *MPK4* in the *MPK12*-deficient background did not noticeably disrupt ABA responses, showing that both MPK4 and MPK12 were not required for ABA-induced stomatal closure (Figure 7; I, Fig. 1e,f).

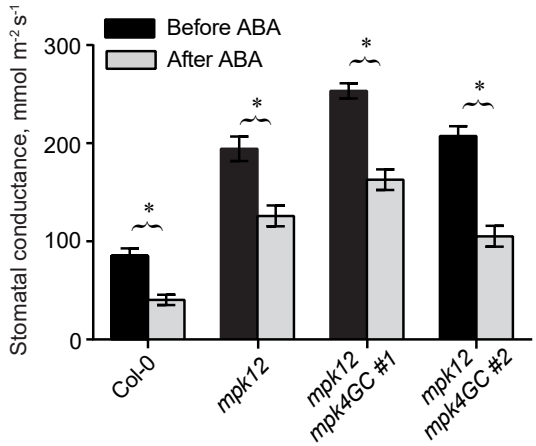

Figure 7. *mpk12 mpk4GC* double mutant plants show intact ABA response.
Stomatal conductance measured in whole rosettes before and 60 minutes after 5 μM ABA treatment of Col-0 wild type, *mpk12* and *mpk12 mpk4GC* double mutant alleles. Error bars indicate ± SEM, n = 6–7. Statistically significant differences are indicated with stars (repeated measures ANOVA with Tukey HSD unequal N *post hoc* test). (Modified from the publication I, Fig. 1d)

Activation of S-type anion channels plays a crucial role in CO_2/bicarbonate- and ABA-induced stomatal closure (Negi *et al.*, 2008, Vahisalu *et al.*, 2008). To determine whether the S-type anion channels could be activated by CO_2/bicarbonate and ABA in guard cells of *mpk12 mpk4GC* plants, we conducted patch-clamp experiments using guard cell protoplasts isolated from wild-type (Col-0) and both *mpk12 mpk4GC* lines (I). Experiments clearly showed that compared with wild-type, bicarbonate-induced activation of the S-type anion channels was impaired in both *mpk12 mpk4GC* lines, whereas there were no significant differences between wild-type and *mpk12 mpk4GC* lines in ABA-induced activation of the S-type anion channels (I, Fig. 4a–d). Thus, MPK4 and MPK12 are critical for CO_2/bicarbonate- but not ABA-induced activation of guard cell S-type anion channels.

To date, only a few recessive mutants (*ca1ca4*, *ht1*, *cbc1cbc2*) that show reduced sensitivity to high [CO_2] but retain ABA sensitivity in stomatal closure have been identified (Hashimoto *et al.*, 2006, Hu *et al.*, 2010, Hiyama *et al.*, 2017). Interestingly, the *mpk12 mpk4GC* double-mutants also retained a strong ABA response and ABA-induced S-type anion channel activation but exhibited abolished response to high [CO_2]. This suggests that MPK4 and MPK12 function upstream of a convergence point of ABA and CO_2 signalling pathways (Merilo *et al.*, 2013, Chater *et al.*, 2015, Hsu *et al.*, 2018) or act in a parallel CO_2 signalling pathway.

4.2. MPK9 is not involved in CO_2- or ABA- induced stomatal movements

To explore whether MPK9 function in stomatal CO_2 signalling, we measured stomatal conductance in full rosettes in intact *mpk9-2* (SALK_064439C) and *mpk9-3* (GK-250D07) T-DNA lines. These *mpk9* alleles displayed wild-type-like stomatal conductance and intact stomatal responses either when CO_2 concentration was changed from 400 p.p.m. to 800 p.p.m. or from 400 p.p.m. to 150 p.p.m. (Figure 8a,b).

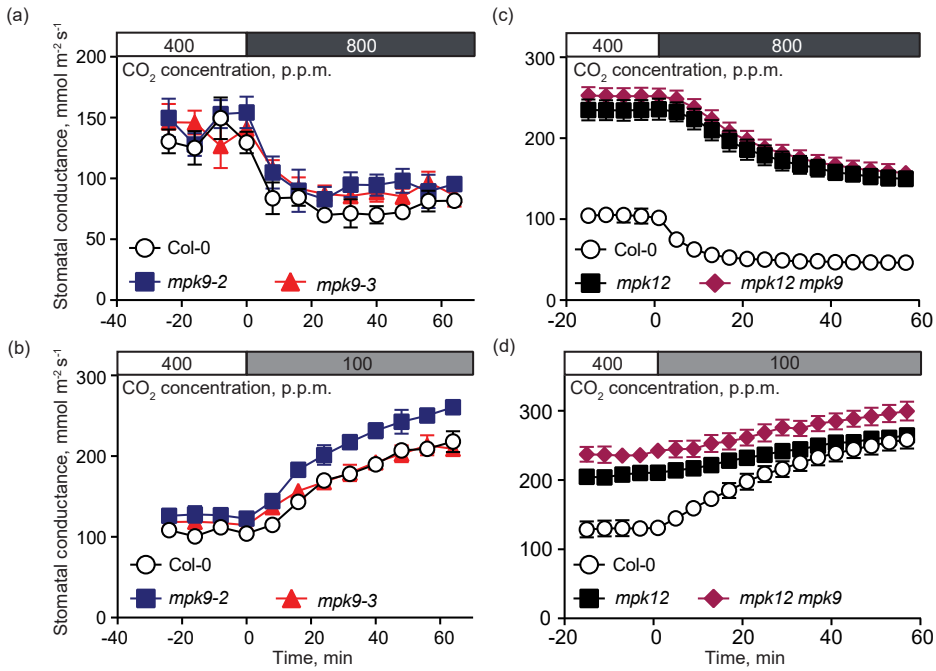

Figure 8. MPK9 does not regulate stomatal CO_2 responses.
(a–b) Time-resolved patterns of stomatal conductance during changing CO_2 concentration from 400 to 800 p.p.m. (a) or from 400 to 100 p.p.m. (b) of Col-0 wild type, *mpk9-2* (SALK_064439C) and *mpk9-3* (GK-250D07) plants. For Col-0 and *mpk9-3*, the experiments have been repeated three times and representative results from one experiment are shown. Error bars indicate ± SEM, n = 3–4.
(c–d) Time-resolved patterns of stomatal conductance during changing CO_2 concentration from 400 to 800 p.p.m. (c) or from 400 to 100 p.p.m. (d) of Col-0 wild type, *mpk12* and *mpk12 mpk9* plants. The experiments have been repeated two times and representative results from one experiment are shown. Error bars indicate ± SEM, n = 6–7. (Modified from the publication I, Fig. 6a–d)

Next, we generated a new *mpk12-4 mpk9-3* double-mutant (referred to as *mpk12 mpk9*), harbouring a deletion of *MPK12* and a T-DNA in *MPK9*, thus including full knockout for both *MPK* transcripts (I, Supplemental Fig. 7). The *mpk12 mpk9* double-mutant and *mpk12* single-mutant plants exhibited an indistinguishable response to increased CO_2 concentration (Figure 8c), whereas *mpk12 mpk9 mpk4GC* triple-mutant responses resembled those of *mpk12 mpk4GC* (I, Fig. 1b and Supplemental Fig. 8a). Furthermore, the *mpk12 mpk9* double-mutant and *mpk12* single-mutant had similar partially inhibited stomatal opening response to decreased CO_2 concentration (Figure 8d; I, Supplemental Fig. 8b), indicating that MPK9 did not have a measurable function in either low- or high [CO_2]-induced stomatal movements.

To clarify the role of MPK9 in ABA signalling, we measured stomatal conductance in full rosettes in intact *mpk9*, *mpk12 mpk9* and *mpk12 mpk9 mpk4GC* plants after ABA application (I). We observed ABA-induced reduction in the stomatal conductance in both single *mpk9* mutant alleles (Figure 9a). Also, stomatal response to ABA was not impaired in *mpk12 mpk9* double- and *mpk12 mpk9 mpk4GC* triple-mutant plants (Figure 9b). However, the *mpk12 mpk9 mpk4GC* triple-mutants displayed clearly larger stomatal conductance than the *mpk12 mpk9* double-mutants (Figure 9b; I, Supplemental Fig. 8a,b), further pointing to the importance of MPK4 and MPK12 in stomatal regulation. In line with previous findings (Jammes et al., 2009), ABA-induced inhibition of light-induced stomatal opening was impaired in the *mpk12 mpk9* double-mutant plants (I, Fig. 6g). However, this was also true for *mpk12* single-mutants (I, Fig. 6g), suggesting that MPK9 did not contribute to stomatal ABA responses and further research will be required to determine the precise role of MPK9 in stomatal regulation.

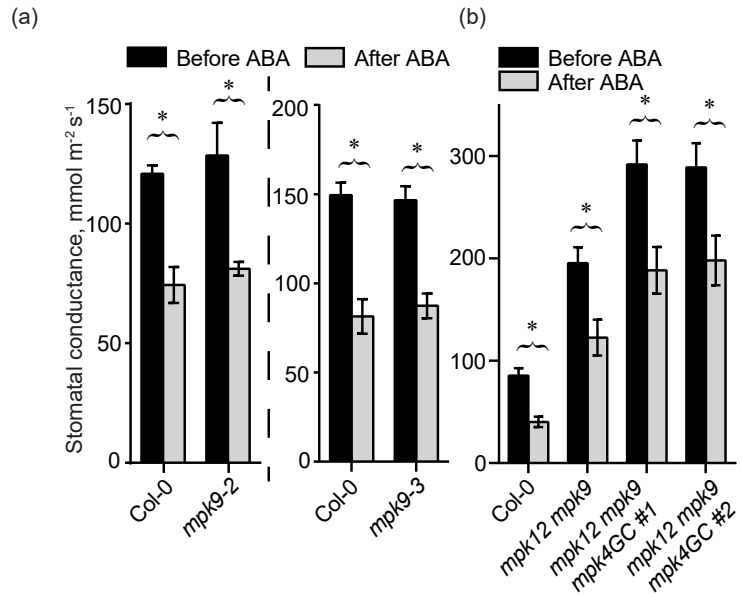

Figure 9. MPK9 does not regulate stomatal ABA response.
(a) Stomatal conductance before and 60 minutes after 5 µM ABA treatment of Col-0 wild type and *mpk9-2*, and Col-0 and *mpk9-3* plants. For Col-0 and *mpk9-3*, the experiments have been repeated three times and representative results from one experiment are shown. Error bars indicate ± SEM, n = 4 for Col-0 and *mpk9-2*, and n = 7 for Col-0 and *mpk9-3*. Statistically significant differences are indicated with stars (repeated measures ANOVA with Tukey HSD *post hoc* test).
(b) Stomatal conductance before and 60 minutes after 5 µM ABA treatment of Col-0 wild type, *mpk12 mpk9* and *mpk12 mpk9 mpk4GC* plants. For Col-0 and *mpk12 mpk9*, the experiments have been repeated two times and representative results from one experiment are shown. Error bars indicate ± SEM, n = 6–7. Statistically significant differences are indicated with stars (repeated measures ANOVA with Tukey HSD unequal N *post hoc* test). (Modified from the publication I, Fig. 6e,f)

4.3. MPK4 and MPK12 are not involved in direct CO_2/HCO_3^- sensing in guard cells

Intracellular CO_2/bicarbonate sensors that mediate the stomatal response remain largely unknown. As described above, MPK4 and MPK12 are critical components of stomatal CO_2 signalling. Therefore, it raises the question of whether these MAPKs could act as CO_2/bicarbonate sensors in plants.

To test whether MPK4 and MPK12 are directly activated by CO_2/HCO_3^-, we performed *in vitro* kinase assays with purified recombinant His-tagged MPK4 and MPK12 (I). The reactions were carried out in assay buffer containing defined concentrations of bicarbonate (0–20 mM) (I). Neither the activity of MPK4 nor MPK12 enhanced by the increased bicarbonate concentrations in the phosphorylation reactions (Figure 10a). Both, the autophosphorylation activity

of MPK4 and phosphorylation of the artificial substrate myelin basic protein (MBP) in bicarbonate-containing reactions remained the same compared with the control reaction, in which bicarbonate was not added (Figure 10a). Also, the weak autophosphorylation activity of MPK12 was not increased by the bicarbonate treatment, and phosphorylation of the substrate was not observed either in bicarbonate-treated or control reactions (Figure 10a).

To explore the possibility that the activity of MPK4 and MPK12 could be affected by the change of the solute pH, which may be caused by dissolution of CO_2, we carried out *in vitro* kinase assays in buffers at a range of defined buffered pH conditions (I). We did not observe an effect on either MPK4 or MPK12 activity (Figure 10b). The activity of enzymes at slightly acidic (pH 6.4) and alkaline (pH 8.8) conditions was similar to the activity at neutral conditions (pH 7.4) (Figure 10b).

Taken together, these data suggest that MPK4 and MPK12 are not involved in direct CO_2/HCO_3^- sensing in guard cells, and the proteins acting as CO_2/HCO_3^- sensors in guard cells remain to be identified. Furthermore, it is not known which MAP3Ks and MAP2Ks act upstream of MPK4 and MPK12 in guard cell CO_2 signalling. Thus, further research will be required to elucidate whether classical MAPK cascades including MPK4 and MPK12 also act in guard cell signalling and to explain how CO_2 sensing is linked to MPK4 and MPK12.

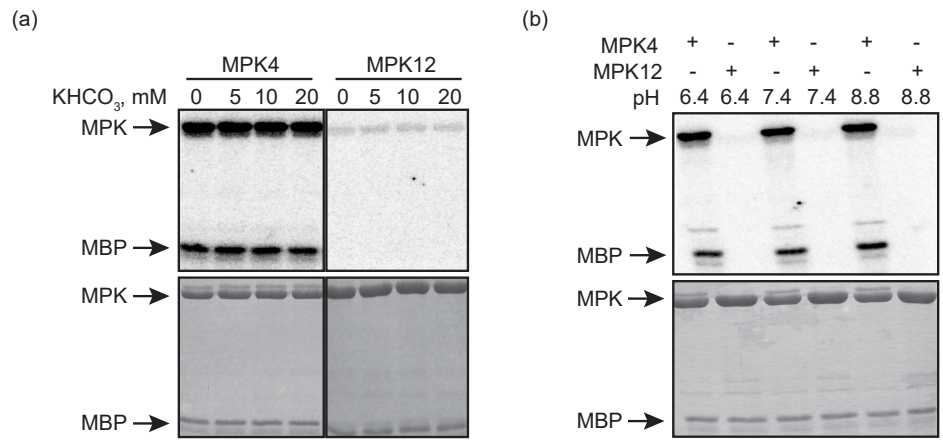

Figure 10. Kinase activities of recombinant MPK4 and MPK12 proteins are not directly CO_2/HCO_3^- regulated.
(a) His-MPK4 and His-MPK12 kinase activity in the presence of the indicated concentrations of $KHCO_3$. No activation of the kinases was observed. Autoradiography (upper panel) and CBB staining (lower panel) are shown.
(b) Effect of pH on His-MPK4 and His-MPK12 kinase activity. The activity of kinases was similar in slightly acidic (pH 6.4), alkaline (pH 8.8) and neutral (pH 7.4) conditions. Autoradiography (upper panel) and CBB staining (lower panel) are shown. (Modified from the publication I, Fig. 5c,d)

4.4. MPK4 and MPK12 but not MPK9 interact with HT1

To identify potential interaction partners of MPK4 and MPK12, and further characterize the role of these MAPKs in CO_2 signalling, we performed split-ubiquitin yeast two-hybrid (Y2H) assays. In a pairwise Y2H assay, we tested whether MPK12 could interact with any of the kinases and phosphatases involved in stomatal signalling (II, Fig. 4a,b and Supplemental Fig. 7a,b). Interestingly, MPK12 interacted with HT1 kinase (II, Fig. 4a,b and Supplemental Fig. 7a,b), identified as a central regulator of CO_2-induced stomatal movements (Hashimoto et al., 2006, Hashimoto-Sugimoto et al., 2016). In addition, HT1 interacted with MPK12 G53R (II, Fig. 4a,b and Supplemental Fig. 7a,b), an MPK12 version that caused higher stomatal conductance and reduced water use efficiency (Des Marais *et al.*, 2014) (II, Fig. 2a–d) as well as partially impaired stomatal responses to [CO_2] changes (II, Fig. 3a,c and Supplemental Fig. 5a,e) and bicarbonate-activation of S-type anion channels (II, Fig. 3e) in Arabidopsis Cvi-0 accession or in a near-isogenic line (NIL) in the Col-0 background (Col-S2). However, MPK12 G53R did not affect stomatal ABA responses in Col-S2 (II, Fig. 3b,c and Supplemental Fig. 5h). The interaction between MPK12 G53R and HT1 was weaker than that between MPK12 and HT1 in yeast (II, Fig. 4a,b and Supplemental Fig. 7a,b). In another Y2H screen carried out in our laboratory, MPK4 was identified as a protein interacting with HT1 (III, Supplemental Fig. 3). In contrast, MPK11, an MPK which belongs to the same subgroup of the MPKs as MPK4 and MPK12 (Ichimura *et al.*, 2002), did not interact with HT1 in yeast (II, Fig. 4a,b; III, Supplemental Fig. 5).

To confirm the identified interactions in plants, we conducted ratiometric bimolecular fluorescence complementation (BiFC) analyses with a split-yellow fluorescent protein (YFP)-tagged MPKs and HT1 in *N. benthamiana* leaves. SLAC1-cyan fluorescent protein (CFP) fusion was used as an internal control in the experiments (II, III). Strong interaction between MPK4 and HT1 as well as between MPK12 and HT1 was observed (II, Fig. 4c–e; III, Fig. 3b–d and Supplemental Fig. 6a,b). Consistent with the Y2H data, the interaction between MPK12 G53R and HT1 was weaker compared with that between MPK12 and HT1 (II, Fig. 4c–e), and MPK11 did not interact with HT1 in plants (II, Fig. 4c–e; III, Fig. 3c,d and Supplemental Fig. 6c). The results of the ratiometric BiFC assay also indicated weaker interaction between MPK12 and HT1 than that between MPK4 and HT1 (III, Fig. 3c).

We also performed microscale thermophoresis (MST) experiments to further confirm the interactions between MPKs and HT1, and to get an insight about dissociation constant (K_d) values of MPK and HT1 complexes. We used purified recombinant 6xHis-tagged HT1 as a target and Strep-tagged MPKs as ligands in the experiments. The interaction between MPK4 and HT1 as well as between MPK12 and HT1 was observed (Figure 11a,b). Preliminary data indicated that the K_d value for HT1 and MPK4 complex was ~200 nM, whereas the K_d value for MPK12 and HT1 complex was ~600 nM. The interaction

between MPK12 G53R and HT1 was not observed in the MST experiments (Figure 11a,b) and therefore the K_d value for MPK12 G53R and HT1 complex could not be determined. Based on the results of Y2H and ratiometric BiFC assays, the interaction between MPK12 G53R and HT1 was weaker than the interaction between MPK12 and HT1 (II, Fig. 4a–d and Supplemental Fig. 7a,b). Thus, the K_d value for MPK12 G53R and HT1 complex should be higher than that for MPK12 and HT1 complex. It is likely that higher concentrations of MPK12 G53R should be used to analyse the interaction between MPK12 G53R and HT1 by MST. Consistent with the results of Y2H and ratiometric BiFC assays, the interaction between MPK11 and HT1 was not detected in the MST experiments (Figure 11a).

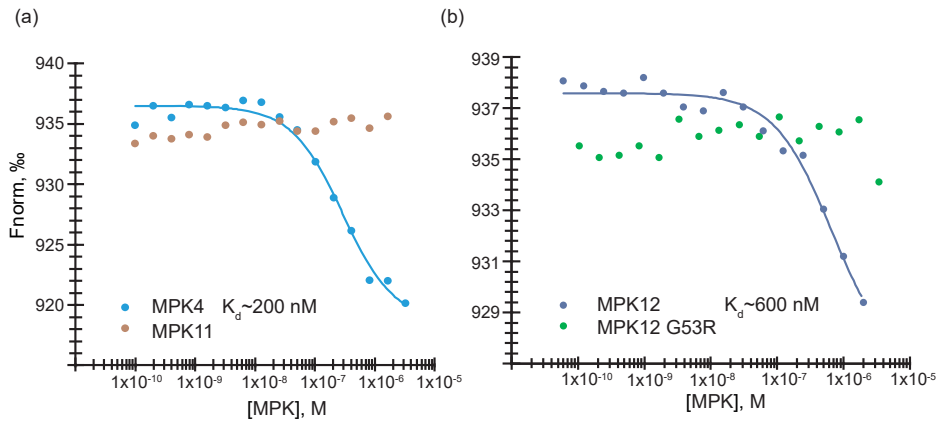

Figure 11. Analysis of binding affinities between MPKs and HT1 by microscale thermophoresis (MST).
(a) Dissociation curve for the interaction of HT1 with MPK4. MPK11 was used as a negative control.
(b) Dissociation curve for the interaction of HT1 with MPK12. MPK12 G53R did not show interaction with HT1 under conditions tested.
Unlabelled MPKs were titrated into a fixed concentration of fluorescently labelled HT1. Results from preliminary experiments are shown.

To test whether MPK9 could interact with HT1, we performed a ratiometric BiFC assay, where MPK9 and HT1 were fused to split-YFPs and SLAC1-CFP was used as an internal reference (I). The results showed no clear interaction between MPK9 and HT1 in contrast to the strong YFP to CFP signal ratio with MPK12 and HT1 (I, Fig. 7a–c). In control experiments with MPK11, the closest homologue to MPK4 and MPK12, only very low YFP signals were observed (I, Fig. 7a–c), supporting a preferential interaction of HT1 with MPK4 and MPK12, but not with MPK9. This further supports a differential role for MPK9 compared with MPK4 and MPK12 in stomatal signalling.

4.5. MPK4 and MPK12 inhibit the activity of HT1

To evaluate the relationship between *MPK12* and *HT1* in CO_2 signalling, we measured stomatal conductance in *ht1-2* (Hashimoto *et al.*, 2006), *mpk12-4*, and *mpk12-4 ht1-2* mutants at ambient CO_2 levels (II). *ht1-2* and *mpk12-4* exhibited opposing stomatal conductance at ambient CO_2 levels: low in the *ht1-2* and high in the *mpk12-4* (II, Fig. 4f). *mpk12-4 ht1-2* double-mutant displayed similar phenotype to *ht1-2* (II, Fig. 4f), suggesting that *HT1* was epistatic to *MPK12*.

To test whether MPK12 could affect the activity of HT1, we performed *in vitro* kinase assays with purified recombinant 6xHis-tagged proteins (II). Casein was used as an artificial substrate for HT1. The strong autophosphorylation activity of HT1 was observed and it phosphorylated casein efficiently (Figure 12a,b). MPK12 showed weak, whereas a hyperactive MPK12 Y122C displayed strong autophosphorylation activity (II, Fig. 5c). In contrast, MPK12 G53R lacked autophosphorylation activity, indicating that the G53R substitution in MPK12 disrupted the kinase activity of the protein (II, Fig. 5c). Neither MPK12 nor MPK12 Y122C, which displayed autophosphorylation activity, did not phosphorylate kinase-dead HT1 K113M (II, Fig. 5c). Addition of defined concentrations (0–30 μM) of MPK12 or the hyperactive MPK12 Y122C into reactions containing a constant amount of HT1 inhibited the autophosphorylation activity of HT1 and phosphorylation of casein by HT1 (Figure 12a,b; II, Fig. 5a,b). MPK12 K70R, designed to remove the kinase activity of MPK12, also inhibited the autophosphorylation activity of HT1 and phosphorylation of casein by HT1, but to a lesser extent than MPK12 (II, Fig. 5a,b). Importantly, MPK12 G53R displayed strongly suppressed inhibition of HT1 activity, probably due to its weaker interaction with HT1 (Figure 12a,b; II, Fig. 5a,b). Thus, the inhibition of HT1 activity by MPK12 might be independent of the MPK12 activity but depends on the Gly53 residue in MPK12.

Since MPK4 and MPK12 are highly similar (Ichimura *et al.*, 2002), we tested whether MPK4 could also inhibit the HT1 kinase activity. We performed *in vitro* kinase assays using purified recombinant 6xHis-tagged MPK4 and HT1 (II). Addition of defined concentrations (0–20 μM) of MPK4 into reactions containing a constant amount of HT1 inhibited the autophosphorylation activity of HT1 and phosphorylation of casein by HT1 (Figure 12c; II, Fig. 6a). Since the mutation of the conserved Gly53 residue blocked MPK12 function, we tested whether a similar mutation would also impair MPK4 function. Indeed, MPK4 harbouring a G55R mutation which corresponds to G53R in MPK12 blocked the MPK4-induced inhibition of HT1 activity (Figure 12c; II, Fig. 6a).

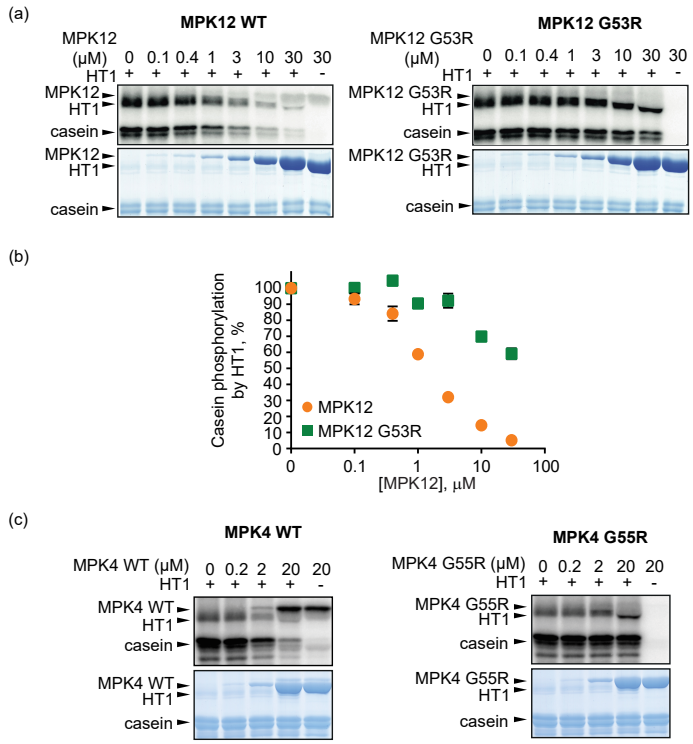

Figure 12. MPK4 and MPK12 inhibit HT1 activity and a conserved glycine in MPK4 and MPK12 is required for efficient inhibition of HT1.
(a) Inhibition of HT1 kinase activity *in vitro* by MPK12 and MPK12 G53R. Autoradiography (upper panel) and Coomassie-stained SDS PAGE (lower panel) are shown.
(b) Casein phosphorylation by HT1 with different MPK12 concentrations (mean ± SEM; n = 3).
(c) Inhibition of HT1 kinase activity in vitro by MPK4 and MPK4 G55R. Autoradiography (upper panel) and Coomassie-stained SDS PAGE (lower panel) are shown. (Modified from the publication II, Fig. 5a,b and Fig. 6a)

To further address the role of the G55R and G53R mutations in MPK4 and MPK12, the structures of MPK4 and MPK12 were modelled (II, Fig. 6b,c) based on the Arabidopsis MPK6 crystal structure (Wang *et al.*, 2016a). The substitution of Gly to Arg in MPK4 and MPK12 caused the protrusion of the arginine sidechain on the surface of the proteins, which altered the structure of the loop region and could affect binding affinities of these MPKs to other proteins (II, Fig. 6b,c).

MPK11 which did not interact with HT1 (II, Fig. 4a–c; III, Fig. 3c,d and Supplemental Fig. 6c), also did not inhibit the kinase activity of HT1 (II, Supplemental Fig. 9). Thus, the function of MPKs as HT1 inhibitors may be restricted to MPK12 and its closest homologue MPK4 (II, Supplemental Fig. 9).

Also, the ability of these MPKs (MPK4 and MPK12) to interact with HT1 and inhibit its kinase activity depends on the conserved residue corresponding to G53 in MPK12. Together, the results presented above (MPK4 and MPK12 interaction with HT1, epistasis between *HT1* and *MPK12*, and inhibition of HT1 activity by MPK4 and MPK12) suggest that the CO_2 signal is transmitted through MPK4 and MPK12 leading to inhibition of HT1.

4.5.1. Tomato MPK12 inhibits the activity of tomato HT1

Tomato is an economically important but highly water-consuming crop. Compared to the amount of biomass produced, water losses by transpiration are very high. A tomato plant can produce up to 25 g of fruit per litre of water used (Soria and Cuartero, 1998, Reina-Sánchez *et al.*, 2005). Since water availability is a major constraint of crop yield and is the single most important factor limiting food production, any improvement in tomato WUE, considered in terms of harvestable biomass per unit of water used would imply significant economic and environmental benefits (Mueller *et al.*, 2012, Sinclair and Rufty, 2012, van Ittersum *et al.*, 2013). However, to date, few studies have aimed to improve WUE in tomato (Cantero-Navarro *et al.*, 2016). CO_2-induced stomatal closure reduces water loss and hence, it may improve plant water use efficiency. Experiments with Arabidopsis MPKs (MPK4 and MPK12) and HT1 suggested that the CO_2 signal was transmitted through MPK4 and MPK12 leading to inhibition of HT1.

To investigate whether MPK12 and HT1 could function similarly in tomato (*Solanum lycopersicum*), we expressed tomato MPK12 (tMPK12) and HT1 (tHT1) in *E. coli* and performed *in vitro* kinase assays with purified recombinant 6xHis-tagged proteins. Based on the sequence alignments, tMPK12 (Solyc01g094960.2) shared 76.2%, and tHT1 (Solyc04g014690.2) 85.6% identity at protein level with Arabidopsis MPK12 and HT1, respectively. Similarly to Arabidopsis HT1, tomato HT1 showed a strong autophosphorylation activity and it phosphorylated the artificial substrate casein (Figure 13). In contrast to Arabidopsis MPK12, tomato MPK12 showed a strong autophosphorylation activity (Figure 13). Like the G53R substitution in Arabidopsis MPK12, a G52R substitution in tomato MPK12 caused a reduction in the kinase activity (Figure 13). Importantly, the addition of defined concentrations of tMPK12 (0–30 μM) into reactions containing a constant amount of tHT1 led to inhibition of the kinase activity of tHT1, whereas addition of tMPK12 G52R did not have an effect on the tHT1 activity (Figure 13). Thus, it is likely that MPK12 and HT1 have similar roles in Arabidopsis and tomato plants. However, further studies, including analysis of stomatal responses of mutants carrying mutations in *MPK12* and *HT1*, are required to characterize the role of MPK12 and HT1 in guard cells of tomato plants.

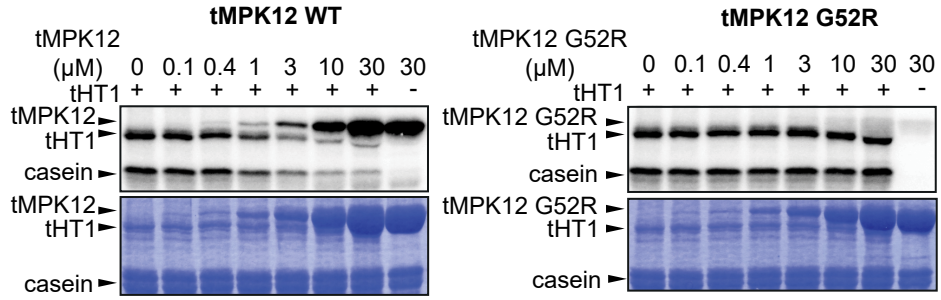

Figure 13. Tomato MPK12 inhibit tomato HT1 activity and a conserved glycine in MPK12 is required for efficient inhibition of HT1.
Inhibition of tomato HT1 kinase activity *in vitro* by tomato MPK12 and MPK12 G52R. Autoradiography (upper panel) and Coomassie-stained SDS PAGE (lower panel) are shown.

4.5.2. Ala109 in HT1 is required for MPK12-induced inhibition of HT1 activity

HT1 kinase activity has been shown to be required for the CO_2 response in plants (Hashimoto *et al.*, 2006). Plants, carrying a recessive *ht1-2* mutation, had a 14-amino-acid deletion in the HT1 kinase domain which abolished the activity of the kinase (Hashimoto *et al.*, 2006). Compared to wild-type plants, stomata of the *ht1-2* mutants were more closed at ambient [CO_2], and showed completely impaired response to low and high [CO_2] (Hashimoto *et al.*, 2006). In contrast, plants harbouring a dominant *ht1-3* mutation had more open stomata but similarly to the *ht1-2* mutants exhibited abolished response to [CO_2] changes (Hashimoto-Sugimoto *et al.*, 2016). The *ht1-3* mutation caused an Arg-to-Lys substitution at the position 102 (R102K) in a non-conserved region in HT1, which did not affect the kinase activity of HT1 but was proposed to affect interactions with its targets (Hashimoto-Sugimoto *et al.*, 2016).

A dominant *ht1-8D* mutant, identified in our laboratory, carries an Ala-to-Val substitution at the position 109 (A109V) which lies close to the ATP binding site of the HT1 kinase domain (III, Fig. 1e). Compared to wild-type plants, the *ht1-8D* mutants had more open stomata (III, Supplemental Fig. 1d) and exhibited higher stomatal conductance at ambient [CO_2] (Figure 14). In contrast, stomata of the recessive *ht1-2* mutants were more closed and displayed low conductance at ambient [CO_2] (Figure 14). However, both *ht1-8D* and *ht1-2* mutants showed completely impaired stomatal responses to low and high [CO_2] (Figure 14a,b), whereas ABA-induced stomatal closure and ABA-induced inhibition of stomatal opening retained functional (III, Fig. 2c and Supplemental Fig. 2a–c).

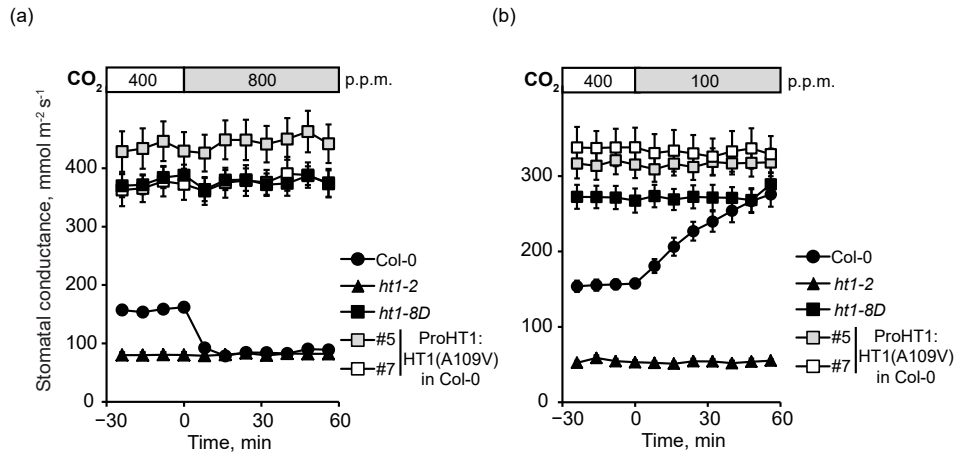

Figure 14. Plants with A109V mutation in HT1 are CO_2 insensitive.
(a–b) The stomatal response of intact plants to CO_2 elevation (from 400 to 800 p.p.m.) and reduction in CO_2 concentration (from 400 to 100 p.p.m.). The stimulus was applied at time point zero and pooled stomatal conductance data from three independent experiments are shown (mean ± SEM, n = 12 to 21). (Modified from the publication III, Fig. 2a,b)

Since the R102K mutation in HT1 was proposed to affect HT1 interactions with other proteins (Hashimoto-Sugimoto *et al.*, 2016), we tested whether Ala-to-Val substitution at the position 109, close to R102, could affect interactions between HT1 and MPKs (MPK4 and MPK12). In contrast to HT1, HT1 A109V interacted neither with MPK4 nor MPK12 in split-ubiquitin yeast two-hybrid assays (III, Supplemental Fig. 5). However, a ratiometric BiFC assay in *N. benthamiana* leaves showed that both versions of HT1 interacted with MPK4 and MPK12, although the interaction between HT1 A109V and MPK4 or MPK12 was weaker compared with the interaction between HT1 and MPK4 or MPK12 (III, Fig. 3b–d and Supplemental Fig. 6a,b). Neither HT1 nor HT1 A109V interacted with MPK11 in the Y2H and ratiometric BiFC assays (III, Fig. 3c,d, Supplemental Fig. 5 and 6c). These data indicated that the A109V substitution in HT1 partially impaired the interaction of HT1 with MPK4 and MPK12.

We also performed *in vitro* kinase assays with purified recombinant 6xHis-tagged HT1 and HT1 A109V to test whether the A109V mutation, which located close to the ATP binding site of the HT1 kinase domain, could affect the kinase activity of HT1 (III, Fig. 4a). Similarly to the R102K mutation (Hashimoto-Sugimoto *et al.*, 2016), the Ala-to-Val substitution at the position 109 did not affect either the autophosphorylation activity of HT1 or phosphorylation of casein by HT1 (III, Fig. 4a). However, inhibition of HT1 A109V by MPK12 was decreased compared with the inhibition of HT1 by MPK12 (Figure 15).

Taken together, these data suggest that the region of the HT1 protein harbouring R102 and A109 is important for the HT1 function in stomatal CO_2 signalling, likely due to protein-protein interactions, since the activity of the kinase is not affected by the mutations at these positions. Both, *ht1-3* (Hashimoto-Sugimoto *et al.*, 2016) and *ht1-8D* dominant mutations, carrying R102K and A109V substitutions in HT1, respectively caused constitutively higher stomatal conductance and a complete lack of CO_2 responses.

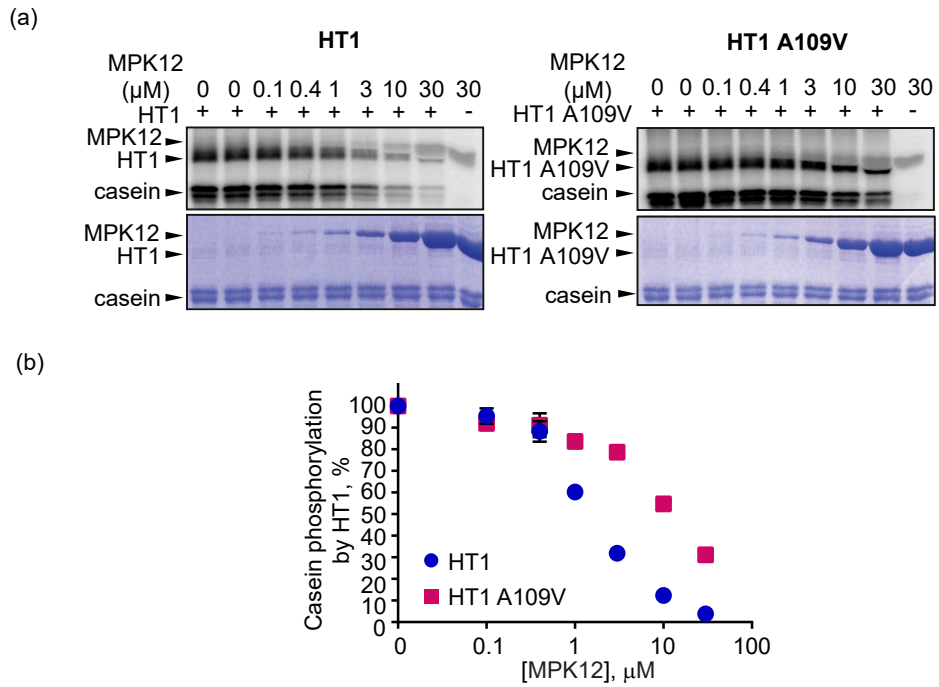

Figure 15. HT1 A109V activity is not inhibited by MPK12 as efficiently as the activity of HT1.
(a) HT1-induced autophosphorylation and casein phosphorylation are inhibited by MPK12, and the inhibition is decreased in HT1 A109V. Autoradiographs (upper panel) and Coomassie-stained SDS PAGE (lower panel) are shown.
(b) Quantification of MPK12-induced inhibition of HT1 and HT1 A109V activity (mean ± SEM; n = 3). (Modified from the publication III, Fig. 4b–d)

4.6. HT1 can inhibit SLAC1 currents induced by OST1 and GHR1

4.6.1. GHR1 functions in stomatal CO_2 signalling

A leucine-rich repeat receptor-like kinase GHR1 is required for ABA-induced stomatal closure (Hua *et al.*, 2012). GHR1 activated SLAC1 in *X. laevis* oocytes and a mutation in *GHR1* resulted in impaired ABA-induced stomatal closure and S-type anion channel activation (Hua *et al.*, 2012). Besides exhibiting an impaired stomatal response to ABA, stomata of *ghr1-3* T-DNA insertion mutants did not respond to elevated [CO_2] (Figure 16; IV, Fig. 2b,c).

OST1 kinase is also required for CO_2- and ABA-induced stomatal closure (Xue *et al.*, 2011, Merilo *et al.*, 2013). *ost1* mutants exhibited impaired CO_2- (Xue *et al.*, 2011, Merilo *et al.*, 2013) and ABA-induced stomatal closure (Mustilli *et al.*, 2002) as well as suppressed HCO_3^-/CO_2- and ABA-induced activation of S-type anion currents (Geiger *et al.*, 2009, Xue *et al.*, 2011). OST1 phosphorylated N- and C-terminal domains of SLAC1 *in vitro* (Geiger *et al.*, 2009, Lee *et al.*, 2009b, Vahisalu *et al.*, 2010) and enhanced SLAC1 currents in *X. laevis* oocytes (Geiger *et al.*, 2009, Maierhofer *et al.*, 2014).

Since the stomata of the *ghr1-3* T-DNA insertion mutants did not respond to elevated [CO_2], but the stomata of *ost1-3* loss-of-function allele (Yoshida *et al.*, 2002) displayed delayed response (Figure 16; IV, Fig. 2b), signalling through GHR1 could be more important than that through OST1 for CO_2-induced stomatal closure.

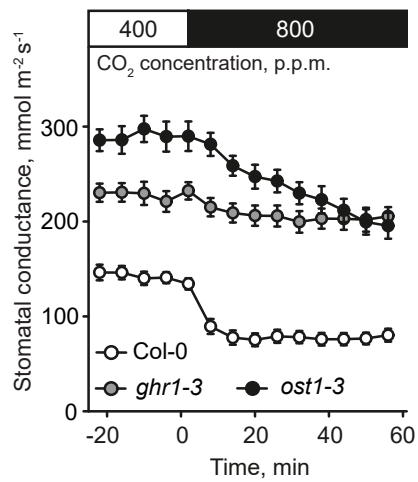

Figure 16. *ghr1-3* plants show impaired stomatal CO_2 response.
Time course of stomatal conductance of Col-0, *ghr1-3* and *ost1-3* plants in response to elevated [CO_2]. The stimulus was applied at time point zero, and pooled stomatal conductance data from three independent experiments are shown (mean ± SEM, n = 8 to 21). (Modified from the publication IV, Fig. 2b)

We also characterized the genetic interaction of *HT1* and *GHR1* in plants (III). We generated an *ht1-2 ghr1-1* double-mutant and analysed stomatal conductance in *ht1-2*, *ht1-2 ghr1-1* and *ghr1-1* plants in response to [CO_2] changes and ABA treatment. *ht1-2 ghr1-1* plants had higher stomatal conductance than *ht1-2* plants, but lower than *ghr1-1* plants at ambient [CO_2] (III, Fig. 7c–e). Both, *ht1-2* and *ht1-2 ghr1-1* mutants were completely insensitive to low and high [CO_2]-induced stomatal movements (III, Fig. 7c,d). However, similar to the *ghr1-1* single-mutant, the *ht1-2 ghr1-1* double-mutant also displayed ABA insensitivity, which was not observed in the *ht1-2* single-mutant (III, Fig. 7e). Therefore, HT1 could act upstream of, or parallel with GHR1.

4.6.2. HT1 inhibits SLAC1 currents induced by OST1 and GHR1 in *X. laevis* oocytes

Since both GHR1 and OST1 mediated [CO_2]-induced stomatal closure, we tested whether HT1 could affect GHR1- or OST1-mediated SLAC1 activation (III). To address this question, we used a heterologous system and expressed SLAC1 fused with C-terminal part of YFP (SLAC1-YC), GHR1 or OST1 fused with the N-terminal part of YFP (OST1-YN or GHR1-YN), and HT1 (or HT1 A109V) in *X. laevis* oocytes (III). Experiments showed that HT1 was able to inhibit S-type anion currents in oocytes when HT1 was coexpressed with GHR1-YN (or OST1-YN) and SLAC1-YC (Figure 17a,b; III, Fig. 5a,b and Supplemental Fig. 7a,b). Interestingly, HT1 A109V also inhibited SLAC1 anion currents in oocytes when coexpressed with GHR1 (or OST1) and SLAC1 (Figure 17a,b; III, Fig. 5a,b and Supplemental Fig. 7a,b). These experiments indicate that HT1 could inhibit SLAC1 anion currents mediated by GHR1 or OST1.

MPK12 inhibited the kinase activity of HT1 *in vitro* (Figure 8). Thus, we tested whether MPK12 could also function as an inhibitor of HT1 in oocytes (III). To address this question, we expressed MPK12 and HT1 together with SLAC1-YC and GHR1-YN in oocytes (III). Indeed, MPK12 restored the GHR1-mediated activation of SLAC1 in the presence of HT1 (Figure 17c). The activity of HT1 A109V was inhibited to a lesser extent by MPK12 compared with that of HT1 *in vitro* (Figure 15a,b). As expected, GHR1-mediated SLAC1 activation was not restored by MPK12 in the presence of HT1 A109V in oocytes (Figure 17c). These results further support a regulatory role for MPK12 in HT1-mediated CO_2 signalling in guard cells. Also, this data can explain the phenotype of dominant *ht1-8D* mutants, which harbour an A109V substitution in HT1. HT1 A109V could not be inhibited by MPK12, which enables HT A109V to inhibit SLAC1 activation by GHR1. Suppression of SLAC1 activation in *ht1-8D* results in higher stomatal conductance and the lack of CO_2-induced stomatal closure.

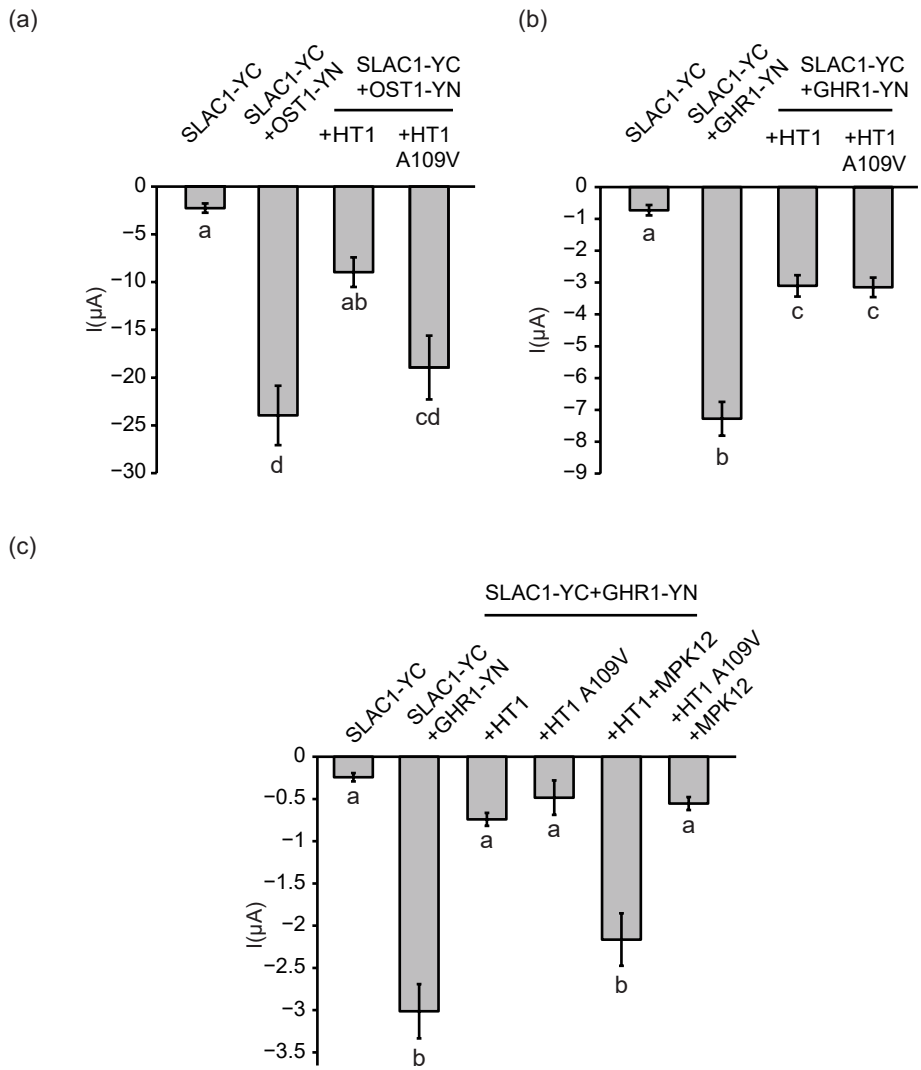

Figure 17. HT1 inhibits OST1- and GHR1-activated SLAC1 anion currents in oocytes and MPK12 counteracts HT1 but not HT1 A109V-induced downregulation of SLAC1 activity in oocytes.
(a) HT1 inhibits OST1-induced SLAC1 activation in oocytes.
(b) HT1 inhibits GHR1-induced SLAC1 activation in oocytes.
(c) MPK12 releases the inhibition of GHR1-induced SLAC1 activation in oocytes caused by HT1, but not by HT1 A109V. In (a) to (c), average currents ± SEM at -140 mV are shown. Statistically significantly different groups are denoted with different letters (ANOVA + Tukey unequal N HSD post hoc test). The sample size was 10 to 17 in (a), 5 to 11 in (b), and 8 to 12 in (c). (Modified from the publication III, Fig. 5a–c)

4.6.3. HT1 phosphorylates GHR1 and SLAC1 *in vitro*

CBC1 and CBC2, which may function as negative regulators of CO_2-induced stomatal closure, have been identified as potential substrates of HT1 (Hiyama *et al.*, 2017). Since SLAC1 activation by GHR1 and OST1 was inhibited by HT1 in oocytes, we tested whether HT1 could phosphorylate GHR1 or OST1, and also whether HT1 could directly phosphorylate N-terminus of SLAC1 *in vitro* (III). We performed *in vitro* kinase assays using purified recombinant 6xHis-tagged HT1, HT1 A109V and N-terminus of SLAC1, GST-tagged full-length GHR1, kinase-inactive OST1 K50N and C-terminus of SLAC1. Both, HT1 and HT1 A109V phosphorylated GHR1 and N-terminus of SLAC1, whereas OST1 and C-terminus of SLAC1 were not phosphorylated by either version of HT1 (Figure 18). Also, MPK4 and MPK12 were able to inhibit phosphorylation of GHR1 by HT1 but not by HT1 A109V *in vitro* (III, Fig. 6b,d). Thus, phosphorylation of GHR1 and N-terminus of SLAC1 by HT1 could prevent activation of SLAC1. However, the molecular details of CO_2 signal transduction downstream of HT1 require further investigation.

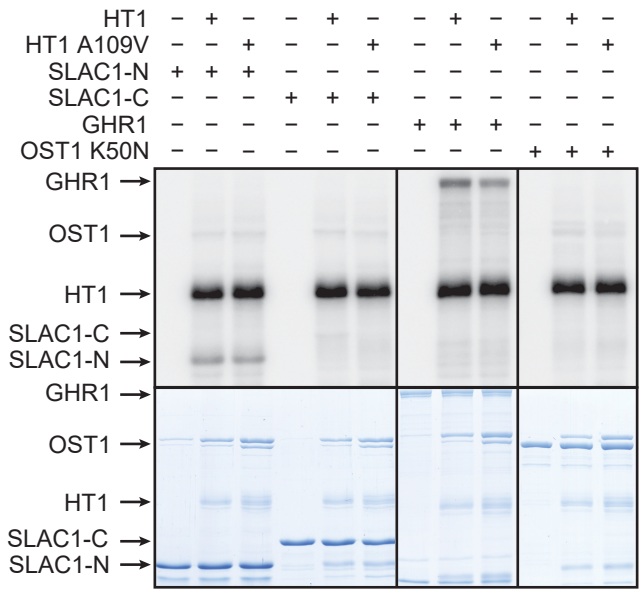

Figure 18. HT1 and HT1 A109V phosphorylate the SLAC1 N-terminus and GHR1. HT1 and HT1 A109V phosphorylate GHR1 and SLAC1 N terminus but not OST1 K50N or SLAC1 C terminus in tested conditions. Autoradiographs (upper panel) and Coomassie-stained SDS PAGE (lower panel) are shown. (Modified from the publication III, Fig. 6a)

4.7. GHR1 does not require kinase activity for SLAC1 activation

Since GHR1 activated SLAC1 in oocytes (III, Fig. 5b and Supplemental Fig. 7b), we tested whether GHR1 could phosphorylate SLAC1 *in vitro* (IV). We performed *in vitro* kinase assays with purified recombinant GST-or 6xHis-tagged full-length GHR1 and 6xHis-tagged N-terminus of SLAC1. Both, GST- and 6xHis-tagged GHR1 did not display autophosphorylation activity and did not phosphorylate N-terminus of SLAC1, whereas OST1 showed autophosphorylation activity and phosphorylated N-terminus of SLAC1 under the same conditions (Figure 19a; IV, Supplemental Fig. 2d).

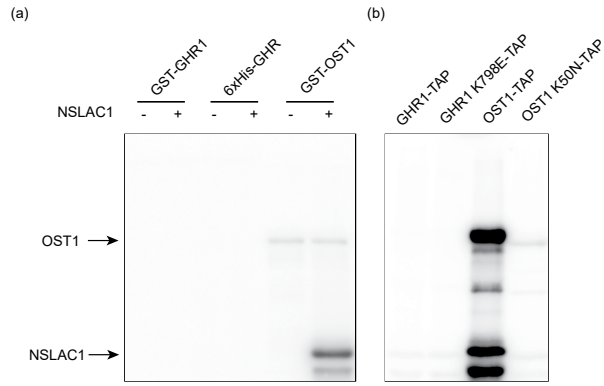

Figure 19. GHR1, purified from *E. coli* and *S. cerevisiae*, is an inactive kinase.
(a) Phosphorylation activity of full-length GST-GHR1, 6xHis-GHR1, and GST-OST1 purified from *E. coli* towards SLAC1 N-terminal fragment. An autoradiograph is shown.
(b) Phosphorylation activity of full-length GHR1-TAP, OST1-TAP and ATP-binding site mutants GHR1 K798E-TAP and OST1 K50N-TAP immunoprecipitated from yeast cell extract towards SLAC1 N-terminal fragment. An autoradiograph is shown. (Modified from the publication IV, Fig. 3d and Supplemental Fig. 2d)

We also tested whether post-translational modifications occurring in eukaryotic cells may be necessary for GHR1 kinase activity (IV). We expressed C-terminally TAP-tagged GHR1 and OST1 in yeast and performed *in vitro* kinase assays with proteins immunoprecipitated from yeast cell extract. OST1 displayed autophosphorylation activity and it phosphorylated N-terminus of SLAC1 efficiently, whereas GHR1 displayed weak residual activity similar to that of negative control GHR1 K798E, in which ATP-binding Lys was substituted by Glu to prevent association of ATP, and kinase-inactive OST1 K50N (Figure 19b; IV, Fig. 3d and Supplemental Fig. 3c).

The leucine-rich repeat (LRR) receptor-like kinase (RLK) GHR1 consists of an extracellular LRR receptor domain, which includes a signal peptide and 19 predicted LRRs, a transmembrane region, and a cytoplasmic kinase domain.

However, two critical residues in the kinase domain, Asp897 and Gly918, required for the kinase function are not conserved and are replaced by Asn and Cys, respectively (IV, Fig. 3a). Introduction of an Asp (GHR1^{ID} N897D), Gly (GHR1^{ID} C918G) or both (GHR1^{ID} N897D/ C918G) into the intracellular domain of GHR1 (GHR1^{ID}; residues 653 to1053) by site-directed mutagenesis did not reconstitute the kinase activity of GHR1 (IV, Fig. 3b and Supplemental Fig. 2a,b).

According to the predicted structure of the catalytic core of GHR1, the key residues for ATP binding (K798 and D916) were present in the active site (IV, Fig. 3c). Nano Differential Scanning Fluorimetry experiments with GHR1 cytoplasmic domain (GHR1^{ID} and GHR1^{ID} K798W) indicated that K798 could bind ATP which led to stabilization of the protein (IV, Fig. 5a–e). However, *ghr1-3* mutants, expressing 35S-GHR1-GFP and 35S-GHR1 K798W-GFP, showed full complementation of the increased water loss and high steady-state stomatal conductance of *ghr1-3* (IV, Fig. 5f,g). O_3- and CO_2-induced stomatal responses of *ghr1-3* were also complemented, although the responses of transgenic lines were slightly weaker than those of the wild-type (IV, Fig. 5h,i and Supplemental Fig. 4a–d). GHR1 versions, in which amino acid residues required for ATP binding were mutated, were also able to activate SLAC1 in *X. laevis* oocytes (IV, Fig. 4a,c). Taken together, GHR1 could be classified as a pseudokinase. It lacks key residues required for the kinase function in its active site, and it does not display *in vitro* kinase activity. Also, the kinase activity of GHR1 is not required for the activation of SLAC1 in oocytes. Although GHR1 can bind ATP *in vitro*, the binding of ATP is not important for its function in plants.

ghr1-17 allele which lacks most of the kinase domain of GHR1 due to introduction of a stop codon (GHR1 W799*) in the kinase domain after ATP binding Lys (GHR1 K798), and *ghr1-3* showed similar stomatal phenotypes, although the phenotypes of *ghr1-17* were not as severe as those of *ghr1-3* (IV, Fig. 7a–d). GHR1-YFP and GHR1 W799*-YFP had similar localization pattern when expressed stably in Arabidopsis or transiently in *N. benthamiana* (IV, Fig. 7e and Supplemental Fig. 5c,d), and abundance of both of the proteins was similar (IV, Supplemental Fig. 5c). Thus, it is likely that the phenotype of *ghr1-17* is caused by the lack of the kinase domain of GHR1.

The phenotype of *ghr1-3* was complemented by the expression of full-length GHR1-GFP, but not by the expression of the kinase domain GHR1^{KD}-GFP (GHR1^{KD}; residues 764-1053) (IV, Fig. 7f,g and Supplemental Fig. 5e,f). Since GHR1^{KD}-GFP appeared to accumulate in multiple locations within the cell, but also at the cell periphery (IV, Supplemental Fig. 5g), there is a possibility that the change in localization could contribute to the lack of complementation. However, the results described above indicate that the kinase domain of GHR1 is required but not sufficient for GHR1 function in plants.

Interestingly, full-length GHR1 and GHR1 W799*, which lacked most of the kinase domain, interacted with SLAC1 (IV, Fig. 8a,b) and with a Ca^{2+}-dependent protein kinase (CPK3) (IV, Fig. 8f,g) in BiFC assays in *N. benthamiana* leaves. Also, both full-length GHR1 and GHR1 W799* were able to activate SLAC1 in oocytes (IV, Fig. 9a–c). Thus, the N-terminal region (in-

cluding the ectodomain, transmembrane domain and intracellular juxtamembrane domain), but not the kinase domain of GHR1 might be required for the interaction with SLAC1 and its activation. Also, the interaction of GHR1 with CPK3, which was previously shown to phosphorylate SLAC1 (Scherzer *et al.*, 2012, Maierhofer *et al.*, 2014), suggests that GHR1 mediates the activation of SLAC1 via interacting proteins. Moreover, loss of CPK3 has been shown to impair stomatal closure (Mori *et al.*, 2006). Since the kinase domains of several pseudokinases interact with a co-receptor (Halter *et al.*, 2014a, Halter *et al.*, 2014b, Hohmann *et al.*, 2017), it is likely that loss-of-function phenotype of *ghr1-17* could be caused by the loss of interaction with thus far unidentified effectors.

GHR1 was phosphorylated by HT1 at multiple sites (Figure 20; IV, Fig. 10). Notably, HT1 phosphorylated 16 Ser, Thr or Tyr residues located on the intracellular juxtamembrane domain of GHR1 and seven of the phosphorylated residues were located in the kinase domain (Figure 20; IV, Fig. 10). Biochemical analyses suggested that GHR1 was a pseudokinase that mediated activation of SLAC1 via interacting proteins rather by direct phosphorylation activity (IV). Thus, GHR1 could function in stomatal closure as a scaffolding component. Since differential modulation of affinities to interacting proteins through phosphorylation is an established regulatory mechanism of receptor-like kinases, phosphorylation of GHR1 by HT1 suggests that HT1 might play a role in regulating these interactions. The interaction of GHR1 with SLAC1 could also itself trigger a conformational change in SLAC1 that modulates the properties of the channel.

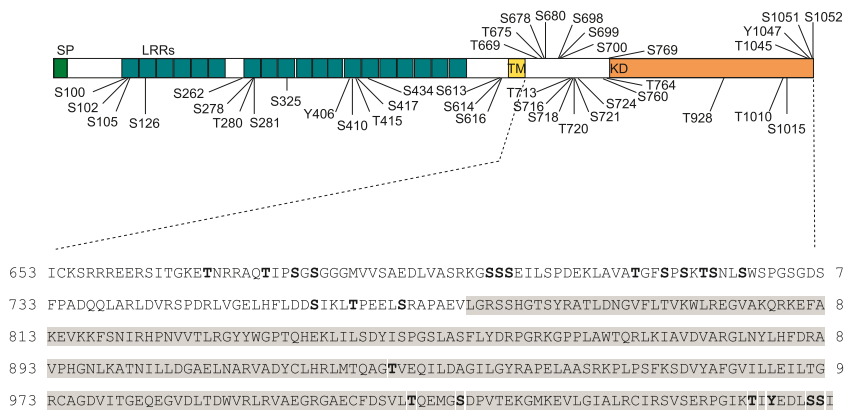

Figure 20. *In vitro* phosphorylation sites of GST-GHR1 by 6xHis-HT1 identified by mass spectrometry.
Purified recombinant GST-GHR1 was phosphorylated by 6xHis-HT1 *in vitro* and the phosphorylation sites were subsequently identified by mass spectrometry. The positions of all identified phosphorylation sites are depicted on the GHR1 protein structure (upper panel). Phosphorylation sites located on the intracellular juxtamembrane domain and pseudokinase domain (grey highlighting) are additionally marked (bold letters) on the GHR1 protein sequence (lower panel). SP, signal peptide; LRR, leucine-rich repeat; TM, transmembrane domain; KD, kinase domain. (From the publication IV, Fig. 10)

4.8. MPK4 has broad functions in plants whereas MPK12 is more specialized in regulation of stomatal responses

As described above, MPK4 and MPK12 function together in the regulation of stomatal responses to CO_2. *MPK4* is expressed in various plant tissues (Petersen *et al.*, 2000) and has different roles in plants (Lin and Chen, 2018). In contrast, *MPK12* is predominantly expressed in guard cells and is involved in the regulation of stomatal aperture in response to abiotic (Jammes *et al.*, 2009, Des Marais *et al.*, 2014) (I, II, III) and biotic stimuli (Jammes *et al.*, 2011).

To address a possible functional redundancy between MPK4 and MPK12 in plants, we tested whether MPK12 could substitute for the function of MPK4 (I). We expressed both proteins under the control of the MPK4 promoter in the *mpk4* mutant background (proMPK4:MPK4 and proMPK4:MPK12). MPK4 and MPK12 in which glycine residues (G55 in MPK4 and G53 in MPK12) were mutated to arginine were also expressed in *mpk4* background (I). Of the various constructs, only proMPK4:MPK4 complemented the dwarf phenotype of *mpk4* plants (Figure 21). These results further emphasize that MPK4 has broad functions in plant signalling pathways, whereas MPK12 is more specific in stomatal CO_2 signalling. Furthermore, the glycine residue G55/G53 was critical for the proper function of both proteins in plants (Figure 21).

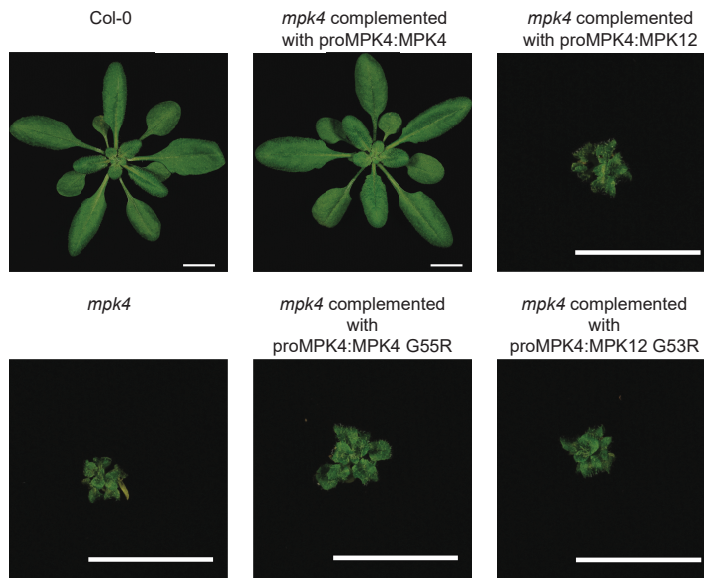

Figure 21. The function of MPK4 in whole plant growth responses.
The *mpk4* mutant was complemented by expression of *MPK4* under its own promoter (proMPK4:MPK4) but not with a point mutation (G55R). Expression of *MPK12* under control of the MPK4 promoter (proMPK4:MPK12 or with the G53R mutation) did not complement the *mpk4* growth phenotype. Shown are photos of representative T2 lines. Scale bars 1 cm. (Modified from the publication I, Fig. 3d)

4.9. A new model for CO_2-induced stomatal closure

Based on previous studies and results presented in this work, we propose a new model for CO_2-induced stomatal closure (Figure 22; V). The elevated CO_2 concentration in leaves could lead to an enhanced influx of CO_2 across the plasma membrane of guard cells through PIP2;1 aquaporins (Wang *et al.*, 2016b). Then, carbonic anhydrases βCA4 and βCA1 accelerate the catalysis of CO_2 molecules to bicarbonate and protons (Hu *et al.*, 2010, Hu *et al.*, 2015). Intracellular bicarbonate ions, in turn, act as signalling molecules which mediate CO_2 signal transduction in guard cells (Xue *et al.*, 2011, Tian *et al.*, 2015, Wang *et al.*, 2016b). However, the primary bicarbonate/CO_2-sensing proteins that mediate the stomatal response remain to be identified. It has been reported that the MATE-type transporter RHC1 might function as a bicarbonate sensing component in CO_2-induced stomatal closing pathway (Tian *et al.*, 2015). However, the role of RHC1 as a bicarbonate sensor is questionable since RHC1 alone was able to induce anion currents, which were independent of bicarbonate, in *X. laevis* oocytes (Wang *et al.*, 2016b).

The S-type anion channel SLAC1 is the central positive mediator of CO_2-induced stomatal closure (Negi *et al.*, 2008, Vahisalu *et al.*, 2008), but the R-type channel ALMT12/QUAC1 also contributes to driving anion efflux during stomatal closure (Meyer *et al.*, 2010). Protein kinases OST1 and GHR1 mediate CO_2 signal transduction in guard cells and activate SLAC1 to trigger stomatal closing (Xue *et al.*, 2011) (III, IV). However, GHR1 could be more important than OST1 for CO_2-induced stomatal closure, since *ghr1* mutants displayed the total lack of response to CO_2, whereas *ost1* mutants showed delayed response (IV). OST1 activates SLAC1 via phosphorylation (Geiger *et al.*, 2009, Lee *et al.*, 2009b) and GHR1 likely mediates SLAC1 activation via interacting proteins or induces anion currents via interaction with SLAC1 (IV). Recently, it was reported that bicarbonate could also directly interact with SLAC1 and up-regulate its activity (Zhang *et al.*, 2018).

HT1 has been shown to be the central regulator of CO_2-induced stomatal movements (Hashimoto *et al.*, 2006, Hashimoto-Sugimoto *et al.*, 2016). It has been reported that HT1 is epistatic to OST1 function during high [CO_2]-induced stomatal movements (Matrosova *et al.*, 2015). We demonstrated that HT1 could function upstream of, or in parallel with, GHR1 in stomatal closure responses (III). Our experiments showed that HT1 could inhibit the activation of SLAC1 by GHR1 and OST1 in *X. laevis* oocytes (III). HT1 could also phosphorylate GHR1 and N-terminus of SLAC1 *in vitro* (III). Whether the phosphorylation of GHR1 by HT1 leads to inhibition of SLAC1 or whether HT1 directly inhibits SLAC1 via phosphorylation remains to be elucidated.

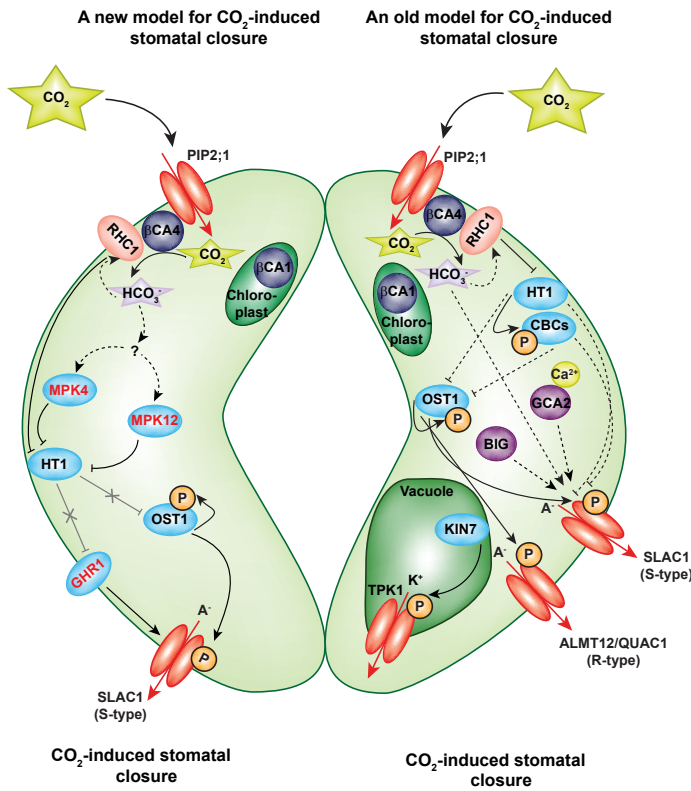

Figure 22. Models for elevated [CO_2]-induced stomatal closure.
(Left) A new model for elevated [CO_2]-induced stomatal closure. The elevated CO_2 concentration in leaves leads to an enhanced influx of CO_2 into guard cells through PIP2;1 aquaporins (Wang *et al.*, 2016b). The PIP2;1 aquaporins interact with the βCA4 carbonic anhydrase (Wang *et al.*, 2016b), which together with the βCA1 accelerate the formation of bicarbonate that acts as an intracellular signalling molecule in guard cells (Hu *et al.*, 2010, Xue *et al.*, 2011, Tian *et al.*, 2015, Wang *et al.*, 2016b). The elevated [HCO_3^-] in guard cells leads to inhibition of HT1 activity by MPK4 and MPK12, which enables the activation of SLAC1 anion channel by OST1 and GHR1, resulting in stomatal closure. New components in CO_2-induced signalling pathway identified in this study are written in red.
(Right) An old model for elevated [CO_2]-induced stomatal closure. The elevated CO_2 concentration in leaves leads to an enhanced influx of CO_2 into guard cells through PIP2;1 aquaporins (Wang *et al.*, 2016b). The PIP2;1 aquaporins interact with the βCA4 carbonic anhydrase (Wang *et al.*, 2016b), which together with the βCA1 accelerate the formation of bicarbonate that acts as an intracellular signalling molecule in guard cells (Hu *et al.*, 2010, Xue *et al.*, 2011, Tian *et al.*, 2015, Wang *et al.*, 2016b). Protein kinases (HT1, OST1, CBCs, KIN7) (Hashimoto *et al.*, 2006, Xue *et al.*, 2011, Hiyama *et al.*, 2017, Isner *et al.*, 2018), intracellular calcium ions (Young *et al.*, 2006) and ion channels (SLAC1, ALMT12/QUAC1, TPK1) (Negi *et al.*, 2008, Vahisalu *et al.*, 2008, Meyer *et al.*, 2010, Xue *et al.*, 2011, Isner *et al.*, 2018) mediate CO_2-induced stomatal closure. However, it remains to be elucidated how these components interact and are integrated into the signalling network. Arrows and blocks represent positive and negative regulation, respectively. Regulation pathways are predicted to be direct (lines) or are unknown and remain to be further investigated (dashed lines).

MPK4 and MPK12 interacted with HT1 in plants and the kinase activity of HT1 was inhibited by MPK4 and MPK12 *in vitro*. However, the kinase activity of MPK4 and MPK12 were likely not required for the inhibition of HT1 activity (II, III). MPK4 and MPK12 could also release SLAC1 from inhibition by HT1 in *X. laevis* oocytes (III). We propose that elevated intracellular HCO_3^-/CO_2 concentration in guard cells leads to inhibition of HT1 by upstream MPKs, MPK4 and MPK12 (Figure 22). Inhibition of HT1, in turn, enables the protein kinases OST1 or GHR1 to activate S-type anion channel SLAC1, which initiates ion efflux from guard cells and triggers stomatal closure in response to elevated CO_2 (Figure 22). It is likely that regulation of HT1 by MPKs also has a role in stomatal opening pathway, since stomatal opening in response to low [CO_2] was also severely impaired in the *mpk12 mpk4GC* double-mutants (I, Fig. 1c), and in *ht1-2* (Hashimoto *et al*., 2006, Matrosova *et al*., 2015) and *ht1-8D* mutants (III, Fig. 2b).

The G53R mutation in MPK12 and the G55R mutation in MPK4 decreased the ability of these MPKs to interact with HT1 and inhibit its kinase activity (Figure 23; II). The A109V mutation in HT1 also caused decreased inhibition of HT1 by MPK4 and MPK12 (Figure 23; III). Lack of inhibition of HT1 in plants could lead to enhanced inhibition of SLAC1 activity which could cause higher stomatal conductance and decreased sensitivity to CO_2 in stomatal closure responses (Figure 23).

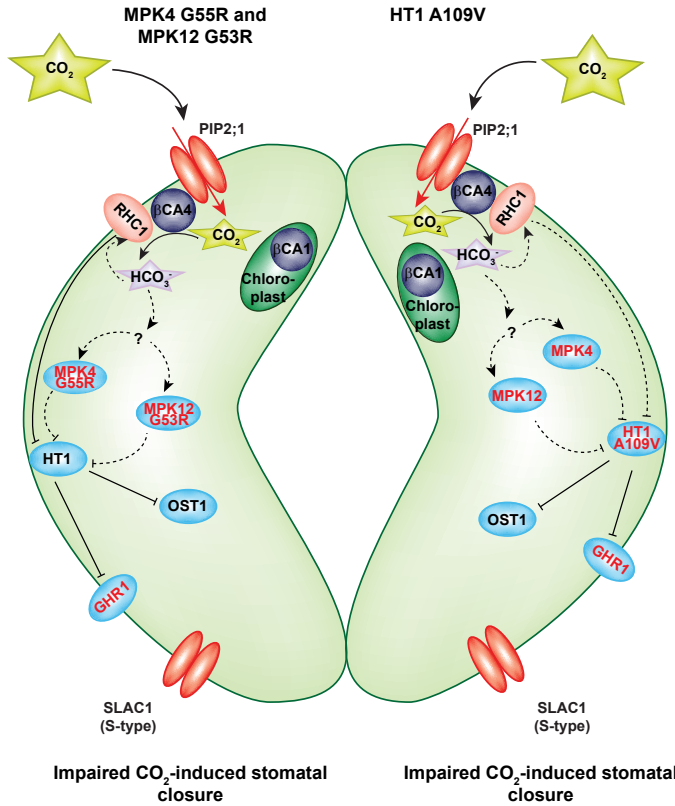

Figure 23. The mutations in *MPK4*, *MPK12* and *HT1* cause impaired CO_2-induced stomatal closure.
(Left) MPK4 G55R and MPK12 G53R do not fully inhibit HT1. Thus, HT1 could inhibit the activation of SLAC1 by OST1 and GHR1 which cause impaired CO_2-induced stomatal closure.
(Right) MPK4 and MPK12 do not fully inhibit HT1 A109V. Thus, HT1 A109V could inhibit the activation of SLAC1 by OST1 and GHR1 which cause impaired CO_2-induced stomatal closure.

CONCLUSIONS

- Both MPK4 and MPK12 are required for CO_2-induced stomatal movements. Stomata of *mpk4GC* and *mpk12* single-mutant plants showed wild-type-like and partially impaired responses to [CO_2] changes, respectively. In contrast, stomata of *mpk12 mpk4GC* double-mutant plants were completely insensitive to both low and high [CO_2] and bicarbonate-induced activation of S-type anion channels was completely impaired in guard cells of the *mpk12 mpk4GC* plants. Stomatal conductance of the above-mentioned mutants (*mpk12*, *mpk4GC* and *mpk12 mpk4GC*) was higher compared with that of wild-type plants at ambient [CO_2]. Higher stomatal conductance in *mpk12 mpk4GC* double-mutants was due to larger stomatal apertures. Since ABA-induced stomatal closure and activation of S-type anion channels retained functional in *mpk12 mpk4GC* plants, MPK4 and MPK12 may function together upstream of the convergence point of CO_2 and ABA signalling pathways or act in a parallel CO_2 signalling pathway.
- The activity of MPK4 and MPK12 was not altered by CO_2/HCO_3^- *in vitro*, suggesting that these MAPKs are not direct CO_2 sensors in plants. Thus, CO_2/HCO_3^- sensing mechanisms in guard cells remain to be elucidated. Also, it remains to be clarified in the future studies whether classical MAPK cascades including MPK4 and MPK12 function in guard cell CO_2 signalling and if so which MAP3Ks and MAP2Ks act upstream of MPK4 and MPK12.
- MPK4 and MPK12 interacted with the HT1 kinase which is the main regulator of CO_2-induced stomatal movements. Both MPK4 and MPK12 inhibited the activity of HT1 *in vitro*. The conserved glycine residue (G55 in MPK4 and G53 in MPK12) but not the kinase activity of MPK4 and MPK12 was required for the inhibition of HT1 activity. Introduction of a G55R mutation in MPK4 or a G53R mutation in MPK12 weakened the interaction between these MPKs and HT1, and strongly suppressed MPK4- and MPK12-induced inhibition of HT1 activity. The G53R mutation in MPK12 caused higher stomatal conductance, reduced water use efficiency and partially impaired CO_2 responses in Arabidopsis Cvi-0 accession or in a near-isogenic line in the Col-0 background (Col-S2).
- Ala109 in HT1 was required for the interaction with MPK4 and MPK12, and also for the inhibition of HT1 activity by these MPKs *in vitro*. The A109V mutation caused constitutively higher stomatal conductance and a complete lack of CO_2 responses in *ht1-8D* plants.
- *HT1* genetically interacted with *GHR1* in CO_2-induced stomatal closure. HT1 inhibited SLAC1 currents induced by GHR1 or OST1 in *X. laevis* oocytes and MPK12 restored GHR1-mediated SLAC1 activation in the presence of HT1. HT1 phosphorylated GHR1 and N-terminus of SLAC1 *in vitro*. However, whether the phosphorylation of GHR1 by HT1 leads to inhibition of SLAC1 activity or whether HT1 directly inhibits SLAC1 via phosphorylation remains to be elucidated.

- GHR1 could be classified as a pseudokinase which likely acts as a scaffolding component in CO_2-induced stomatal closure. Since the stomata of the *ghr1-3* mutants did not respond to elevated [CO_2], but the stomata of *ost1-3* mutants displayed delayed response, signalling through GHR1 could be more important than that through OST1 for CO_2-induced stomatal closure.
- MPK9 was not involved in CO_2- or ABA-induced stomatal movements.
- Taken together, the present study revealed that MPK4 and MPK12 but not MPK9 are among the earliest CO_2 signalling components presently known in guard cells. It suggested that CO_2-signal is transmitted through MPK4 and MPK12, leading to inhibition of HT1 which, in turn, enables SLAC1 activation by its activators, OST1 and GHR1. The G53R mutation in MPK12 and the G55R mutation in MPK4 decrease the ability of these MPKs to interact with HT1 and inhibit its kinase activity. The A109V mutation in HT1 also decreases MPK4- and MPK12-induced inhibition of HT1. Lack of inhibition of HT1 in plants could lead to enhanced inhibition of SLAC1 activity which could cause higher stomatal conductance and decreased sensitivity to elevated [CO_2]. With the continuous increase in atmospheric CO_2 levels and the need for breeding crops that would display optimal water use efficiencies, these findings are important for developing strategies for breeding crops suitable for future climates by specific modulation of CO_2-dependent stomatal movements.

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SUMMARY IN ESTONIAN

Mitogeen-aktiveeritud proteiinkinaaside, MPK4 ja MPK12, roll õhulõhede vastuses CO_2 kontsentratsiooni muutustele

Taimed on elu aluseks Maal. Kasutades Päikeselt tulevat valgusenergiat, toodavad nad süsihappegaasist ja veest fotosünteesi käigus orgaanilisi ühendeid ning kõrvalsaadusena vabaneb selle käigus ka loomseks eluks vajalik hapnik. Taimed on olulisel kohal nii inimese kui loomade toidulaual. Kuna maailm seisab silmitsi kliimamuutustega, mida põhjustab järjest suurenev süsihappegaasi kontsentratsioon atmosfääris, on tulevikukliima tingimustes saagikate toidutaimede aretamiseks oluline mõista, kuidas taimed reageerivad erinevate keskkonnatingimuste, sealhulgas õhu CO_2 kontsentratsiooni, muutumisele.

Õhulõhed on taimelehel asuvad avad, mis reguleerivad taime gaasivahetust ümbritseva keskkonnaga. Õhulõhede kaudu siseneb taime süsihappegaas ja väljub veeaur ning hapnik. Iga õhulõhe on ääristatud kahe sulgrakuga, mille ruumala muutus kutsub esile õhulõhe avanemise või sulgumise. Kui õhulõhesid ümbritsevatesse sulgrakkudesse sisenevad ioonid ja vesi, mille tulemusel nende rakkude ruumala ja turgorrõhk suurenevad, siis õhulõhed avanevad. Ioonide ja vee väljavool põhjustab aga sulgrakkude ruumala ja turgorrõhu vähenemise, mis viib omakorda õhulõhede sulgumiseni. Õhulõhede avanemise põhjustab valgus, madal CO_2 kontsentratsioon ja kõrge õhuniiskus, sulgumise aga pimedus, kõrge CO_2 kontsentratsioon, madal õhuniiskus, taimehormoonid (nt abstsiishape (ABA)), patogeenid ja õhu saasteained. Õhulõhede avanemine soodustab taime kasvu, kuna avatud õhulõhede kaudu jõuab taime fotosünteesiks vajalik CO_2, samas aga suureneb avatud õhulõhede kaudu ka vee aurustumine taimest, mis põuatingimustes võib viia taime närbumiseni. Seetõttu on õhulõhede avatuse täpne regulatsioon ülioluline, optimeerimaks taime kasvu erinevates stressitingimustes.

Valguse käes CO_2 kontsentratsioon rakuvaheruumides väheneb fotosünteesi tulemusel, mistõttu õhulõhed avanevad, lehte pääseb rohkem CO_2, et fotosüntees saaks jätkuda. Pimedas aga õhulõhed sulguvad, kuna rakuhingamise tagajärjel CO_2 kontsentratsioon rakuvaheruumides suureneb. Atmosfääris oleva CO_2 kontsentratsiooni suurenemine vähendab õhulõhede avatust isegi valges, mistõttu vee aurustumine taimest väheneb. Mõistes, kuidas toimub CO_2 mõjul õhulõhede sulgumine, oleks võimalik aretada toidutaimi, mille õhulõhed on piisavalt avatud, et taime pääseks vajalik kogus CO_2, kuid samal ajal veekadu aurustumise näol oleks minimaalne. See võimaldaks parandada saagikust ja kokku hoida vett piirkondades, kus veevarud on piiratud. Piirkondades, kus aga veevarud on piisavad, võiks parandada saagikust selliste toidutaimede aretamine, mille õhulõhed reageeriksid atmosfääri kõrgemale CO_2 kontsentratsioonile vähem ning oleksid seega rohkem avatud ja soodustaksid läbi selle fotosünteesi.

Praegusel ajal pole täpselt teada, kuidas toimub CO_2 mõjul signaaliülekanne, mis viib ioonide ja vee väljumiseni sulgrakkudest ning seejärel õhulõhede sulgumiseni. Teada on vaid mõned valgud, mis osalevad õhelõhede avatuse reguleerimisel, kuid kuidas need valgud omavahel seotud on, pole päris täpselt selge. Lisaks osaleb suurem osa tuvastatud valkudest õhulõhede vastuses nii CO_2-le kui ABA-le, kuid valke, mis osalevad üksnes õhulõhede vastuses CO_2-le, on teada väga vähe. Käesolev töö annab panuse mõistmaks, kuidas CO_2 kutsub esile õhulõhede sulgumise ning pakub välja uue mudeli õhulõhede sulgumise kohta kõrge [CO_2] mõjul.

Antud töös uuriti mitogeen-aktiveeritud proteiinkinaaside, MPK4 ja MPK12, rolli õhulõhede vastuses CO_2 kontsentratsiooni muutustele. Mitogeen-aktiveeritud proteiinkinaasid (MAP-kinaasid) on seriini/treoniini-spetsiifilised proteiinkinaasid, mis modifitseerivad sihtmärkvalke fosfaatrühma lisamise (fosforüleerimise) teel, mõjutades nii nende aktiivsust, rakus paiknemist või sidumisomadusi. MAP-kinaaside rolli näiteks taime arengus ja immuunreaktsioonides on laialdaselt uuritud, kuid vähe on teada nende osalusest õhulõhede avanemisel ja sulgumisel. Kirjandusest on teada, et MAP-kinaase, MPK4 ja MPK12, kodeerivad geenid on sulgrakkudes kõrgelt ekspresseeritud, kuid ainult *MPK12* ekspressioon on sulgraku-spetsiifiline. *MPK4* ekspresseeritakse lisaks sulgrakkudele ka teistes taimekudedes. Samuti leidub vihjeid, et MPK4-l ja MPK12-l võib olla roll hariliku müürlooga ning tubakataimede õhulõhede avatuse reguleerimisel. Uurimaks, kas MPK4 ja MPK12 osalevad õhulõhede avatuse regulatsioonis CO_2 ja ABA mõjul, konstrueeriti antud töö käigus hariliku müürlooga taimeliinid, kus *MPK12* ekspressioon puudus ning samaaegselt oli ka *MPK4* ekspressioon sulgraku-spetsiifiliselt vaigistatud (*mpk12 mpk4GC* kaksikmutandid), ja analüüsiti nende taimede õhulõhede juhtivust. Analüüsiti ka selliste taimede, kus puudus ainult *MPK12* ekspressioon (*mpk12* üksikmutandid) või oli ainult *MPK4* ekspressioon sulgraku-spetsiifiliselt vaigistatud (*mpk4GC* üksikmutandid), õhulõhede juhtivust. Selgus, et *mpk12 mpk4GC* topeltmutantide õhulõhed on normaaltingimustel rohkem avatud ning seetõttu on nende juhtivus kõrgem, võrreldes metsiktüüpi taimede õhulõhedega. Samuti täheldati, et vastupidiselt metsiktüüpi taimede õhulõhedele, mis sulgusid kõrge [CO_2] ja avanesid madala [CO_2] mõjul, ei reageerinud *mpk12 mpk4GC* taimede õhulõhed CO_2 kontsentratsiooni muutustele. Samas aga oli neil sarnaselt metsiktüüpi taimede õhulõhedele säilinud võime sulguda ABA mõjul. Võrreldes *mpk12 mpk4GC* kaksikmutantidega, reageerisid *mpk12* üksikmutantide õhulõhed osaliselt CO_2 kontsentratsiooni muutustele ning sarnaselt *mpk12 mpk4GC* taimede õhulõhedele oli nende reageerimisvõime ABA-le säilinud. Sulgrakkude spetsiifiliste *mpk4GC* taimede õhulõhed aga avanesid madala [CO_2] ja sulgusid kõrge [CO_2] mõjul sarnaselt metsiktüüpi taimede õhulõhedele, kuid *mpk4GC* taimede õhulõhede juhtivus oli normaaltingimustel kõrgem. Analüüsiti ka õhulõhede sulgumisel olulist rolli omavate S-tüüpi (aeglaste) anioonkanalite aktiveerumist CO_2/HCO_3^- ja ABA mõjul *mpk12 mpk4GC* sulgrakkude protoplastides. Selgus, et anioonkanaleid ei aktiveerita *mpk12 mpk4GC* sulgrakkude protoplastides CO_2/HCO_3^- mõjul, kuid aktiveeritakse ABA mõjul. Eelpool-

kirjeldatud tulemustest võib järeldada, et nii MPK4 kui MPK12 osalevad õhulõhede sulgumisel kõrge [CO_2] ning avanemisel madala [CO_2] mõjul ning MPK4 ja MPK12 on vajalikud S-tüüpi anioonkanalite aktiveerimiseks CO_2/HCO_3^- mõjul. Samuti järeldub neist eksperimentidest, et MPK4 ja MPK12 ei ole olulised õhulõhede sulgumiseks ABA toimel.

Kuna kirjanduses on vihjatud, et MPK9-l võib sarnaselt MPK4-le ja MPK12-le olla roll müürlooga õhulõhede avatuse regulatsioonis, analüüsiti ka selliste taimede, kus puudus *MPK9* ekspressioon, õhulõhede juhtivust. Selgus, et *mpk9* üksikmutantide õhulõhed avanevad madala [CO_2] ja sulguvad kõrge [CO_2] toimel sarnaselt metsiktüüpi taimede õhulõhedele. *mpk12 mpk9* kaksikmutantide õhulõhed reageerisid CO_2 kontsentratsiooni muutustele aga sarnaselt *mpk12* üksikmutantide õhulõhedele ning *mpk12 mpk9 mpk4GC* kolmikmutantide õhulõhed sarnaselt *mpk12 mpk4GC* kaksikmutantide õhulõhedele. Nii *mpk9* üksik-, *mpk12 mpk9* kaksik- kui *mpk12 mpk9 mpk4GC* kolmikmutantide õhulõhed sulgusid ABA mõjul sarnaselt metsiktüüpi taimede õhulõhedele. Antud tulemustest võib järeldada, et MPK9 ei osale õhulõhede avatuse reguleerimisel CO_2 ning ABA mõjul.

Kuna CO_2 retseptorid/sensorid taimedes pole teada, püüti välja selgitada, kas MPK4 ja MPK12 võiks toimida CO_2 sensoritena. Katsetest rekombinantsete valkudega selgus, et nii MPK4 kui ka MPK12 ei aktiveeru CO_2/HCO_3^- toimel *in vitro*, viidates, et need MAP-kinaasid ei toimi CO_2 sensoritena.

Pärmi kaksikhübriid-meetodil MPK4 ja MPK12-ga seonduvate valkude tuvastamiseks läbiviidud skriinimise käigus leiti, et nii MPK4 kui ka MPK12 interakteeruvad kinaasiga HT1, mis omab olulist rolli õhulõhede avatuse regulatsioonis CO_2 mõjul. MPK4 ning MPK12 interakteerusid HT1-ga ka taimerakkudes (tubaka lehe epidermiserakud) ning eksperimentides rekombinantsete valkudega. Lisaks täheldati, et konserveerunud glütsiinijääk (G55 MPK4-s ja G53 MPK12-s) on oluline MPK4 ja MPK12 interaktsiooniks HT1-ga. Antud glütsiinijäägi asendamine arginiinijäägiga (R) viis MPK4 või MPK12 ja HT1 vahelise interaktsiooni nõrgenemiseni. Katsed rekombinantsete valkudega näitasid, et MPK4 ning MPK12 inhibeerivad HT1 autofosforüleerumist ning HT1-vahendatud kaseiini fosforüleerimist *in vitro*. Lisaks selgus, et HT1 inhibitsioon ei sõltu MPK4 ja MPK12 aktiivsusest, kuid konserveerunud glütsiinijääk, mis oli oluline MPK4 ning MPK12 interaktsiooniks HT1-ga, oli vajalik ka HT1 efektiivseks inhibeerimiseks MPK4 või MPK12 poolt. Võrreldes MPK4- ja MPK12-vahendatud HT1 inhibitsiooniga, inhibeerisid MPK4 G55R ja MPK12 G53R HT1 autofosforüleerumist ja HT1-vahendatud kaseiini fosforüleerimist nõrgemini. MPK12 G53R valku ekspresseerivate taimede õhulõhede juhtivus oli kõrgem ning nende taimede veekasutuse efektiivsus madalam, võrreldes MPK12 valku ekspresseerivate taimedega. Samuti põhjustas G53R asendus MPK12-s õhulõhede nõrgenenud reaktsiooni CO_2 kontsentratsiooni muutustele, kuid reageerimisvõime ABA-le säilis. Lisaks tehti kindlaks, et mittekonserveerunud alaniinijääk HT1 valgus (A109) on vajalik HT1 interaktsiooniks MPK4 ja MPK12-ga. Antud alaniinijäägi asendamine valiinijäägiga (V) põhjustas HT1 ja MPK4/MPK12-vahelise interaktsiooni nõrgenemise.

A109V asendus ei mõjutanud HT1 kinaasi aktiivsust *in vitro*, kuid võrreldes HT1 inhibitsiooniga, inhibeerisid MPK4 ning MPK12 HT1 A109V autofosforüleerumist ning HT1 A109V-vahendatud kaseiini fosforüleerimist nõrgemini. Dominantne mutatsioon *HT1* geenis, mis põhjustas A109V asenduse HT1 valgus, tõi kaasa õhulõhede kõrge juhtivuse ning kaotas nende reageerimisvõime CO_2 kontsentratsiooni muutustele, kuid mitte ABA-le.

Samuti näidati, et kinaasid OST1 ja GHR1, mis on vajalikud õhulõhede sulgumiseks ABA mõjul, osalevad ka õhulõhede sulgumisel kõrge [CO_2] mõjul, kuid GHR1 omab õhulõhede sulgumisel kõrge [CO_2] mõjul suuremat tähtsust kui OST1. HT1 ja HT1 A109V takistasid suure kannuskonna (*Xenopus laevis*) ootsüütides S-tüüpi anioonkanali (SLAC1) aktiveerimist GHR1 ja OST1 poolt. MPK12 takistas aga GHR1 inhibeerimist HT1 poolt, võimaldades GHR1-l aktiveerida SLAC1. MPK12 ei takistanud GHR1 inhibeerimist HT1 A109V poolt, mistõttu SLAC1 aktivatsioon polnud võimalik. Lisaks näidati, et GHR1 ei aktiveeri SLAC1-te fosforüleerimise teel, vaid vahendab SLAC1 aktivatsiooni, interakteerudes teiste kinaasidega, mis fosforüleerivad SLAC1-te. Pakuti ka välja, et GHR1 interakteerub ise SLAC1-ga ning aktiveerib selle, kutsudes esile muutuse SLAC1 konformatsioonis.

Võttes arvesse ülalkirjeldatud tulemusi, pakuti välja mudel MPK4 ja MPK12 rolli kohta õhulõhede vastuses kõrgele CO_2 kontsentratsioonile. Mudeli kohaselt interakteeruvad MPK4 ning MPK12 kõrge [CO_2] toimel HT1 kinaasiga ning inhibeerivad selle aktiivsust. Kuna HT1 takistab SLAC1 anioonkanali aktiveerimist GHR1 ja OST1 poolt ning õhulõhede sulgumist, siis HT1 inhibeerimine MPK4 ja MPK12 poolt võimaldab SLAC1 aktivatsiooni ning õhulõhede sulgumise kõrge [CO_2] mõjul. G55R asendus MPK4-s ja G53R asendus MPK12-s viivad nende MPK-de ja HT1-vahelise interaktsiooni nõrgenemiseni. Samuti inhibeerivad MPK4 G55R ja MPK12 G53R HT1 aktiivsust vähem. Seetõttu saab HT1 jätkuvalt takistada SLAC1 aktivatsiooni GHR1 ning OST1 poolt ja õhulõhede sulgumist. Ka A109V asendus HT1 valgus vähendab HT1 ja MPK4/MPK12-vahelise interaktsiooni tugevust. Samuti ei inhibeeri MPK4 ja MPK12 efektiivselt HT1 A109V aktiivsust, mistõttu on SLAC1 aktivatsioon GHR1 ja OST1 poolt ning õhulõhede sulgumine kõrge [CO_2] mõjul takistatud. MPK4 G55R ja MPK12 G53R ning HT1 A109V valke ekspresseerivate taimede õhulõhed on seega kõrge [CO_2] keskkonnas rohkem avatud kui metsiktüüpi taimede õhulõhed.

Kokkuvõtteks, antud töö tulemused aitavad mõista, kuidas taimed reguleerivad õhulõhede avatust CO_2 kontsentratsiooni muutuse toimel. Töö käigus tehti kindlaks uued valgud signaaliülekande rajas, mis viib kõrge [CO_2] mõjul S-tüüpi anioonkanalite aktiveerimiseni sulgrakkudes ning õhulõhede sulgumiseni. Samuti pakuti välja mudel tuvastatud valkude rolli kohta õhulõhede vastuses kõrgele CO_2 kontsentratsioonile. Saadud tulemused aitavad kaasa selliste taimede aretamisele, mille õhulõhed oleks kõrgema atmosfääri CO_2 kontsentratsiooni tingimustes piisavalt avatud, et taime pääseks vajalik kogus CO_2, kuid samal ajal oleks veekadu aurustumise näol minimaalne, võimaldades parandada saagikust ja hoida kokku vett piirkondades, kus veevarud on piiratud. Samuti on

saadud tulemustest kasu, aitamaks kaasa selliste taimede aretamisele, mille õhulõhed reageeriksid atmosfääri kõrgemale CO_2 kontsentratsioonile vähem ning oleksid seega rohkem avatud, võimaldades parandada saagikust piirkondades, kus veevarud on piisavad.

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