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CHUNG-YUEH YEH

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Characterization of MPK and HT1 kinases in CO_2-induced stomatal movements

UNIVERSITY OF TARTU
Press
1632

Institute of Bioengineering, Faculty of Science and Technology, University of Tartu, Estonia

The dissertation was accepted for the commencement of the degree of Doctor of Philosophy in Biomedical Engineering on April 25, 2024, by the Joint Council of the Doctoral Program of Engineering and Technology of the University of Tartu.

Supervisors: Yuh-Shuh Wang, PhD
Associate Professor of Molecular Plant Biology
Institute of Bioengineering, University of Tartu, Estonia

Hannes Kollist, PhD
Professor of Plant Biology
Institute of Bioengineering, University of Tartu, Estonia

Reviewer: Kristiina Laanemets, PhD
Senior Researcher and Head of Department
The Centre of Estonian Rural Research and Knowledge, Estonia

Opponent: Professor Taishi Umezawa
Tokyo University of Agriculture and Technology
Koganei, JAPAN

Commencement: Auditorium 121, Nooruse 1, Tartu, Estonia, at 14.15 on May 17th, 2024

Publication of this thesis is granted by the Institute of Bioengineering, Faculty of Science and Technology, University of Tartu.

ISSN 2228-0855 (print)
ISBN 978-9916-27-518-4 (print)
ISSN 2806-2620 (pdf)
ISBN 978-9916-27-519-1 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

I. **Chung-Yueh Yeh**, Yuh-Shuh Wang, Yohei Takahashi, Katarina Kuusk, Karnelia Paul, Triinu Arjus, Oleksii Yadlos, Julian I Schroeder, Ivar Ilves, Alfonso T Garcia-Sosa, Hannes Kollist (2023). MPK12 in stomatal CO_2 signaling: function beyond its kinase activity. New Phytoloist, 239(1): 146–158. doi: 10.1111/nph.18913.

II. Yohei Takahashi, Krystal C Bosmans, Po-Kai Hsu, Karnelia Paul, Christian Seitz, **Chung-Yueh Yeh**, Yuh-Shuh Wang, Dmitry Yarmolinsky, Maija Sierla, Triin Vahisalu, Cezary Waszczak, J Andrew McCammon, Jaakko Kangasjärvi, Li Zhang, Hannes Kollist, Thien Trac, Julian I Schroeder (2022). Stomatal CO_2/bicarbonate sensor consists of two interacting protein kinases, Raf-like HT1 and non-kinase-activity requiring MPK12/MPK4. Science Advance, 8(49). doi: 10.1126/sciadv.abq6161.

III. Tõldsepp, Kadri; Zhang, Jingbo; Takahashi, Yohei; Sindarovska, Yana; Hõrak, Hanna; Ceciliato, Paulo H.O.; Koolmeister, Kaspar; Wang, Yuh-Shuh; Vaahtera, Lauri; Jakobson, Liina; **Yeh, Chung-Yueh**; Park, Jiyoung; Brosche, Mikael; Kollist, Hannes; Schroeder, Julian I. (2018). Mitogen-activated protein kinases MPK4 and MPK12 are key components mediating CO_2-induced stomatal movements. The Plant Journal, 96 (5), 1018–1035. doi: 10.1111/tpj.14087.

Author's contribution

I. I performed most of gas exchange experiments, western blot analysis and transgenic lines screening. I also participated in analyzing the data, visualizing the results and writing the manuscript.

II. I isolated *ht1* mutants (*ht1-G89R* and *ht1-R173Q*) and analyzed their gas exchange performance in various conditions, edited parts of the manuscript.

III. I performed gas exchange experiments, BiFC experiments, western blot analysis, qPCR experiments, edited parts of the manuscript.

ABBREVIATIONS

ABA	Abscisic acid
ABI1	ABA INSENSITIVE 1
AHA1	Arabidopsis H^+-ATPASE 1
ALMT12	ALUMINUM-ACTIVATED MALATE TRANSPORTER 12
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
At	*Arabidopsis thaliana*
BiFC	Bimolecular fluorescence complementation
βCA1/βCA4	β-carbonic anhydrases 1 and 4
CBC1/ CBC2	CONVERGENCE OF BLUE LIGHT AND CO_2 1/2
CFP	Cyan Fluorescent Protein
Col-0	An *Arabidopsis thaliana* accession from Germany
CPK	Calcium-dependent kinase
Cvi-0	An *Arabidopsis thaliana* accession from Cape Verde islands
GFP	Green Fluorescent Protein
GHR1	GUARD-CELL HYDROGEN PEROXIDE RESISTANT 1
GST	Glutathione S-transferase
HT1	HIGH LEAF TEMPERATURE 1
KAT1/KAT2	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1/2
KIN7	KINASE7
MAPKKK	Mitogen-activated protein kinase kinase kinase
MAPKK	Mitogen-activated protein kinase kinase
MPK	Mitogen-activated protein kinase
NCED3/ NCED5	NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 and 5
NRT1.1	NITRATE TRANSPORTER 1.1
OST1	OPEN STOMATA 1
OST2	OPEN STOMATA 2
PATROL1	PROTON ATPASE TRANSLOCATION CONTROL 1
PP2C	Protein phosphatase type 2C
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RAF	Rapidly accelerated fibrosarcoma
RBOHD/RBOHF	RESPIRATORY BURST OXIDASE D / F
RHC1	RESISTANT TO HIGH CARBON DIOXIDE 1
ROS	Reactive oxygen species
SA	Salicylic acid
SEM	Standard error of the mean
SLAC1	SLOW ANION CHANNEL 1
SnRK2	Sucrose-non-fermenting kinase 2
Y2H	Yeast two-hybrid
YFP	Yellow Fluorescent Protein

INTRODUCTION

Plants are the primary producers i.e. by using solar energy and soil water they are capable to convert atmospheric carbon dioxide (CO_2) into energy rich organic compounds and in the course of this release oxygen. Both organic compounds and oxygen are essential for human beings and other heterotrophs. Concentrations of atmospheric CO_2 are increasing and this drives climate change that affects plant growth and agricultural food production. Therefore, it is important to study how plants deal with and adapt to the changing environment.

Stomata are tiny pores in the epidermis of leaves which are surrounded by two specialized guard cells that control plant gas exchange with the surrounding environment. Plants absorb CO_2 from the atmosphere for photosynthesis and lose water through transpiration via stomata. Guard cells sense many environmental signals such as light, CO_2, volatiles, pathogens, temperature, and humidity to adjust the stomatal aperture to balance the CO_2 uptake and water loss by transpiration. The opening and closing of stomatal pores are caused by a change in guard cell turgor pressure that is triggered by osmotically active ions entering or leaving the guard cell. This rapid and precise change in guard cell size is regulated by complex molecular signaling pathways in the guard cells that include many protein kinases as well as plant hormones such as abscisic acid (ABA). Therefore, unlocking the molecular signaling pathways in the guard cells triggered by different stimuli would help us to design and breed crops with good water use efficiency.

The molecular mechanisms of plants CO_2 sensing are not fully understood although many key components had been identified. This thesis studied molecular mechanism of stomatal CO_2 signaling and provides a model for CO_2 sensing in guard cells.

1. REVIEW OF LITERATURE

1.1 Ion transporters and channels in stomatal movements

1.1.1 Ion inward during stomatal opening

Guard cell plasma membrane H^+ ATPases are integral to the process of stomatal opening (Kinoshita and Shimazaki, 1999). These proton pumps utilize ATP derived from photosynthetic activity, lipid droplet breakdown, and starch breakdown to generate an electrochemical gradient across the guard cell plasma membrane, activating voltage-gated inward rectifying K^+ channels (Daloso et al., 2016; McLachlan et al., 2016; Schroeder et al., 1987). Notably, the primary route for K^+ influx during stomatal opening is through the voltage-gated K^+ channel POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1) (Lebaudy et al., 2008; Nakamura et al., 1995; Pilot et al., 2001). Additionally, several H^+ coupled symport channels have been identified, facilitating the transport of K^+, $Cl^–$, malate$^{2–}$, and other solutes into guard cells (Jezek and Blatt, 2017). Once transported across the plasma membrane and into the cytoplasm, these ions are sequestered in the vacuole/tonoplast (Andrés et al., 2014; Eisenach and De Angeli, 2017), which constitutes most of the intracellular space in guard cells and undergoes significant size changes during stomatal movements (Gao et al., 2005). While the mechanisms of ion transport across the tonoplast membrane are less understood, H^+ coupled antiporters, such as CHLORIDE CHANNEL (CLC), NA^+/H^+ EXCHANGER (NHX), and ALUMINIUM-ACTIVATED MALATE TRANSPORTER (ALMT) family members, are known to play crucial roles in facilitating solute/ion transport into the tonoplast during stomatal opening (Eisenach and De Angeli, 2017).

1.1.2 Ion outward during stomatal closure

Stomatal closure is extensively studied in response to the plant hormone abscisic acid (ABA), yet various closure signals seem to converge on similar downstream targets (Jezek and Blatt, 2017). Early closure events involve alterations in the voltage sensitivities of Ca^{2+} channels, leading to an influx of Ca^{2+} from both extracellular and endomembrane stores (Grabov and Blatt, 1998). Subsequently, activated signaling pathways and elevated cytoplasmic Ca^{2+} levels collaborate to inhibit the activity of plasma membrane H^+ ATPases (Merlot et al., 2007) and inward-rectifying K^+ channels (KAT1) (Grabov and Blatt, 1997; Grabov and Blatt, 1999). Additionally, they activate outward-rectifying anion channels, such as SLOW ANION CHANNEL-ASSOCIATED (SLAC) and ALMT family members, causing an efflux of anions (Geiger et al., 2009; Grabov et al., 1997; Meyer et al., 2010; Schmidt et al., 1995). This efflux of anions leads to the depolarization of the guard cell membrane, further activating the outward- rectifying K^+ channel GATED OUTWARDLY RECTIFYING K^+ CHANNEL (GORK) (Blatt, 1990; Blatt and Armstrong, 1993; Suhita et al., 2004). Although the events on

the plasma membrane are relatively well-understood, our knowledge of the processes on the tonoplast membrane remains limited. The mechanisms of Ca^{2+} efflux from the tonoplast are not entirely clear, and one channel, TWO PORE CHANNEL 1 (TPC1), has been identified with the capability to transport Ca^{2+} into the cytoplasm. However, its recorded stomatal closing phenotype is restricted to an inability to respond to extracellular Ca^{2+} (Islam et al., 2010; Peiter, 2011; Ranf et al., 2007). During closure, the tonoplast undergoes acidification, a process mediated by vacuolar proton pumps on the tonoplast membrane (Bak et al., 2013). TWO PORE K^+ CHANNEL 1 (TPK1) has been shown to be activated by increased cytoplasmic Ca^{2+} concentrations and signaling cascades, playing a role in tonoplast K^+ release during stomatal closure (Gobert et al., 2007; Latz et al., 2007; Latz et al., 2013). Additionally, channels such as CLC, NHX, and ALMT on the tonoplast membrane have been associated with the release of ions from the tonoplast (Eisenach and De Angeli, 2017).

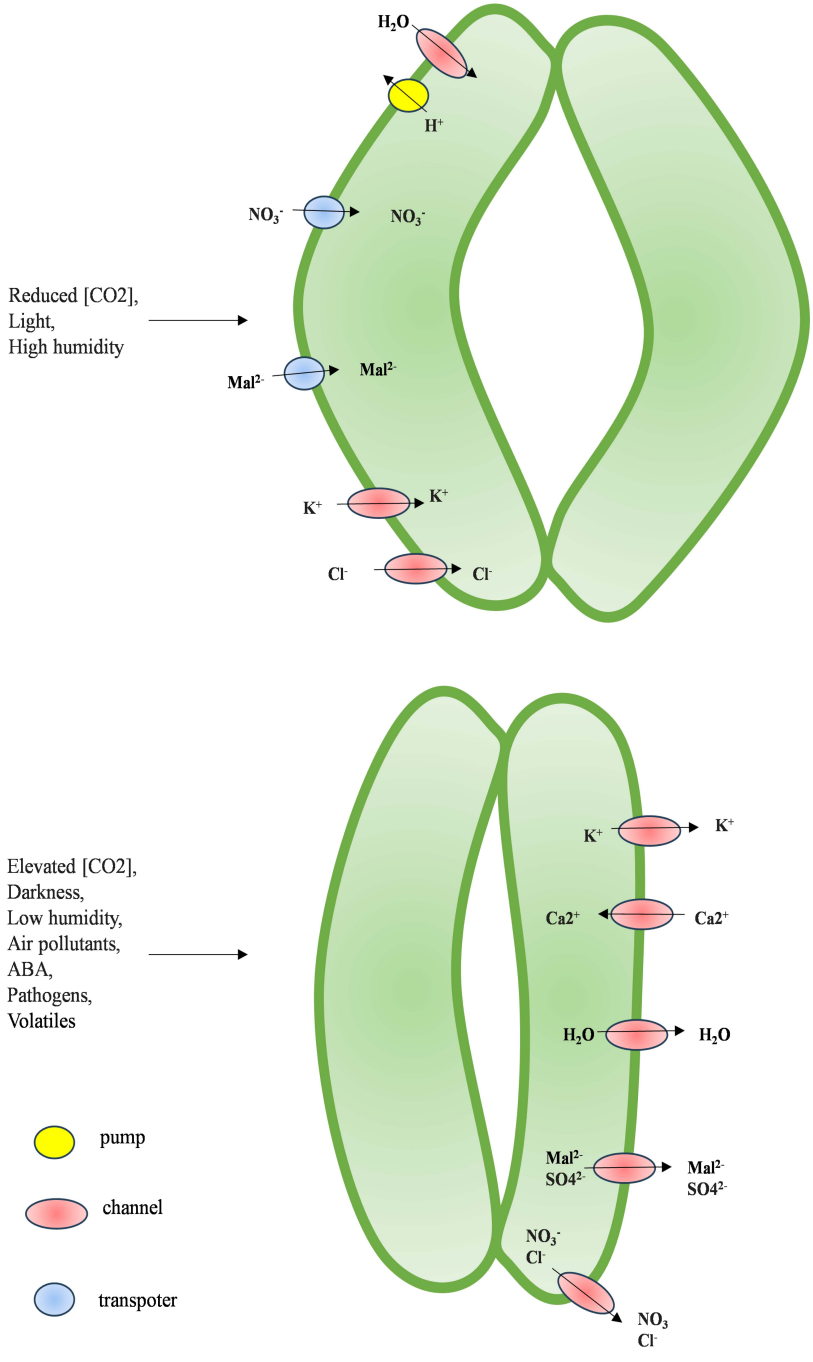

Figure 1. Guard cells response to diverse stimuli that induce the opening or closing of stomata. Stomatal opening results from the efflux of protons and the influx of cations, anions, and water (upper). Conversely, the influx of Ca^{2+} and the release of cations, anions, and water contribute to stomatal closure (lower).

1.2 Stomatal ABA signaling in guard cells

1.2.1 Abscisic acid

Abscisic acid (ABA), a multifunctional plant hormone, was initially extracted from cotton fruit (Ohkuma et al., 1963) and named so due to its association with abscission induction. ABA is classified as a sesquiterpenoid, belonging to the isoprenoid or terpenoid class of organic compounds (Nambara and Marion-Poll, 2005). As one of the crucial phytohormones, ABA plays a significant role in regulating plant growth, development, and orchestrating defensive responses against abiotic stresses, such as drought, cold, and salinity (Cutler et al., 2010). The biosynthesis of ABA occurs de novo, starting from C40 carotenoids that stem from isopentenyl pyrophosphate synthesized within plastids through the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Nambara and Marion-Poll, 2005). While vascular tissues are likely the primary sites of ABA biosynthesis, it can also be synthesized in guard cells, where it, along with transported ABA, triggers downstream signaling pathways culminating in stomatal closure (Nambara and Marion-Poll, 2005). The level of endogenous ABA is governed by a delicate balance between biosynthesis and catabolism, as well as the rate of transport to its target sites. ABA can be biologically inactivated either through hydroxylation and subsequent catabolic pathways or by conjugation with glucose (Nambara and Marion-Poll, 2005; Okamoto et al., 2008)

1.2.2 ABA signaling pathway

The discovery of ABA receptors was a breakthrough in plant biology. A family of 14 cytoplasmic proteins known as PYRABACTIN LIKE (PYL) or REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) proteins unveiled a critical role in ABA sensing and defined a mechanism how plants respond to abiotic stresses (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009). The fundamental characteristics of the ABA signaling pathway are as follows: upon ABA binding, the PYL/RCAR receptors undergo conformational changes, facilitating their interaction with a group of phosphatases called PROTEIN PHOSPHATASE 2C (PP2C). This interaction inhibits the PP2C phosphatase activity, thus preventing them from inhibiting a set of kinases known as SNF1- RELATED PROTEIN KINASE 2 (SnRK2), including SnRK2.6/OPEN STOMATA 1 (OST1) (Umezawa et al., 2009).

Intriguingly, SnRK2 kinases have been found to target not only ion channels but also transcription factors, primarily the ABA INSENSITIVE 5 (ABI5)/ABA RESPONSIVE ELEMENT BINDING PROTEIN (AREB)/ABRE BINDING FACTOR (ABF) family of transcription factors, resulting in altered gene expression (Bensmihen et al., 2005; Yoshida et al., 2010).

In *Arabidopsis*, there are 48 Raf (Rapidly accelerated fibrosarcoma)-like protein kinases, which can be further divided into different groups based on their sequence homology (Ichimura et al., 2002; Jonak et al., 2002). Arabidopsis

B-group Raf-like kinases play crucial roles in osmotic stress (Lozano-Juste et al., 2020). It has been shown that the interaction between SnRK2 and B-group RAFs, resulting in the activation of both ABA-independent and ABA-dependent SnRK2s (Katsuta al., 2020; Lin et al., 2020; Lin et al., 2021; Takahashi et al., 2020; Wang et. al., 2023). For example, a recent proteomic study showed that RAF15(B-group) and RAF19/HT1(C-group) can directly phosphorylate OST1 (Wang et. al., 2023). In this scenario, RAFs serve as upstream kinases that activate SnRK2s. Hence, B-group RAFs positively modulate SnRK2s through phosphorylation (Lozano-Juste et al., 2020).

Recently, C-group RAFs were shown to act as inhibitors of ABA-activated SnRK2s under normal growth conditions. However, under stress conditions, these RAFs serve as substrates for SnRK2s, leading to their degradation promoted by SnRK2-dependent phosphorylation (Kamiyama et al., 2021; Sun et al., 2022). In osmotic stress response, two C-group RAFs, RAF22 and RAF36 were demonstrated to inhibit the activity of ABA-responsive SnRK2s (Kamiyama et al., 2021; Sun et al., 2022).

Recently, in ABA signaling, a three-component network involving RAF22, ABI1, and OST1 was proposed (Sun et al., 2022). The reciprocal interaction between RAF22 and ABI1 triggers activation of both proteins. Phosphorylation of ABI1 by RAF22 enhances its phosphatase activity, leading to inhibition of OST1 and ABA signaling, while ABI1-mediated dephosphorylation of RAF22 boosts the kinase activity of RAF22 (Sun et al., 2022). OST1, in turn, phosphorylates RAF22, inhibiting its kinase activity and consequently activating ABA signaling (Sun et al., 2022).

1.3 CO_2 signaling in guard cells

1.3.1 Elevated CO_2-induced stomatal closure

Plants close stomata in response to elevated levels of CO_2. During stomatal closure, ß carbonic anhydrases (ßCAs) play a crucial role in the initial stages of CO_2 signaling (Hu et al., 2010). When CO_2 levels are high, ßCAs facilitate the production of bicarbonate ions (HCO_3^-), which were suggested to be sensed by the MATE-type transporter RESISTANT TO HIGH CO_2 1 (RHC1). Elevated HCO_3^- concentration induces a conformational change in RHC1, allowing it to bind and inhibit the HIGH LEAF TEMPERATURE 1 (HT1) kinase (Tian et al., 2015). Nevertheless, the expression of RHC1 alone in oocytes also led to ion currents that were independent of bicarbonate (Wang et al., 2016). Therefore, further investigation is needed to understand the role of RHC1 as a potential bicarbonate sensor.

Additionally, Mitogen-activated Protein KINASE 4 (MPK4) and MAP KINASE 12 (MPK12) are also thought to inhibit HT1 under elevated CO_2 conditions (Hõrak et al., 2016). Due to their central roles in the guard cell CO_2 signaling and being the main subjects of this thesis, the Raf-like kinases, which include the HT1, and the MPKs are described further in the later sections. HT1

kinase acts as a negative regulator of CO_2 signaling. Under high CO_2 conditions, inhibition of HT1 releases OPEN STOMATA 1 (OST1) and GUARD-CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1) to promote stomatal closure through activation of SLAC1 and ALUMINUM-ACTIVATED MALATE TRANSPORTER 12 (ALMT12) ion channels (Hashimoto et al., 2006; Meyer et al., 2010; Vahisalu et al., 2008). Similar to ABA-induced stomatal closure, Ca^{2+} also plays a significant role in CO_2-induced stomatal movements (Webb et al., 1996). Plants carrying mutations in high-order calcium-dependent protein kinases (CPKs) have been found to influence the speed of both elevated CO_2-induced closure and low CO_2-induced opening, suggesting functionally redundant roles for CPKs in CO_2-induced stomatal movements (Schulze et al., 2020).

The closure of stomata requires the efflux of K^+ from guard-cell vacuoles through vacuolar K^+ channels, which are encoded by TPK genes. A recent investigation unveiled a protein kinase called KINASE7 (KIN7) responsible for activating the guard-cell vacuolar K^+ channel associated with TPK1 (Isner et al., 2018). Mutant forms of *kin7* show disruptions in both abscisic acid and CO_2-induced stomatal closure, implying a shared mechanism between the CO_2 and abscisic acid signaling pathways. Notably, KIN7 was observed to traffic from the plasma membrane to the tonoplast (vacuolar) membrane, indicating a potential mechanism for CO_2-mediated regulation of ion fluxes across the vacuolar membrane during stomatal movements (Isner et al., 2018).

Apart from the proposed RHC1-mediated inhibition of HT1, studies have indicated that ABA signaling upstream of OST1 is also necessary for elevated CO_2-induced stomatal closure. Mutants with alterations in ABA biosynthesis genes (*nced3/5*), ABA receptor genes (*pyr1pyl1pyl2pyl4*), PP2C phosphatase genes (*abi1, abi2*), and genes involved in ROS production (*rbohd/f*) display defective stomatal closure in response to elevated CO_2 (Chater et al., 2015; Webb and Hetherington, 1997). Additionally, the ABA catabolism mutant *cytochrome p450 family 707 a1/3* (*cyp707a1/3*) exhibits a hypersensitive response to CO_2-induced stomatal closure (Movahedi et al., 2021). Notably, recent findings demonstrate that expression of the ABA receptor PYL4 or PYL5 is adequate to rescue elevated CO_2-induced stomatal closure in higher order ABA receptor mutants (*pyr1pyl2pyl4pyl5pyl8*) (Dittrich et al., 2019).

Beyond its central role in ABA signaling, OST1 also participates in CO_2 signal transduction within guard cells (Xue et al., 2011). Notably, *ost1* mutants displayed impaired CO_2-induced stomatal closure and HCO_3^-/CO_2-induced S-type anion current activation (Xue et al., 2011). Furthermore, it was proposed that HT1-mediated phosphorylation inhibited OST1's kinase activity, thereby preventing SLAC1 activation by OST1 (Tian et al., 2015).

Leucine-rich repeat receptor-like pseudokinase GUARD-CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1) has been demonstrated to play a crucial role in ABA-induced stomatal movements (Hua et al., 2012). GHR1 activated S-type anion channel SLAC1 in X. laevis oocytes, which is independent of its kinase activity (Hua et al., 2012; Sierla et al., 2018). In addition

to impaired response to ABA, *ghr1* mutants also displayed an insensitive response to elevated CO_2 (Sierla et al., 2018). *ghr1 slac1* double mutants showed similar water loss rate to *slac1* single mutants, indicating that GHR1 acts upstream of SLAC1 (Hua et al., 2012). A very recent study showed that maize ortholog of GHR1, PAN2, plays a crucial role in regulating both subsidiary cell development and stomatal closure. Due to the abnormally shaped subsidiary cells, *pan2* mutants showed an impaired response to elevated CO_2 (Liu et al., 2024). Moreover, the GHR1 ortholog from rice can restore *ghr1* mutant phenotype (Hua et al., 2012). This suggests that in maize PAN2 might play similar functions as GHR1 to regulate stomatal movements by activating SLAC1.

A recent discovery for studying high CO_2-mediated stomatal closure and stomatal development was made by using infrared thermal imaging (He et al., 2018). This study identified a single point mutation in the *BIG* gene, which resulted in a partially reduced response to elevated CO_2, leading to diminished stomatal closure and the activation of S-type anion channels. Additionally, BIG was found to be essential for reducing stomatal density in response to elevated CO_2 (He et al., 2018). Intriguingly, BIG doesn't play a role in inhibiting stomatal opening when CO_2 is low. These findings point toward BIG as a critical signaling component that distinguishes between low CO_2-induced stomatal opening and the stomatal closure induced by high CO_2 (He et al., 2018).

1.3.2 Reduced CO_2-induced stomatal opening

Stomatal opening is triggered by various environmental factors, including light, low CO_2 concentration, and increased air humidity. A critical factor for effective stomatal opening is the activation of the plasma membrane H^+ ATPase OPEN STOMATA 2 (OST2/AHA1) (Assmann et al., 1985; Merlot et al., 2007), which leads to proton efflux, membrane hyperpolarization, and subsequent activation of voltage-dependent K^+ uptake channels, including KAT1 and KAT2 (Pilot et al., 2001; Schachtman et al., 1992). Additionally, guard cells import nitrate ions through the NITRATE TRANSPORTER 1.1 (NRT1.1), causing ion flux and resulting in water influx and increased turgor pressure, ultimately leading to stomatal opening (Guo et al., 2003).

Despite the extensive knowledge about stomatal closure mechanisms, the molecular processes governing stomatal opening are less well understood. Only a limited number of mutants with impaired low CO_2-induced stomatal opening have been identified so far. For instance, plants lacking PROTON ATPase TRANSLOCATION CONTROL 1 (PATROL), a protein responsible for regulating the translocation of H^+ ATPase OST2/AHA1 in the plasma membrane, exhibit impaired low CO_2-induced stomatal opening (Hashimoto-Sugimoto et al., 2013). Additionally, in plants lacking the nitrate transporter NRT1.1, low CO_2-induced stomatal opening in darkness occurs at a slower rate compared to the wild type (Guo et al., 2003). Furthermore, plants deficient in HT1 show impairments in both low CO_2-induced stomatal opening and high CO_2-induced stomatal closure (Hashimoto et al., 2006). Similarly, plants deficient in the car-

bonic anhydrases βCA1 and βCA4 also display significant impairments in low CO_2-induced stomatal opening (Hu et al., 2010). However, the exact roles of these components in the signal transduction pathway that leads to H^+ ATPase activation and stomatal opening in response to a decrease in CO_2 concentration are currently unknown.

1.3.3 Important Raf-like protein kinases in CO_2 signaling within guard cells

HIGH LEAF TEMPERATURE1 (*HT1*), a gene predominantly expressed in guard cells, encodes a Ca^{2+}-independent Ser/Thr kinase that relies on Mg^{2+} for its functionality (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016). This plasma membrane-associated HT1 kinase, resembling a Raf-like Group C MAPKKK (Ichimura et al., 2002), has been identified as having a specific role in CO_2 responses within guard cells (; Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). Both recessive *ht1-2* and dominant *ht1-3/ht1-8D* mutants demonstrated disrupted responses to varying CO_2, while maintaining normal ABA responsiveness (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). The *ht1-2* mutant carried a deletion within the kinase domain, leading to a loss of HT1 kinase activity. Consequently, these plants exhibited constitutively high CO_2 responses (Hashimoto et al., 2006). Conversely, the dominant *ht1-3* and *ht1-8D* mutants, characterized by widely open stomata, harbored the R102K and A109V mutation, respectively, in the non-conserved region (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). These mutations minimally impacted kinase activity but were postulated to influence HT1's target interactions (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016).

Epistasis analyzes indicated HT1's dominance over ßCA1 and ßCA4, as evident in the constitutive high CO_2 response of the *ca1ca4ht1-2* triple mutant (Hu et al., 2010). Similarly, *ost1-3ht1-2* double mutants exhibited comparable constitutive high CO_2 responses, highlighting *HT1*'s epistatic relationship with *OST1* (Matrosova et al., 2015).

HT1's involvement extends beyond CO_2 responses, potentially affecting light-induced stomatal opening (Hashimoto et al., 2006; Matrosova et al., 2015). Notably, *ht1-2* mutants displayed impaired red light-induced stomatal opening, while retaining responsiveness to blue light (Hashimoto et al., 2006; Matrosova et al., 2015).

Additionally, CONVERGENCE OF BLUE LIGHT AND CO_2 1 (CBC1) and CBC2, also belonging to Raf-like kinases, interacted with HT1 and were phosphorylated by HT1 (Hiyama et al., 2017). Loss-of-function *cbc1 cbc2* double mutants exhibited constitutively closed stomata unresponsive to CO_2 or blue light, yet retained a normal ABA response (Hiyama et al., 2017). The suggested role of CBCs involved integrating signals from blue light and low CO_2 to induce stomatal opening, potentially through S-type anion channel inhibition, either directly or indirectly via kinase and phosphatase activity (Hiyama et al., 2017). The downstream components of CBC1 and CBC2 remain to be identified.

1.4 Mitogen-activated protein kinases in plants

1.4.1 MAPK cascade

MAPK cascades are present in all eukaryotic cells and consist of ubiquitous signaling mechanisms. MAPK cascades are essential for several functions in plants, including hormonal regulation, pathogen defense and developmental processes (Lee et al.,2016). Approximately 10% of plant kinases are believed to participate in MAPK signaling pathways (Colcombet and Hirt, 2008). MAPK cascades consist of at least three types of kinases: mitogen-activated protein kinase kinase kinases (MAPKKKs, also termed MAP3Ks or MEKKs), mitogen-activated protein kinase kinases (MKKs, also termed as MAP2Ks or MEKs), and MAPKs (or MPKs). These proteins show a high degree of conservation across all eukaryotic cells (Colcombet and Hirt, 2008; Ichimura et al., 2002). The initial step in signal transduction involves the activation of MAPKKKs upon stimulation of plasma membrane receptors (Colcombet and Hirt, 2008; Ichimura et al., 2002). Then MAP3Ks phosphorylate two amino acid residues of downstream MAP2Ks at a conserved Ser/Thr-X5-Ser/Thr motif (X represents any amino acid residue) of the MAP2K activation loop (Colcombet and Hirt, 2008; Ichimura et al., 2002). After activation, the MAP2ks phosphorylate MAPKs on the threonine and tyrosine residues within the Thr-X-Tyr motif of activation loops (Colcombet and Hirt, 2008; Ichimura et al., 2002). Subsequently, phosphorylated MAPKs forward these signals to the effector proteins in the cytoplasm or nucleus, including transcription factors or other kinases, thereby modulating their functional activity, turnover, and localization (Colcombet and Hirt, 2008; Ichimura et al., 2002). These phosphorylation events are termed MAPK cascades.

1.4.2 MPK4 and MPK12 in guard cell signaling

Arabidopsis MPK4 exhibits a broad expression pattern in various plant tissues, with particularly high expression levels in guard cells. MPK4 was identified for its role in negatively regulating plant defense responses against pathogens (Petersen et al., 2000). Loss of MPK4 activity resulted in constitutive activation of defense responses, characterized by elevated salicylic acid (SA) levels, increased resistance to virulent pathogens, and upregulated expression of pathogenesis-related (PR) genes (Brodersen et al., 2006; Petersen et al., 2000). The involvement of MPK4 in plant pathogen defenses was later investigated (Gao et al., 2008; Qiu et al., 2008). However, while exploring the function of MPK4 in plant immunity, it was found that its role was likely not linked to the regulation of stomatal closure, as *mpk4-2* lines expressing a constitutively active MPK4 exhibited wild-type-like stomatal responses to pathogens (Berriri et al., 2012).

In *Nicotiana tabacum*, NtMPK4, which shares close orthology with Arabidopsis MPK4 and MPK12, along with its homologue NtMPK4L(MPK4-like), has been proposed to play a role in regulating stomatal movements (Gomi et al., 2005; Marten et al., 2008; Yanagawa et al., 2016). Silencing of either *NtMPK4*

or *NtMPK4L* in *N. tabacum* plants resulted in enlarged guard cell size and wider stomatal aperture (Gomi et al., 2005; Marten et al., 2008; Yanagawa et al., 2016). Furthermore, *NtMPK4*- and *NtMPK4L*-silenced plants exhibited reduced stomatal closure in response to ozone exposure (Gomi et al., 2005; Yanagawa et al., 2016). In addition to these findings, silencing of *NtMPK4* impaired stomatal responses to elevated CO_2, while ABA sensitivity remained unaffected (Gomi et al., 2005; Marten et al., 2008). It was observed that darkness failed to activate plasma membrane S-type anion channels in *NtMPK4*-silenced guard cells, indicating that NtMPK4 is also essential for darkness-induced stomatal closure (Marten et al., 2008).

Two Arabidopsis MAPKs, MPK9 and MPK12, display significant and preferential expression in guard cells, suggesting potential roles in the regulation of stomatal movements (Jammes et al., 2009). However, single mutants *mpk9-1* and *mpk12-1* did not exhibit any discernible alterations in stomatal movements in response to ABA (Jammes et al., 2009). Interestingly, mutations in both *MPK9* and *MPK12* led to increased transpirational water loss and significantly impaired ABA-induced inhibition of stomatal opening (Jammes et al., 2009). Furthermore, plants carrying G53R mutation in MPK12 or deletion of MPK12 showed constitutively more open stomata and CO_2-insensitivity phenotype (Jakobson et al., 2016). Bicarbonate-induced activation of S-type anion channels was impaired in *MPK12*-deficient plants (Jakobson et al., 2016).

1.4.3 Involvement of other MAP kinases in stomatal movements

MPK3's involvement in ABA-induced stomatal opening inhibition has been suggested (Gudesblat et al., 2006). In Arabidopsis, the specific inhibition of *MPK3* expression in guard cells using RNA technology led to a partial reduction in ABA-induced inhibition of stomatal opening, without affecting ABA's promotion of stomatal closure (Gudesblat et al., 2006). The role of MPK3 likely lies downstream of H_2O_2 in ABA signaling, as evidenced by the unaffected ABA-induced H_2O_2 synthesis in *MPK3*-silenced lines (Gudesblat et al., 2006).

Recently, MPK11 has been reported for its inhibitory role in red light-induced stomatal opening (Li et al., 2023). Notably, MPK11 exhibited significant expression within guard cells, with MPK11-GFP signals detected in both nuclear and cytoplasmic compartments (Li et al., 2023). The *MPK11* transcript levels in guard cells were elevated by white light, correlating with wider stomatal apertures in *mpk11* mutants compared to the wild type (Li et al., 2023). Furthermore, red light was found to upregulate *MPK11* transcript levels in guard cells, aligning with the observation of enlarged stomatal apertures in *mpk11* mutants under red light (Li et al., 2023). Collectively, these findings underscore the inhibitory role of red light-induced MPK11 in the context of stomatal opening, a mechanism that fine-tunes stomatal apertures and mitigates excessive transpiration-induced water loss under light exposure (Li et al., 2023).

There is indication that the MKK1-MPK6 module could participate in an ABA-dependent signaling pathway leading to H_2O_2 production (Xing et al.,

2008). H_2O_2 production in response to ABA was absent in guard cells of *mkk1* and *mpk6* mutants, while MKK1 overexpression resulted in H_2O_2 production (Xing et al., 2008). MPK6's activity was boosted by ABA treatment in wild-type plants but not in *mkk1* mutants, suggesting the role of MKK1 in ABA-induced activation of MPK6 (Xing et al., 2008). Additionally, another research has suggested the involvement of the MKK1-MPK6 module in darkness-induced stomatal closure, operating downstream of H_2O_2 (Zhang et al., 2017). Mutants *mkk1* and *mpk6* exhibited a complete inability for darkness-induced stomatal closure, despite H_2O_2 production being observed in their guard cells under darkness (Zhang et al., 2017). In this context, darkness-triggered MPK6 activity was heightened in wild-type plants but not in *mkk1* mutants, implying MKK1's mediation of darkness-induced MPK6 activation (Zhang et al., 2017).

In stomatal immunity, MPK3 and MPK6, along with their upstream MAPK kinases MKK4 and MKK5 are centrally implicated regulators (Su et al., 2017). Double mutants *mpk3mpk6* and *mkk4mkk5* displayed impaired stomatal closure when exposed to flg22, a peptide involved in plant immune response (Su et al., 2017). Flg22-induced activation of MPK3 and MPK6 was observed in wild-type plants but not in mkk4 mkk5 double mutants, suggesting that MKK4 and MKK5 act upstream of MPK3 and MPK6 in this context (Su et al., 2017).

Another potential player in ABA-induced stomatal closure is the MAPKK kinase MKKK20 (Li et al., 2017). Mutations in the gene encoding MKKK20 led to a compromised stomatal response to ABA (Li et al., 2017). Notably, ABA treatment enhanced MPK6 kinase activity in *MKKK20* overexpression lines but not in *mkkk20*, *mkk5*, and *mkkk20mkk5* mutants. This points to potential regulation of MKKK20's protein kinase activity by ABI1, a PP2C, as implied from its ability to reduce MKKK20 activity in vitro (Li et al., 2017).

Furthermore, MKKK18, another MAPKK kinase, is also considered a potential contributor to guard cell ABA signaling (Mitula et al., 2015). In response to ABA, *mkkk18* mutants displayed an impaired stomatal response, while *MKKK18* overexpression lines exhibited heightened sensitivity to ABA-induced stomatal closure (Mitula et al., 2015). ABI1 was shown to decrease MKKK18's kinase activity in vitro, indicating a potential regulatory relationship, similar to MKKK20 (Mitula et al., 2015).

Very recently, a *high-temperature sensitive* (*hts*) mutant was identified in the ethyl methane sulfonate (EMS)-induced maize (*Zea mays*) mutant library. This mutation is associated with a single base change in the *MITOGEN-ACTIVATED PROTEIN KINASE 20* (*ZmMPK20*) gene (Cheng et al., 2023). The *hts* mutant displayed markedly elevated stomatal opening and increased water loss rates, along with reduced thermotolerance, in comparison to wild-type plants under high-temperature conditions. *ZmMPK20*-knockout mutants displayed comparable phenotypes to *hts* mutant (Cheng et al., 2023). ZmMPK20 was found to interact with MAP KINASE KINASE 9 (ZmMKK9) and the E3 ubiquitin ligase RPM1 INTERACTING PROTEIN 2 (ZmRIN2). The ZmMKK9–ZmMPK20–ZmRIN2 cascade could downregulate high-temperature–induced stomatal opening by maintaining a balance between water loss and leaf temperature, thereby improving plant thermotolerance (Cheng et al., 2023).

2. AIM OF THE STUDY

The main aim of this thesis was to investigate the transmission of CO_2 signals within guard cells. The specific focus was placed on studying the involvement of MPKs and HT1 in the regulation of stomatal movements induced by CO_2 changes. To achieve these objectives, the following inquiries were examined:

1. Do MAP kinases (MPK4, MPK9, MPK11, MPK12) play a role in CO_2-induced stomatal movements?
2. Is kinase activity of MPK12 required for CO_2-induced stomatal movements?
3. Could MAP kinases (MPK4, MPK12) sense CO_2/bicarbonate directly?
4. Could MPK4 substitute the MPK12 function in the guard cells?

Utilizing the findings derived from the exploration of the aforementioned queries, a novel framework for CO_2 signal transduction in guard cells was proposed.

3. MATERIALS AND METHODS

The specific methodologies are outlined in the Materials and Methods sections of publications I–III.

4. RESULTS AND DISCUSSION

4.1 MPK12 and MPK4 are required for CO_2-induced stomatal movements

Our previous study demonstrated that Arabidopsis plants carrying mutations in *MPK12* showed partially impaired stomatal movements in response to CO_2 changes (Jakobson et al., 2016). Moreover, employing antisense suppression of *NtMPK4* in tobacco plants resulted in an impaired CO_2 response (Marten et al., 2008). The Arabidopsis *mpk4* mutants exhibit significant growth defects attributed to constantly activated stress genes and an overproduction of salicylic acid (Petersen et al., 2000). Due to this severe dwarfed phenotype, it is impossible to measure the stomatal conductance in these *mpk4* mutant plants. In order to investigate the involvement of MPK4 and MPK12 in stomatal movements, we established stable homozygous double-mutant lines by using a guard cell-specific promoter to selectively inhibit *MPK4* expression within the guard cells of the *mpk12-4* line, which lacks the *MPK12* gene. We employed two distinct homozygous *mpk12-4 mpk4GC* lines, denoted as *mpk12 mpk4GC* #1 and *mpk12 mpk4GC* #2, for conducting further physiological experiments and signaling assessments (III).

To investigate the potential roles of MPK4 and MPK12 in CO_2 signaling within stomata, we measured stomatal conductance in both whole intact plants and intact leaves when subjected to changes in CO_2 concentrations. Wild-type (Col-0) plants closed stomata (stomatal conductance decreased) when the CO_2 concentration was elevated from 400 ppm to 800 ppm, and opened stomata (stomatal conductance increased) in response to CO_2 changes from 400 ppm to 100 ppm **(Figure 2A, B; III, Fig. 1)**.

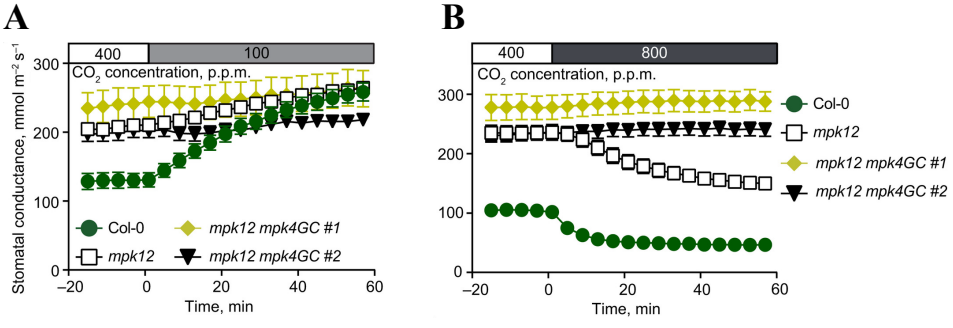

Figure 2. *mpk12mpk4GC* double mutants show abolished stomatal CO_2 response.

(**A)(B)** Time-resolved patterns of whole-plant stomatal conductance in response to changes in CO_2 concentrations from 400 to 100 ppm **(A)** or from 400 to 800 ppm **(B)** of Col-0, *mpk12-4* and *mpk12mpk4GC* double mutants. Error bars indicate ± SEM, n = 6–7. (Modified from the publication III, Fig. 1b, c)

In contrast to abolished responses in the double-mutant *mpk12 mpk4GC* plants, the single-mutant *mpk12-4* plants exhibited a partial reduction in CO_2 responses **(Figure 2A, B; III, Fig. 1)**. Hence, the suppression of *MPK4* in the *mpk12-4* background resulted in significant impairments in CO_2 responses compared to the *mpk12-4* single mutant **(Figure 2A, B; III Fig. 1)**, confirming the involvement of both MPK4 and MPK12 in CO_2-triggered stomatal movements.

4.2 Function of MPK4 in stomatal signaling

To investigate the distinct role of MPK4, independent of MPK12, in stomatal CO_2 signaling, we created *mpk4GC* lines in the wild-type (Col-0) background, where *MPK4* expression was selectively suppressed in guard cells (III). We further confirmed the inhibition of *MPK4* expression within guard cells in two distinct *mpk4GC* lines (denoted to as *mpk4GC #1* and *mpk4GC #2*) using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). In contrast to the severe growth impairment observed in *mpk4* mutants (Petersen et al., 2000), the *mpk4GC* lines exhibited normal growth and were only slightly smaller than wild-type plants (III, Fig. 3a, d). We measured whole-plant stomatal conductance in *mpk4GC* mutant plants in response to varying CO_2 levels, both low (400 to 100 ppm) and high (400 to 800 ppm). Both *mpk4GC #1* and *mpk4GC #2* lines showed stomatal responses to changes in CO_2 levels similar to those of wild-type plants (**Figure 3A, B; III, Fig. 3**).

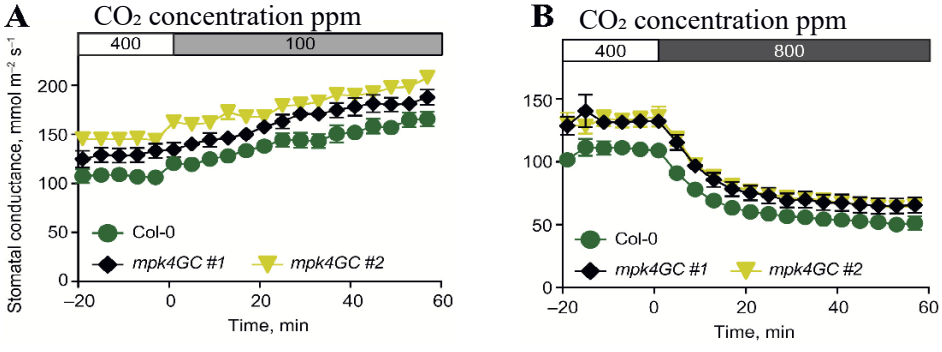

Figure 3. *mpk4GC* lines show wild-type-like stomatal CO_2 response.

(A)(B) Time-resolved patterns of whole-plant stomatal conductance in response to changes in CO_2 concentrations from 400 to 100 ppm **(A)** or from 400 to 800 ppm **(B)** of Col-0, *mpk12-4* and *mpk12mpk4GC* double mutants. Error bars indicate ± SEM, n = 10–14. (Modified from the publication III, Fig. 3b, c).

4.3 MPK4 performs diverse functions in plants, while MPK12 is primarily specialized in the regulation of stomatal responses

As mentioned earlier, MPK4 and MPK12 collaborate in governing stomatal responses to CO_2 changes. MPK4 is more ubiquitous and has broader functions (Lin and Chen, 2018; Petersen et al., 2000). Conversely, MPK12 exhibits predominant expression in guard cells and plays a pivotal role in modulating stomatal aperture in response to various abiotic and biotic stimuli (Des Marais et al., 2014; Jakobson et al., 2016; Jammes et al., 2009; Jammes et al., 2011).

To explore potential functional redundancy between MPK4 and MPK12 in Arabidopsis, we carried out experiments to determine whether MPK12 could serve as a substitute for MPK4's function (III). We introduced both MPK12 and MPK4 proteins into the *mpk4* mutant background, driven by the *MPK4* promoter (proMPK4:MPK4 and proMPK4:MPK12*)*. Kinase-dead version of MPK4 and MPK12, with specific mutations replacing glycine residues (G55 in MPK4 and G53 in MPK12) with arginine, were also introduced into the *mpk4* mutant background. Out of the different constructs, only proMPK4:MPK4 successfully rescued the growth defect phenotype in the *mpk4* mutant plants **(Figure 4; III Fig. 3)**.

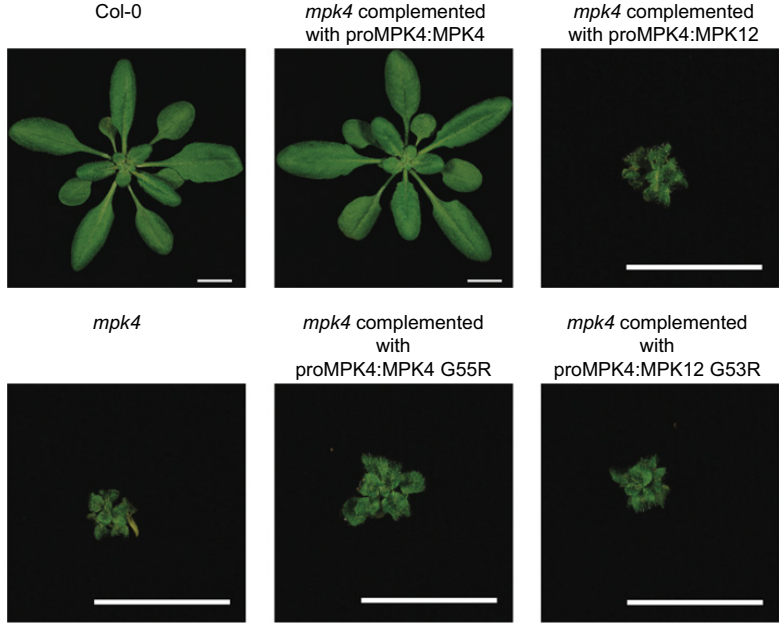

Figure 4. The growth phenotype of *mpk4* and its complementation lines.

The growth defect phenotype of *mpk4* mutant was rescued by introducing *MPK4* expression driven by its native promoter but not with a point mutation (G55R). The expression of *MPK12* under the regulation of the *MPK4* promoter, whether with or without the G53R mutation, did not restore the normal growth phenotype observed in the *mpk4* mutant plants. (Taken from the publication III, Fig. 3d).

These complementation assays underscore the diverse roles of MPK4 within plant signaling networks, whereas MPK12 exhibits a higher specificity in stomatal CO_2 signaling in guard cells. Moreover, the glycine residue at position 55 (G55) in MPK4 and G53 in MPK12 was found to be essential for effective functioning of both proteins in plants.

4.4 MPK9 does not play a role in CO_2-induced stomatal movements

A previous study indicated that MPK12 and MPK9 participated in the stomatal responses to ABA (Jammes et al., 2009). To investigate the role of MPK9 in stomatal CO_2 signaling, we measured stomatal conductance in intact plants of the *mpk9-2* (SALK_064439C) and *mpk9-3* (GK-250D07) T-DNA lines. These *MPK9* mutants showed similar stomatal responses to wild-type plants when transitioning from 400 ppm to 800 ppm or from 400 ppm to 100 ppm CO_2 concentrations (III, Figure 6a, b). Subsequently, we created the double mutant, denoted as *mpk12 mpk9*, by crossing the *mpk12-4* with the *mpk9-3*, resulting in the simultaneous knockout of both *MPK12* and *MPK9* transcripts (III, Supplemental Figure 7). The plant stomatal response to elevated or reduced CO_2 concentration was almost virtually identical in both the *mpk12 mpk9* double-mutant and the *mpk12* single-mutant plants **(Figure 5A, B; III Fig. 6)**. These results suggest that MPK9 does not participate in CO_2-induced stomatal movements.

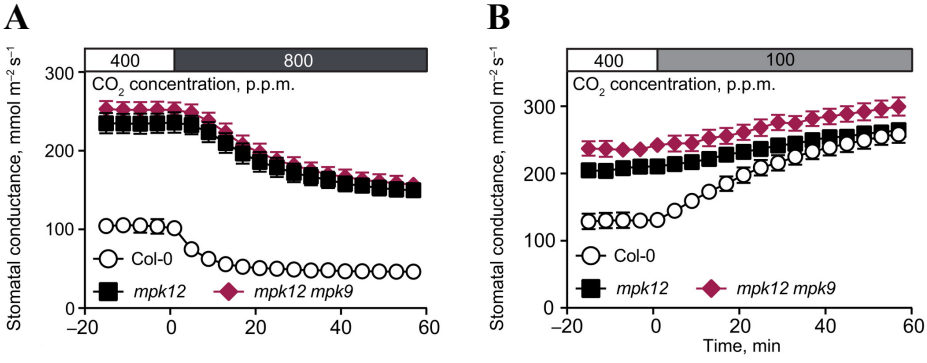

Figure 5. Stomatal CO_2 responses are not regulated by MPK9.

(A)(B) Time-resolved patterns of stomatal conductance in response to changes in CO_2 concentrations (in parts per million; ppm) as indicated on top of the panels of *mpk12-4*, *mpk12 mpk9* and Col-0 plants. These experiments have been repeated twice, and representative results from one experiment are shown. Error bars indicate ±SEM, n = 6–7. (Taken from the publication III, Fig. 6).

4.5 As opposed to MPK11, MPK4 restores the phenotype associated with the deletion of MPK12

The Arabidopsis genome contains a total of 20 MPKs, which can be categorized into four distinct subgroups (A to D) based on their sequence homology (Hamel

et al., 2006; Ichimura et al., 2002). MPK12 bears a striking resemblance to MPK4 and MPK11 within the subgroup B. We reported that MPK12 may have diverged from MPK4 and is unique to the Brassicaceae family among angiosperms (III, Fig. S5). Introducing MPK12 under the control of the *MPK4* promoter failed to rescue the growth defect phenotype in *mpk4* mutant plants (III). This suggests that MPK12 has evolved into a more specialized regulator of stomatal function. Nonetheless, the investigation of whether MPK4 or similar close homologs like MPK11 can serve as substitutes for MPK12 in guard cells remains unexplored. To determine whether MPK4 or MPK11 could serve as a substitute for MPK12's function in guard cells (I), we fused the coding sequence of *MPK4* or *MPK11* to *mVenus-HA* and placed it under the control of *MPK12* promoter and terminator. Every plant harboring the MPK4 construct that exhibited mVenus fluorescence specifically within guard cells successfully reinstated the CO_2 responses in the *mpk12-4* mutant background **(Fig. 6; I Fig. 5)**. Conversely, none of the transgenic plants carrying MPK11, even though they displayed distinct mVenus fluorescence within their guard cells, were able to restore the *mpk12-4* phenotype. Therefore, MPK11 does not play a role in CO_2-induced stomatal movements.

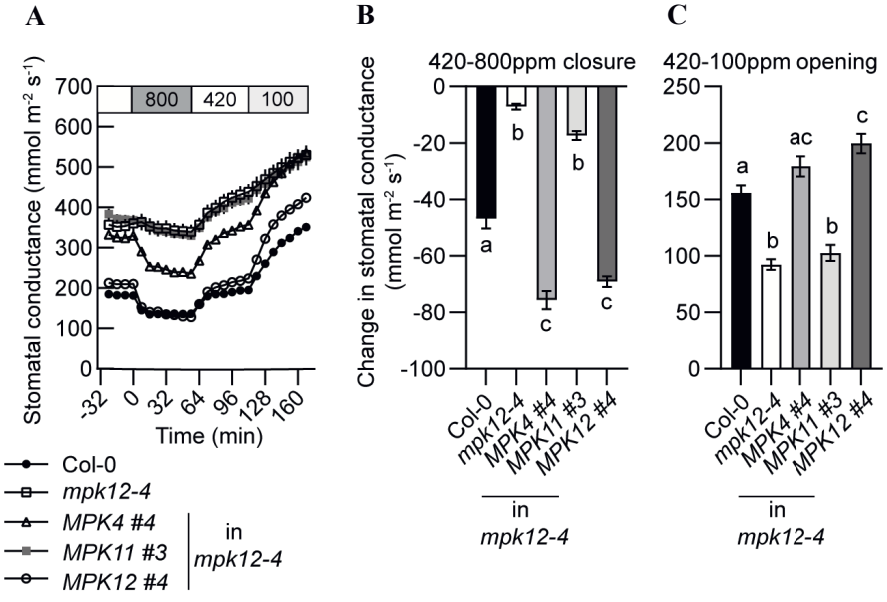

Figure 6. Expression of MPK4 in guard cells can restore *mpk12-4* CO_2 responses.

(A) Time-course patterns of stomatal conductance in response to changes in CO_2 concentrations (in parts per million; ppm) as indicated on top of the panels of *mpk12-4* plants complemented with *pMPK12:MPK4/11/12-mVenus-HA* constructs and Col-0 plants (n = 4–5).

(B)(C) Stomatal closure at 16 min after application of 800 ppm CO_2; stomatal opening after application of 100 ppm CO_2 for 56 min; statistically significant groups are denoted with different letters (ANOVA with Tukey HSD unequal N post hoc test, $P < 0.05$). (Taken from the publication I, Fig. 5).

4.6 MPK4 and MPK12 cannot sense CO_2/HCO_3^- directly in guard cells

The ongoing increase in atmospheric carbon dioxide (CO_2) levels leads to the closure of stomata, which has significant impacts on the processes like transpiration, photosynthesis, and plant growth. Nonetheless, the intracellular primary CO_2 sensor remains unidentified. As mentioned earlier, MPK4 and MPK12 play important roles in the stomatal CO_2 signaling pathways. This leads to the query of whether MPK4 or MPK12 could potentially serve as CO_2 or bicarbonate (HCO_3^-) sensors in plants.

To investigate whether CO_2/HCO_3^- directly activates MPK4 or MPK12, we conducted in vitro kinase assays utilizing purified His-tagged MPK4 and MPK12 proteins (III). The experiments were conducted in an assay buffer with specified bicarbonate concentrations ranging from 0 to 20 mM. The presence of different bicarbonate concentrations did not lead to an increase in the kinase activity of either MPK4 or MPK12 in the phosphorylation levels **(Figure 7; III Fig. 5)**.

We also investigated whether the activity of MPK4 and MPK12 could be influenced by alterations in the solution's pH, potentially due to the dissolution of CO_2, by in vitro kinase assays using buffers with various predetermined pH levels (III). We did not detect any impact on the activity of either MPK4 or MPK12 (III, Fig. 5d).

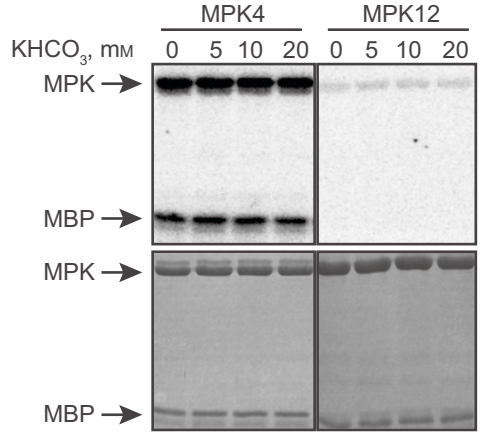

Figure 7. The kinase activities of the recombinant MPK4 and MPK12 proteins are not directly regulated by CO_2/HCO_3^-.

The kinase activity of His-MPK4 and His-MPK12 in the presence of the specified concentrations of $KHCO_3$. There was no observed activation of the kinases. The upper panel displays autophosphorylation, while the lower panel shows Coomassie Brilliant Blue (CBB) staining. (Taken from the publication III, Fig. 5c)

In summary, these findings indicate that MPK4 and MPK12 do not participate in the direct sensing of CO_2/HCO_3^- in guard cells. What are the proteins responsible for CO_2/HCO_3^- sensing in guard cells remains an open question. Additionally, the specific MAP3Ks and MAP2Ks that function upstream of MPK4 and MPK12 in guard cell CO_2 signaling are yet to be identified. Hence, additional investigations are necessary to clarify whether classical MAPK cascades play a role in guard cell signaling.

4.7 The HT1 protein kinase activates the Raf-like kinase CBC1 through phosphorylation

In a prior investigation, it was documented that the HT1 kinase phosphorylates the CBC1 and CBC2 kinases in vitro (Hiyama et al., 2017). It is currently unclear whether this phosphorylation has any impact on the kinase activity of the CBC1 and CBC2. We validated the phosphorylation of CBC1 and CBC2 by HT1 through in vitro phosphorylation assays. These assays utilized recombinant His-HT1 and GST-CBC1 or GST-CBC2 proteins and tested for their ability of incorporating radioactive ^{32}P-ATP (II, Fig. S1). Additionally, the phosphorylation levels of histone, which serves as an artificial kinase substrate, exhibited a concurrent increase with phosphorylated CBC1, but independent of HT1 (II, Fig. S1). This indicated that histone is not a substrate of HT1. Based on these data, it can be inferred that phosphorylation of CBC1 by HT1 might play a role in activating CBC1 kinase. In-gel kinase assays were conducted to explore this hypothesis and provide direct proof of HT1-triggered activation of CBC1 kinase **(Figure 8A; II, Fig. 1)**. Conversely, the kinase-dead HT1-K113W mutant did not activate CBC1 **(Figure 8B; II, Fig. 1)**. The CBC1-D253A variant exhibited a diminished level of phosphorylation in comparison to the wild-type CBC1. Furthermore, there was no evident phosphorylation of histone when HT1 was present. These data indicate that once HT1 activates CBC1 via phosphorylation, the CBC1 protein kinase then initiates phosphorylation of histone **(Figure 8B; II, Fig. 1)**. We also detected phosphorylation sites within CBC1 by using mass spectrometry. This analysis involved examining the recombinant CBC1 protein in the presence and absence of HT1 (II, Fig. 1C). Two HT1-dependent phosphorylation sites, Thr-256 and Ser-280, which are situated within or in close proximity to the activation loop of CBC1, were identified. Phosphorylation assays conducted in vitro indicate that these two phosphorylation sites, which are dependent on HT1, play a crucial role in the activation of CBC1 by HT1 (II, Fig. 1D). Conversely, the blue light-dependent phosphorylation sites (Ser-43 and 45) (Hiyama et al., 2017) do not appear to have a distinct role in the activation of CBC1 by HT1 (II, Fig. 1D). We created transgenic lines carrying wild-type CBC1 or mutated CBC1(T256A/S280A) in the *cbc1 cbc2* background. Gas exchange experiments demonstrated that CBC1(T256A/S280A)/*cbc1 cbc2* plants exhibited reduced stomatal conductance and a lack of sensitivity to CO_2 changes, resembling the characteristics of the original *cbc1 cbc2* double mutant

(II, Fig. 1E). These findings indicate that the phosphorylation of CBC1 at Thr-256 and Ser-280 is essential for CBC1's role in mediating the stomatal response to CO_2 changes.

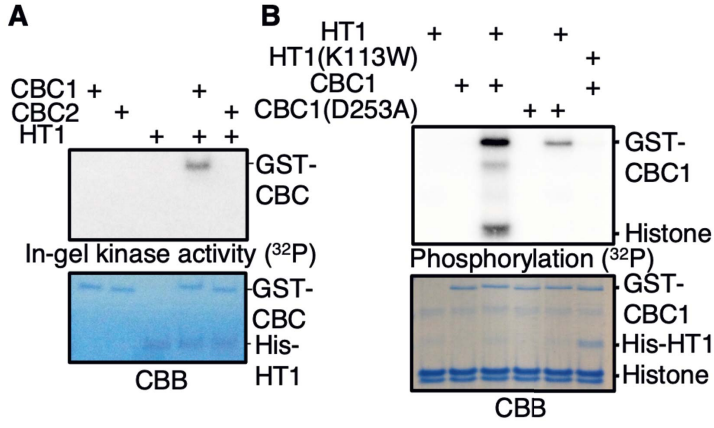

Figure 8. The CO_2 signaling Raf-like kinase CBC1 is activated by the HT1 protein kinase through phosphorylation.

(A) Recombinant CBC1 and CBC2 proteins were incubated with or without HT1 proteins for 30 min with ATP, and *in-gel* kinase assays were performed.
(B) The kinase inactive CBC1-D253A and HT1-K113W protein isoforms were used for in vitro phosphorylation analyzes with recombinant CBC1 and HT1 proteins as indicated. Histone was used as an artificial phosphorylation substrate of CBC1. (Taken from the publication II, Fig. 1A, B)

4.8 Elevated $NaHCO_3$ levels leads to the inhibition of HT1-mediated CBC1 kinase phosphorylation in the presence of MAP kinases MPK4 and MPK12

We examined whether the activation of CBC1 by HT1 is hindered by CO_2/bicarbonate by introducing $NaHCO_3$ into the in vitro phosphorylation assays. However, our findings did not show a distinct impact of $NaHCO_3$ on the HT1-mediated CBC1 phosphorylation levels **(Figure 9A, control lanes 1 & 2; II, Fig. 2)**. Interestingly, upon the inclusion of MPK4 or MPK12 in the kinase assay, it was observed that the presence of $NaHCO_3$, rather than NaCl, hindered both CBC1 and histone phosphorylation in vitro **(Figure 9A, MPK4/MPK12 lanes 4 & 6; II, Fig. 2)**. Nonetheless, we did not see a clear effect of MPK4 or MPK12 without addition of $NaHCO_3$ **(Figure 9A, MPK4/MPK12 lanes 3 & 5; II, Fig. 2)**. On the other hand, the cytosolic domain of the pseudo kinase GHR1 did not exhibit a distinct effect, providing further evidence for the specific function of MPK4 and MPK12.

The reduction in CBC1 kinase activity exhibited a dependence on the concentration of $NaHCO_3$ **(Figure 9B; II, Fig. 2)**. The concentration at which 50% of the activity was inhibited (EC50) was approximately 7.1 +/– 1.0 mM of $NaHCO_3$ in vitro.

This value is in line with the bicarbonate sensing systems found in other organisms, such as the cyanobacterium adenylyl cyclase bicarbonate sensor and the mammalian soluble adenylyl cyclase bicarbonate sensor (Cann et al., 2003; Chen et al., 2000)

We also investigated whether the down-regulation of CBC1 activity could be influenced by alterations in the solution's pH by conducting in vitro kinase assays using buffers with various predetermined pH levels (II). We observed that under conditions of higher pH, specifically at pH values of 7.5 or above, $NaHCO_3$ had an inhibitory effect on CBC1 phosphorylation when MPK4 and HT1 were present (II, Fig. 2D and E). This observation suggests that bicarbonate ions are the primary inorganic carbon signaling molecules. It is important to highlight that these findings don't necessarily rule out a potential secondary role of CO_2, especially considering its higher abundance at lower pH conditions. Therefore, it is possible that both CO_2 and bicarbonate ions act as signaling molecules in the MPK4/12:HT1-mediated CBC1 phosphorylation.

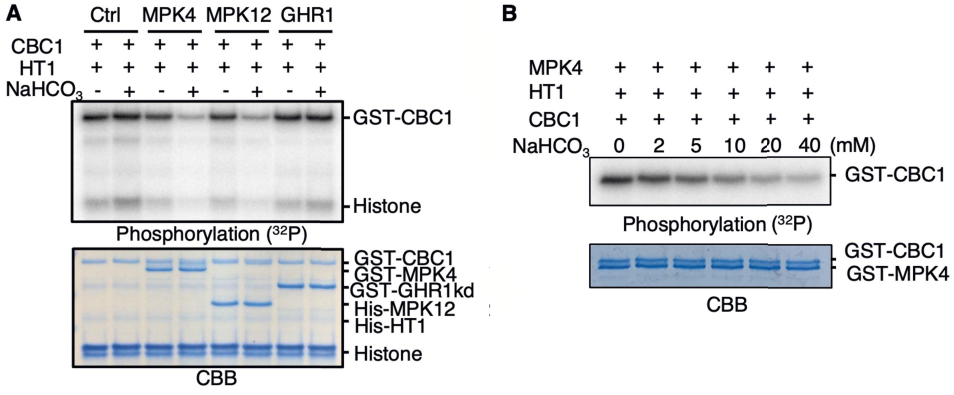

Figure 9. In vitro, MAP kinases MPK4 and MPK12 suppress HT1-mediated CBC1 kinase phosphorylation with elevated $NaHCO_3$ levels.

(A) Recombinant HT1 and CBC1 proteins were incubated with MPK4, MPK12 or the (pseudo)-kinase domain of GHR1 in the presence or absence of 20 mM $NaHCO_3$ for 30 min, and *in vitro* phosphorylation assays were performed. Histone was used as an artificial protein kinase substrate.
(B) MPK4, HT1 and CBC1 proteins were incubated with $NaHCO_3$ at the indicated concentrations for 30 min, and *in vitro* phosphorylation assays were performed. (Taken from the publication II, Fig. 2)

In our *in vitro* phosphorylation assays, MPK11, a member of the Arabidopsis MPK subfamily shared with MPK4 and MPK12, did not facilitate $NaHCO_3$-induced inhibition of CBC1 phosphorylation (II, Fig. S2A). This contrasts with MPK12 and MPK4, which demonstrated inhibitory effects on CBC1 phosphorylation in the presence of $NaHCO_3$ (II, Fig. S2A, B). These findings align with previous research suggesting that MPK11 does not play a role in CO_2 signaling in guard cells (Jakobson et al., 2016; II). Additionally, we also examined MPK3 from a different Arabidopsis MPK subfamily, known for its varied roles in plant stress signal transduction pathways, functioning redundantly with MPK6. Our findings indicate that MPK3 does not participate in the inhibition of CBC1 phosphorylation, unlike MPK4 and MPK12 (II, Fig. S2B).

4.9 The dominant HT1 mutations interfere with the bicarbonate-dependent downregulation of CBC1 protein kinase activity

A genetic screen was conducted to identify Arabidopsis mutants sensitive to ozone, potentially characterized by increased stomatal conductance phenotypes that facilitate the entry of damaging ozone into intercellular leaf spaces (Vahisalu et al., 2008). Screening of over 50,000 M2 Arabidopsis lines, generated through EMS mutagenesis, resulted in identification of potential mutants showing impaired responses in the high CO_2-induced stomatal closure. Among these mutants, six were found to have mutations in the HT1 gene. This set included new alleles, *ht1-G89R* and *ht1-R173Q*, as well as re-isolations of the previously known *ht1-A109V*(*ht1-8D*) (Hõrak et al., 2016). The *ht1-G89R* and *ht1-R173Q* mutants exhibited dominance and showed reduced sensitivity of stomata to elevated CO_2 **(Figure 10A, B; II, Fig. 3A, B)**. Analysis of whole-plant gas exchange indicated that the *ht1-G89R* mutant exhibited higher stomatal conductance at ambient CO_2 levels, with an unresponsive behavior to changes in CO_2 concentration **(Figure 10A; II, Fig. 3A**). Stomatal conductance of the *ht1-G89R* mutant was lower than that observed in wild-type plants at low CO_2 (100 ppm CO_2) **(Figure 10A; II, Fig. 3A**). The *ht1-R173Q* mutant exhibited partially impaired stomatal responses to changes in CO_2 levels **(Figure 10B; II, Fig. 3B)**.

In vitro assays showed that both recombinant HT1-G89R and HT1-R173Q proteins activated CBC1 protein **(Figure 10C, lanes 4 and 6; II, Fig. 3C**). Nevertheless, the activation of CBC1 protein by HT1-G89R was observed to be lower than that by the wild-type HT1 protein **(Figure 10C**, **lane 2 vs. lane 4; II, Fig. 3C**). Interestingly, in *in vitro* phosphorylation assays, we observed that the HT1-G89R isoform did not exhibit $NaHCO_3$-mediated inhibition of CBC1 activity, which contrasted with the WT HT1 protein **(Figure 10C; II, Fig. 3C)**. Moreover, the R173Q mutation showed partially impaired $NaHCO_3$-dependent downregulation of CBC1 **(Figure 10C, lane 6 and 7; II, Fig. 3C**). These findings

aligned with the stomatal phenotypes observed in the mutant plants (**Figure 10A and B; II, Fig. 3A, B**). The reduced stomatal conductance observed in the *ht1-G89R* mutant plants compared to WT plants under low CO_2 conditions (100 ppm CO_2) **(Figure 10A; II, Fig. 3A**) is in accordance with the diminished kinase activity of the HT1-G89R isoform at low CO_2/bicarbonate concentrations **(Figure 10C**, **lane 2 vs. lane 4; II, Fig. 3C**). In our previous study, whole-plant gas exchange analyzes demonstrated that the *ht1-A109V* mutant plants, which exhibited a more pronounced dominance of CO_2 insensitivity (Hõrak et al., 2016), displayed a higher stomatal conductance compared to the *ht1-G89R* mutant plants (II, Fig. S3A). Under the experimental conditions imposed, these mutants with reduced sensitivity to CO_2 changes did not exhibit any clear impact on the photosynthesis-mediated uptake of CO_2 (II, Fig. S3B). We investigated additional *ht1* mutants displaying dominance in CO_2-induced stomatal movements. In opposition to recessive *ht1* kinase mutants (Hashimoto et al., 2006), two potent dominant *ht1* mutations, *ht1-R102K* (*ht1-3*) (Hashimoto-Sugimoto et al., 2016) and *ht1-A109V* (*ht1-8D*) (Hõrak et al., 2016), induce a consistently open and high CO_2-insensitive stomata phenotype (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). These results suggest that these dominant mutations may be caused by an unregulated HT1 function in guard cells. Nevertheless, these mutations did not significantly enhance HT1 kinase activity (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). In our phosphorylation assays employing recombinant MPK4/12, HT1, and CBC1 proteins, the R102K and A109V mutations in HT1 intriguingly interfere with the $NaHCO_3$-induced reduction of CBC1 phosphorylation and CBC1 activity (II, Fig. 3D and E).

Our *in vitro* analyzes provide insight into how these specific point mutations in HT1 contribute to their CO_2-insensitive stomatal phenotypes. Based on the discoveries outlined earlier, a model can be proposed where the low CO_2-induced activation of the CBC1 kinase requires the phosphorylation by HT1. The activity of CBC1, in turn, is attenuated by the down-regulation of HT1 induced by high CO_2/bicarbonate levels in the presence of the MPK4/12. This model predicts that the presence of the CBC kinases would be necessary for the constitutively open stomatal phenotypes observed in the dominant *ht1-A109V* mutant. Hence, we created *cbc1 cbc2 ht1-A109V* triple mutant plants for further investigation. Analysis of whole-plant stomatal conductance revealed a closed stomatal phenotype in the triple mutant, resembling to some extent the characteristics of *cbc1 cbc2* mutant plants. In contrast, the *ht1-A109V* single mutant exhibited constitutively open stomata (II, Fig. 3F). This genetic evidence reinforces the model proposing that HT1 serves as a pivotal upstream regulator linking CO_2 sensing to the downstream regulation of CBC kinase activity.

Further experiments, including analysis of the recessive *ht1-2* mutant (Hashimoto et al., 2006), indicated that the *cbc1 cbc2* double mutant and *cbc1 cbc2 ht1-A109V* triple mutant had slightly higher stomatal conductance compared to the *ht1-2* mutant. Stomatal conductance of the *ht1-2* mutants remained consistently very low (II, Fig. S4). The *cbc1 cbc2* double mutant plants exhibited a

minimal response to changes in CO_2 concentrations (II, Fig. S4). These findings hint at the possibility that other members in the C7 subgroup of the Raf-like kinase family that includes CBC1 and CBC2 (Hiyama et al., 2017) could share a similar function, possibly offering genetic redundancy, alongside the CBC1 and CBC2 in regulating stomatal opening in response to low CO_2 levels.

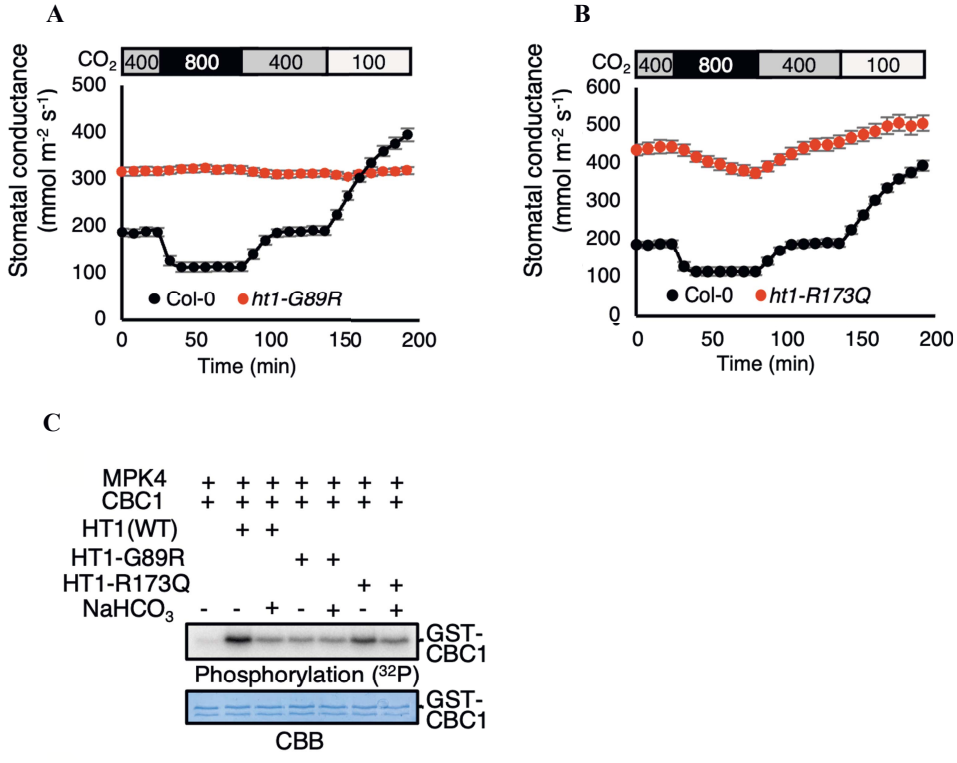

Figure 10. The dominant HT1 mutations interfere with the HCO_3^--dependent down-regulation of CBC1 protein kinase activity.

(**A**)(**B**) Whole-plant gas exchange analyzes using *ht1-G89R* (**A**) and *ht1-R173Q* (**B**). Ambient CO_2 concentrations are indicated by the top bars. n = 7 experiments, error bars show ± SEM.
(**C**) Recombinant HT1 (wild type, HT1-G89R or HT1-R173Q) and CBC1 proteins were incubated with MPK4 in the presence or absence of 20 mM $NaHCO_3$ or 20 mM NaCl ("-" controls) for 30 min, and *in vitro* phosphorylation assays were performed. (Taken from the publication II, Fig. 3)

4.10 Bicarbonate deactivates HT1 kinase by stabilizing the interaction between HT1 and MPK4/12

We continued with experiments aimed at uncovering the molecular mechanism underlying CO2/bicarbonate sensing. Upon individual exposure of CBC1, HT1, or MPK4 to elevated $NaHCO_3$, their respective kinase activities remained unaffected (II, Fig. 4A), especially in the case of the MPKs. This aligns with our previous findings indicating no direct activation of MPK4 and MPK12 by increased CO_2 or $NaHCO_3$ (III). Intriguingly, exposure to $NaHCO_3$ led to the inhibition of the HT1 kinase activity in the presence of either MPK4 or MPK12 (II, Fig. 4B). Conversely, CBC1 activity remained unaffected when MPK4 or MPK12 and CBC1 were included in the reaction without the HT1 protein (II, Fig. 4B), indicating that MPK4, MPK12, and HT1 could constitute the bicarbonate-sensing module. We explored potential interactions between MPKs and HT1, along with the influence of HCO_3^-. *In vitro* pull-down assays revealed a substantial enhancement in the binding between MPK4 and HT1 in the presence of HCO_3^- **(Figure 11A; II, Fig. 4C)**. Comparable experiments also showed an interaction between MPK12 and HT1 in a bicarbonate-dependent manner, while other MPKs (MPK3 and MPK11) exhibited no binding to the HT1 protein (II, Fig. 4D). The HCO_3^--dependent binding was absent upon HCO_3^- removal, suggesting a reversible interaction between MPK4 and HT1 based on the bicarbonate concentration (II, Fig. 4E). Notably, the HT1-G89R mutation impaired the HCO_3^--dependent interaction of HT1 with MPK4 **(Figure 11B; II, Fig. 4F)**. The HT1-R173Q variant, associated with a milder CO_2-insensitive phenotype (II, Fig. 3B), retained partial interaction with MPK4 upon HCO_3^- addition, though with reduced strength compared to the WT HT1 protein **(Figure 11B; II, Fig. 4F)**. Moreover, the strong dominant HT1-R102K and HT1-A109V mutant protein did not exhibit an interaction induced by bicarbonate between HT1 and MPK4 **(Figure 11C, D; II, Fig. 4G and H)**. These results align with the findings from our *in vitro* phosphorylation assays (II, Fig. 3C to E) and analyzes of stomatal conductance (II, Fig. 3A, B, and F).

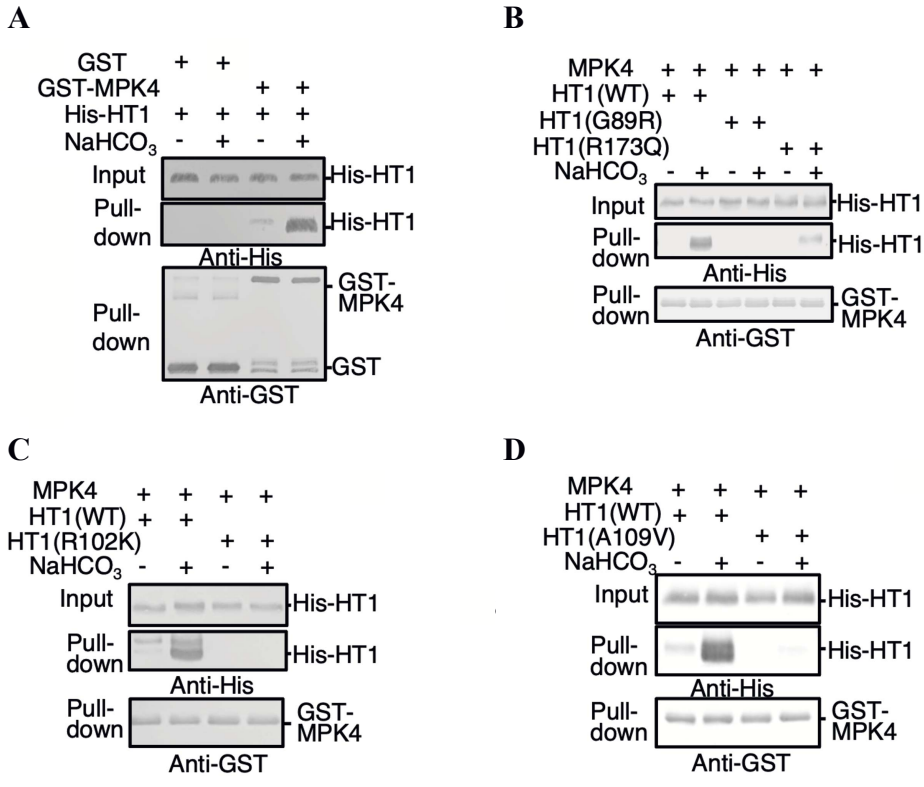

Figure 11. Bicarbonate enhances the interaction between HT1 and MPK4/12

(**A**) His-HT1 and GST-MPK4 or GST control proteins were used for *in vitro* pull-down assays with or without 20 mM $NaHCO_3$. $NaHCO_3$ or 20 mM NaCl ("-" controls) were supplemented in all buffers throughout the pull-down assay procedures including the washing step.
(**B**) *In vitro* pull-down assays were performed using recombinant HT1 (wild type, HT1-G89R and HT1-R173Q) proteins.
(**C**) *In vitro* pull-down assays were performed using HT1-R102K isoform.
(**D**) *In vitro* pull-down assays were performed using HT1-A109V isoform.
(Taken from the publication II, Fig. 4)

4.11 The interaction between MPK12 and HT1 does not require MPK12 kinase activity; instead, it relies on specific amino acids

In a previous study, we reported that the interaction between MPK12 and HT1 was notably disrupted when a G53R substitution was present in MPK12 (Jakobson et al., 2016). This substitution was initially identified in the Cvi-0 natural accession of *Arabidopsis thaliana*. We observed that MPK12 displayed autophosphorylation activity in *in vitro* kinase assays, but this activity was not evident in the G53R mutant. This suggests that loss of kinase activity might influence the interaction. Nonetheless, when we introduced a kinase-inactive MPK12 with an arginine substitution to a conserved lysine (K70) necessary for ATP binding, inhibition of HT1 was only marginally affected compared to when the wild-type MPK12 and the hyperactive MPK12(Y122C) were added (Jakobson et al., 2016). These findings prompted us to inquire whether kinase activity of the MPK12 is essential for its interaction with HT1. To investigate this question, we created an additional MPK12 mutant with D196G and E200A substitutions, denoted as MPK12(DE). In a manner similar to MPK12(Y122C), the design of MPK12(DE) was guided by conserved substitutions that induce the constitutive activation of kinases in Arabidopsis MPK3/4/6 (Berriri et al., 2012). Nevertheless, in the *in vitro* kinase assay, purified recombinant MPK12(DE) protein displayed only modest, though comparable, autophosphorylation activity to the wild-type MPK12. In contrast, MPK12(Y122C) exhibited a robust autophosphorylation signal in this assay **(Figure 12A; I, Fig. 1)**. Conversely, the G53R and K70R mutants did not exhibit any discernible autophosphorylation activity.

Subsequenty, we carried out interaction assays between HT1 and all five variants of MPK12. Because of the membrane association characteristic of HT1, we used the split-ubiquitin yeast-two-hybrid (Y2H) system to investigate the interaction between HT1 and MPK12 (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). We observed strong interaction between wtMPK12 and HT1, while its close homolog, MPK11, showed no interaction. This result is in line with our earlier findings (Jakobson et al., 2016). However, the kinase-dead version, K70R, and the hyperactive Y122C, showed interactions with HT1 **(Figure 12B; I, Fig. 1b)**. We conducted western blot analysis to exclude the possibility that lack of interaction was due to poor or no expression of MPK12 proteins in yeast. This western blot analysis confirmed that all the five MPK12 variants and MPK11 were expressed well in yeast (I, Fig. S2a).

We also studied the interaction between HT1 and the MPK12 variants in *Nicotiana benthamiana* by using ratiometric bimolecular fluorescence complementation (BiFC) assays. Consistent with the findings from the Y2H experiments, along with wtMPK12, the K70R and Y122C variants displayed strong interaction with HT1 **(Figure 12C; I, Fig. 1c)**. In contrast, the G53R and DE variants of MPK12, along with MPK11, showed minimal YFP/CFP signals **(Figure 12C; I, Fig. 1c)**. Western blot analysis confirmed the well expression of

all split YFP fusion proteins (I, Fig. S2b). These findings, obtained from different approaches, prove that the interaction between HT1 and MPK12 is independent of MPK12 kinase activity. Furthermore, the G53, D196, and E200 amino acids might be localized at the interaction interface.

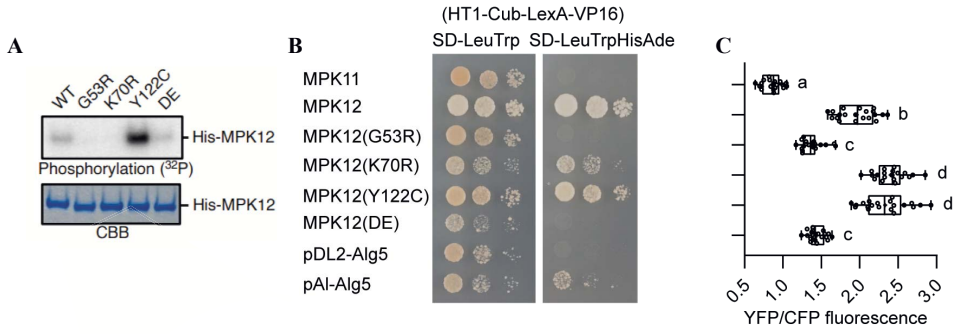

Figure 12. MPK12 interaction with HT1 does not depend on MPK12 kinase activity.

(A) The kinase activity of MPK12 and its variants: WT and DE versions of MPK12 exhibited modest autophosphorylation, while the Y122C variant demonstrated elevated activity; no autophosphorylation activity was observed for the G53R and K70R variants: MPK12 autophosphorylation levels (upper panel) and Coomassie brilliant blue (CBB)-stained gels (lower panel) are shown; the illustrated experiment was repeated 3 times with similar results.
(B) Split-ubiquitin yeast two-hybrid (Y2H) assay: yeast growth with serial dilution on a SD-LeuTrp plate indicates presence of both bait (HT1-Cub-LexA-VP16) and prey (NubG-HA-X) plasmids, and on a SD-LeuTrpHisAde plate to select for interaction; only MPK12, MPK12(K70R) and MPK12(Y122C) interact with HT1; pDL2-Alg5 and pAI-Alg5 are negative and positive prey controls, respectively; bar graph shows relative β-galactosidase activities normalized by the positive control; error bars are SEM (n=3)
(C) Ratiometric bimolecular fluorescence complementation (BiFC) assay in *Nicotiana benthamiana*; box & whisker plot shows stronger interaction between HT1 and MPK12, MPK12(K70R) and MPK12(Y122C); y-axis depicts the split-YFP pairs (HT1-YFPn + MPK-YFPc) used for leaf infiltration, along with *Agrobacterium* carrying a *SLAC1-CFP* construct as a control; x-axis is the ratio of YFP and CFP fluorescence, which indicates interaction strength normalized by the SLAC1-CFP reference control; 20 confocal images taken from three independent leaves in one experiment were analyzed for each interaction pair; more than 3 independent experiments were performed with similar results; statistically significant groups are denoted with different letters (One-Way ANOVA with Tukey HSD post hoc test, $P < 0.05$)
(Taken from the publication I, Fig. 1)

4.12 MPK12 kinase activity is not required for its function in CO_2-dependent stomatal regulation

As we could not discern any evidence of HT1 phosphorylation by MPK12, it is tempting to hypothesize that the function of MPK12 involves sequestering HT1, preventing its phosphorylation of downstream effectors, irrespective of MPK12's phospho-transfer activity. The *mpk12-4* mutant, carrying a deletion of the *MPK12* locus, along with the five MPK12 variants employed in the interaction and kinase assays, offer a robust platform and tools for investigating this inquiry. Our rationale was that by introducing these MPK12 variants into the *mpk12-4* plants, we could acquire insights into the mechanisms underlying MPK12's role in CO_2 signaling. We formulated and produced three sets of binary constructs (I, Fig. S6a). Every set comprised five constructs featuring distinct *MPK12* variants, accompanied by a seed-specific red fluorescent protein marker. The initial set included a 2.5 kb segment of the *MPK12* genomic sequence without any tag (*gMPK12*) to prevent potential interference with the native gene function. The second set involved attaching *mVenus* and an *HA* tag to the genomic sequence of *MPK12* (*gMPK12-mVenus-HA*) to facilitate the confirmation of guard cell-specific MPK12 expression. The final set sought to produce the MPK12-mVenus-HA fusion protein in a broad range of cell types. This was achieved by inserting the coding sequence between an *Arabidopsis ubiquitin 10* promoter (*pUBQ10*) and an *Agrobacterium tumefaciens NOS* terminator (*tNOS*) (*pUBQ10:cMPK12-mVenus-HA_tNOS*). All three sets of constructs were employed for the development of transgenic plants in the *mpk12-4* background. From each of the 15 plant lines, a number of T1 seeds exhibiting distinct red fluorescence were chosen, and their responses to changes in CO_2 were measured by using a comprehensive plant gas-exchange device (I, Fig. S6b-d). In line with our earlier findings (Jakobson et al., 2016), the *mpk12-4* plant exhibited markedly diminished reactions to elevated and reduced CO_2 concentrations. Notably, its stomatal conductance was approximately 1.5 times greater than that of Col-0 under ambient CO_2 conditions (420 ppm) (I, Fig. S6b). The majority of T1 plants carrying the wtMPK12 in all three construct sets exhibited stomatal conductance similar to Col-0 (I, Fig. S6b) and responded comparably to both elevated and reduced CO_2 conditions (I, Fig. S6c, d). This suggests that the addition of the mVenus-HA tag does not significantly impact the functionality of MPK12. Among the four MPK12 mutants, only K70R and Y122C capable of interacting with HT1, irrespective of their kinase activities, were able to reinstate CO_2 responses in the *mpk12-4* background (I, Fig. S6c, d).

Next, we focused on the group of *gMPK12-mVenus-HA* plants and picked two distinct T2 lines from each MPK12 variant for more in-depth investigation. In line with our findings in T1 plants, only those plants carrying the wtMPK12, K70R, and Y122C mutants showed a restoration of stomatal responses to CO_2 and the wild-type steady-state conductance at ambient CO_2 in the *mpk12-4* background **(Figure 13A, B; I, Fig. 3a, d)**. The guard cell-specific expression of MPK12 variants was confirmed by confocal microscopy based on the mVenus

fluorescence. The expression pattern aligned with the anticipated guard cell specificity of the native *MPK12* promoter (I, Fig. 3c, f). The findings strongly indicate that MPK12 plays a role in guard cell CO_2 signaling that is independent of its catalytic activity.

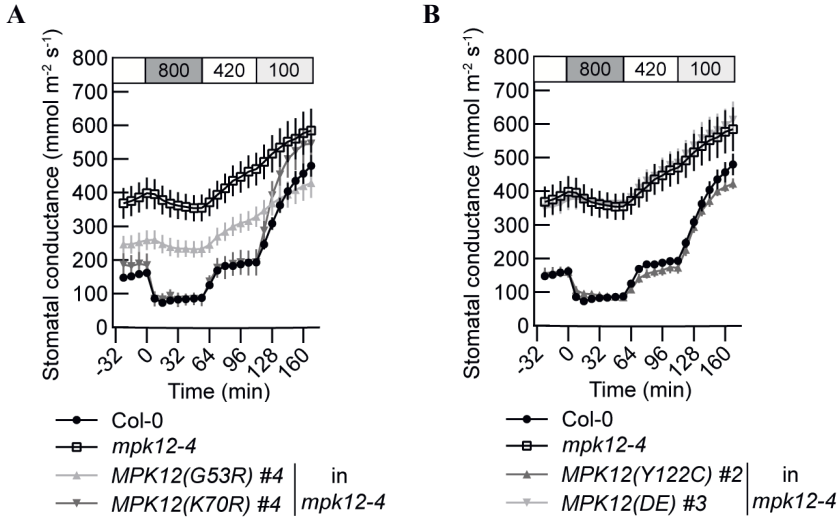

Figure 13. The catalytic function of MPK12 is dispensable for CO_2-induced stomatal movements

(A)(B) Time-course patterns of whole-plant stomatal conductance in response to changes in CO_2 concentrations (in parts per million; ppm) as indicated on top of the panels; intact plants of Col-0 (n=13), *MPK12* deletion line *mpk12-4* (n=12), and *mpk12-4* transformed with *MPK12(G53R)* (n=7), *MPK12(K70R)* (n=5) in **(A)** and *MPK12(Y122C)* (n=4), *MPK12(DE)* (n=5) in **(B)** were analyzed; data of Col-0 and *mpk12-4* controls are the same in (A) & (B) as they were obtained in the same experimental set. (Taken from the publication I, Fig. 3a, d)

4.13 In the presence of bicarbonate, purified MPK12 and HT1 form a heterodimeric structure

In pursuit of more direct binding assays to investigate the interaction between MPK12 and HT1 and its reliance on CO_2/HCO_3^- concentration, alternative protein purification systems were explored due to challenges in expressing HT1 protein in *Escherichia coli*.

We simultaneously expressed HT1 and MPK12 proteins in insect cells using the baculovirus expression system, and subsequently employed tandem purification methods (I, Fig. S1d). Throughout the purification procedure, 20mM of $NaHCO_3$ was included to facilitate formation of the MPK12:HT1 complex. The purified complex was subsequently analyzed by size exclusion chromatography, with or without the addition of $NaHCO_3$. When $NaHCO_3$ was present, the

principal peak closely aligned with the elution volume (Ve) of the 158 kD standard, indicating that it corresponded to the MPK12:HT1 heterodimer (147.5 KD) **(Figure 14A; I, Fig. 2d).** This peak showed a noticeable reduction, and in the absence of $NaHCO_3$, accompanied by a second more pronounced peak with a larger Ve, indicating a smaller size (75 kD). This fraction likely represented a mixture of both monomers. Confirmation of the presence of both MPK12 and HT1 in the larger size fraction was achieved via SDS-PAGE **(Figure 14B; I, Fig. 2e).** These data furnish more direct proof of the CO_2/HCO_3^--dependent interaction between MPK12 and HT1, suggesting a 1:1 stoichiometry in the MPK12:HT1 complex.

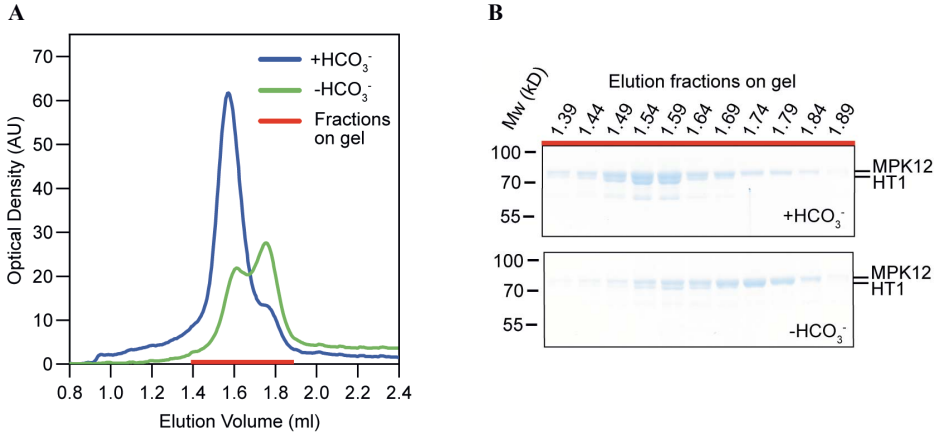

Figure 14. MPK12 and HT1 form a heterodimer in the presence of bicarbonate

(A) Elution profiles of the Superdex 200 5/150 GL size exclusion chromatography of the His-mCherry-HT1 + twin strep-mVenus-MPK12 protein complex; the same tandem affinity purified protein preparation, purified in the presence of 20mM $NaHCO_3$, was used in both $+HCO_3^-$ and $-HCO_3^-$ runs.
(B) Coomassie brilliant blue stained 10% polyacrylamide-SDS gels showing total proteins in the peak fractions of the chromatography runs depicted in (A); the starting elution volumes of the sampled chromatography fractions are indicated on top of each lane; the molecular weight (Mw) standards are indicated on the left; expected Mw of the His-mCherry-HT1 and twin strep-mVenus-MPK12 proteins are 72.9 kD and 74.6 kD, respectively. (Taken from the publication I, Fig. 2)

4.14 Kinase activity-independent interaction between MPK12:HT1 depends on CO_2/HCO_3^- concentration

Both BiFC and Y2H assays enable investigations into interactions with a fixed perspective as they possess irreversibility. Despite their limitations, the irreversibility inherent in these assays may augment the study of transient interactions. We adapted the BiFC experiments by subjecting *Nicotiana benthamiana* plants to either reduced (100 ppm) or elevated (800 ppm) CO_2 conditions post-infiltration to capture any potential CO_2-dependent interaction between MPK12 and HT1. In our adapted confocal microscopy-based ratiometric BiFC assays, we observed an elevation in the YFP/CFP signal for wtMPK12 and HT1 under 800 ppm CO_2 compared to conditions of 100 ppm CO_2. The signal elevation induced by high CO_2 was not observed for MPK12(G53R), which exhibited weak interaction signals under both high and low CO_2 conditions **(Figure 15A; I, Fig. 2a)**. Likewise, epifluorescence microscopy-based BiFC assays were performed with initial inoculation and subsequent growth under low, 100 ppm CO_2 conditions. Continuous gassing with low CO_2 air was maintained during fluorescence imaging for the samples treated with low CO_2. These experiments also unveiled MPK12:HT1 interaction induced by high CO_2, a response nullified by the G53R mutation in MPK12 **(Figure 15B; I, Fig. 2b)**.

In our previous study, we documented that HT1 repressed SLAC1 activity needed for stomatal closure, and this inhibition was counteracted by the interaction of MPK12:HT1 (Hõrak et al., 2016). In this study (I & II), we revealed the inhibition of CBC1 phosphorylation by HT1 in response to CO_2/bicarbonate (HCO_3^-) when MPK12 was introduced into the reaction. Our proposition is that MPK12 and MPK4, in conjunction with HT1, constitute the primary sensor for CO_2/HCO_3^- in guard cells (II). To investigate deeper into whether the inhibition of CBC1 phosphorylation by HT1 induced by MPK12 necessitates MPK12 kinase activity or solely its interaction with HT1, we conducted *in vitro* kinase assays using the five variants of MPK12 under conditions of both elevated and low CO_2/HCO_3^- (I). In these experiments, it was observed that 20 mM of CO_2/HCO_3^- led to inhibition of CBC1 phosphorylation in the presence of HT1 along with wtMPK12, aligning with findings from our recent study (II). Notably, both the MPK12(K70R) and MPK12(Y122C) mutant proteins were able to inhibit CBC1 kinase activity, contrasting with the G53R and DE variants **(Figure 15C; I, Fig. 2c)**. These findings support the essential contribution of the MPK12:HT1 interaction to the CO_2/HCO_3^--sensing mechanism, underscoring its independence of MPK12 kinase activity.

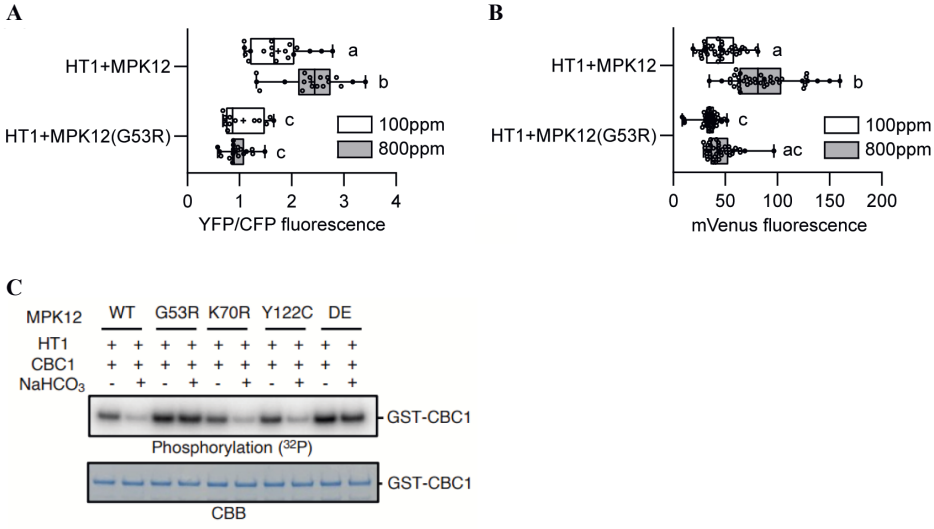

Figure 15. MPK12 interacts with HT1 and inhibits CBC1 phosphorylation by HT1, which are dependent on CO_2/HCO_3^- concentrations and specific amino acids in MPK12

(A) Ratiometric confocal microscopy-based BiFC assays in *N. benthamiana* plants kept under low (100 ppm) and high (800 ppm) CO_2 conditions after infiltration: MPK12 exhibits enhanced interaction with HT1 under 800 ppm CO_2, whereas MPK12(G53R) does not; y-axis indicates split YFP pairs, and x-axis is YFP signal normalized to the SLAC1-CFP reference control; statistically significant groups are denoted with different letters (two-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.005$, n=12 images for MPK12(G53R) at 100 ppm CO_2, and 18 for all others in a single experiment).
(B) Epifluorescence microscopy-based BiFC was conducted in *N. benthamiana* plants, which were infiltrated and grown at 100 ppm CO_2 for 3 days. Subsequently, they were either exposed to 800 ppm or maintained at 100 ppm CO_2: MPK12 displays a significant increase in interaction with HT1 under high CO_2 conditions, but G53R does not; y-axis shows split mVenus pairs, and x-axis is the mVenus fluorescence intensity; experiments were repeated twice with similar results; statistically significant groups are denoted with different letters (two-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$; n=40 for randomly selected non-overlapping regions from 10 images for each treatment).
(C) Under elevated CO_2/HCO_3^- conditions, CBC1 phosphorylation by HT1 is reduced by the WT, K70R, and Y122C variants of MPK12, whereas the G53R and DE variants show no reduction.; The recombinant proteins His-MPK12s, His-HT1 and GST-CBC1 were incubated with or without 20 mM $NaHCO_3$ for 30 min. Subsequently, *in vitro* phosphorylation analyses were conducted by adding ^{32}P-ATP; CBC1 phosphorylation levels and Coomassie brilliant blue (CBB)-stained gels are shown. (Taken from the publication I, Fig. 2a, b, c)

4.15 A MPK12:HT1 structural modeling explains important MPK12 amino acids at the interaction interface with HT1

We developed a theoretical model of the MPK12:HT1 complex utilizing AlphaFold2 (Jumper et al., 2021). The forecasted configuration of the MPK12: HT1 complex exhibited favorable per-residue confidence metrics, indicating high precision for the residues within the interface region (depicted in deep blue) **(Figure 16A; I, Fig. 4a).** The anticipated aligned errors indicated minimal values for the relevant residue pairs, forecasting clearly defined relative positions. The structure is folded adequately, devoid of excessively exposed regions. Crucial secondary and tertiary structures of MPK12 are identifiable, including a smaller N-lobe and a larger C-lobe (**Figure 16B; I, Fig. 4b).** The residues in MPK12 predicted to play a crucial role in binding at the interface include G53, D196 (donating a hydrogen bond from the side-chain oxygen of D196 to the side-chain nitrogen of K91 in HT1), and E200 (forming a hydrogen bond from the backbone oxygen of E200 to the side-chain NH2 of R102 in HT1), in addition to residues in the substrate binding region: N263, Y277, and P285. Removing D196, E200, and G53 led to an anticipated unfavorable binding scenario, marked by the absence of intermolecular contacts and an impact on the function of MPK12. K70 and Y122 are predicted not to be part of the contact surface between MPK12 and HT1 (**Figure 16C; I, Fig 4c)**, and changing them does not influence physiological functions (I, Fig. 3).

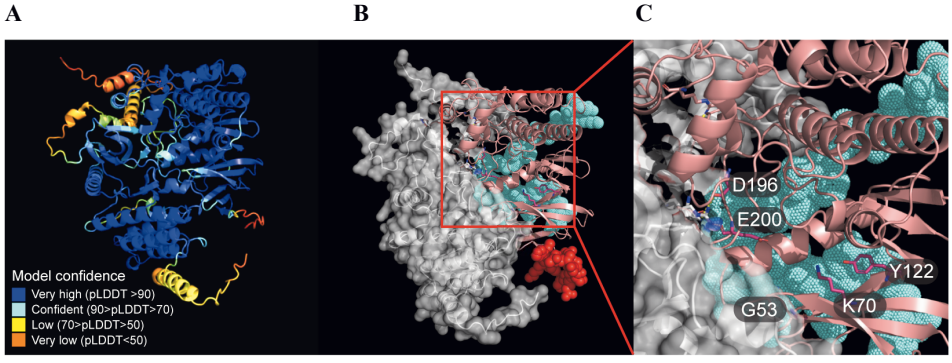

Figure 16. AlphaFold2 structural prediction model for the MPK12-HT1 complex

(A) Color-coded model confidence indicates a very good overall confidence, with very high accuracy at the interface area indicated by the per-residue confidence metrics (pLDDT).
(B) HT1 (gray, surface-filled structure) in complex with MPK12 (pink, ribbon structure) with highlights in the N-terminal region (15 amino acids, red spheres), C-terminal region (41 amino acids, cyan dots), and residues G53, K70, Y122, D196, and E200 of MPK12.
(C) Closeup of the interaction interface highlighting MPK12 interface residues G53, D196, and E200, and MPK12 non-interface residues K70 and Y122, of MPK12. (Taken from the publication I, Fig. 4)

4.16 A new model for CO2-induced stomatal movements

In this thesis, we elucidate the fundamental mechanisms of stomatal CO_2 sensing and early signaling at the biochemical, genetic, and physiological levels, involving three distinct protein kinases: MPK4/MPK12, HT1, and CBC1 (I, II, III). Although approximately 20% of the activity of downstream S-type anion channels can be influenced by HCO_3^- as a secondary sensing mechanism (Zhang et al., 2018), the primary sensors for CO_2/bicarbonate that regulate the necessary upstream phosphorylation events and, consequently, stomatal closure, have not been clearly identified. The present data, which demonstrate the role of the MPK4/MPK12-HT1 complex as a bicarbonate sensor, along with the robust genetic CO_2-insensitive characteristics observed in *ht1* and *cbc1 cbc2* mutants **(Figure 10; II, Fig. 3**) (Hashimoto et al, 2006; Hiyama et al, 2017), propose a model for how plant cells convey and perceive the CO_2 signal to initiate stomatal closure. Under conditions of low CO_2/bicarbonate concentrations, HT1 kinase phosphorylates and activates CBC1 protein kinase, resulting in the inhibition of stomatal closure mechanism. Nevertheless, in the presence of elevated CO_2 concentrations, carbonic anhydrases expedite the intracellular conversion of CO_2 to bicarbonate (Hu et al, 2010). The higher concentration of bicarbonate ions can prompt the binding between MPK4/12 and HT1 **(Figure 11; II, Fig. 4) (Figure 14A; I, Fig. 2d)**, resulting in the inhibition of HT1 kinase activity. Inhibition of HT1 kinase subsequently leads to the down-regulation of CBC1 kinase activity, facilitating the initiation of stomatal closure **(Figure 17; II, Fig. 7C)**.

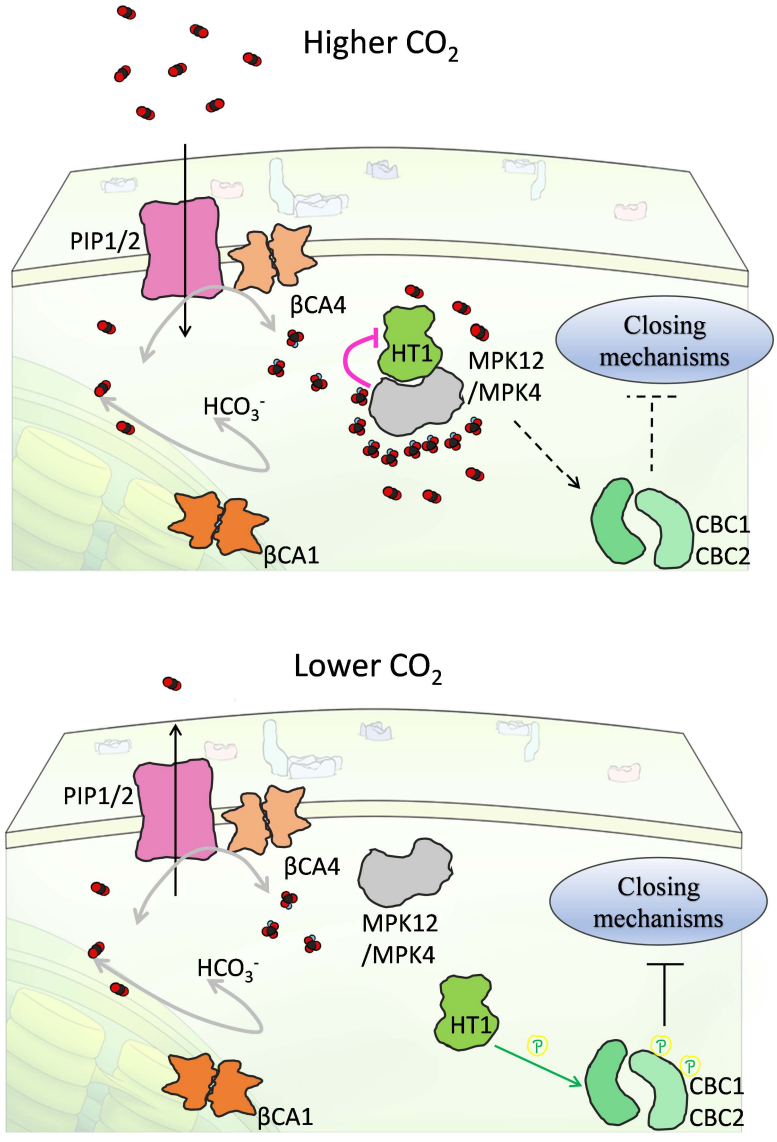

Figure 17. Models for CO_2-induced stomatal movement.

(Upper) The increased concentration of CO_2 in leaves results in an augmented entry of CO_2 into guard cells. Carbonic anhydrases accelerate the intracellular transformation of CO_2 into bicarbonate. The accumulated bicarbonate ions can initiate the interaction between MPK4/12 and HT1, resulting in the suppression of HT1 kinase activity. Suppression of HT1 kinase activity consequently results in the reduction of CBC1 kinase activity, promoting the onset of stomatal closure.
(Lower) In situations characterized by low CO_2/bicarbonate concentrations, the HT1 kinase phosphorylates and activates the CBC1 protein kinase, leading to the suppression of the stomatal closure mechanism. (Graphics was originally designed by Triinu Arjus. Modified from the publication II, Fig. 7C.)

CONCLUSIONS

- CO_2-induced stomatal movements require the involvement of both MPK4 and MPK12. The stomatal responses to changes in CO_2 were akin to wild-type in *mpk4GC* plants, while in *mpk12* single-mutant plants, the responses were partially impaired. On the contrary, stomata in *mpk12 mpk4GC* double-mutant plants exhibited complete insensitivity to both low and high CO_2.
- Complementation analyzes demonstrate that MPK4 and MPK12 serve different roles in plant functions. Introducing *MPK12* under the regulation of the *MPK4* promoter did not successfully restore the growth defect phenotype in *mpk4* mutant plants, thus MPK4 has an indispensable role in the regulation of plant defense systems. However, introducing *MPK4* under the control of *MPK12* promoter restores the CO_2 responses in the *mpk12-4* background suggesting that MPK12 and MPK4 have overlapping roles in guard cells.
- The kinase activity of MPK4 and MPK12 remained unchanged in the presence of CO_2/HCO_3^- in vitro, indicating that these MAPKs do not directly function as CO_2 sensors in planta.
- MPK9 and MPK11 do not play a role in stomatal movements induced by CO_2 changes.
- The bicarbonate ions can trigger the association between MPK4/12 and HT1, leading to the inhibition of HT1 kinase activity. Inhibition of HT1 kinase activity subsequently leads to the decrease in CBC1 kinase activity, facilitating the initiation of stomatal closure.
- The MPK12 isoform lacking or enhancing kinase activity alone is effective in sensing stomatal CO_2 and complementing the *in vivo* response to stomatal CO_2 changes. This implies an unforeseen, phosphorylation-independent function of MAP kinase in planta.
- The reversible binding between MPK4/MPK12 and HT1 aligns with the swift reversibility observed in stomatal opening and closing in leaves when exposed to fluctuating CO_2 concentrations.
- Despite the similar kinase activity of HT1-R102K and HT1-A109V to WT HT1 kinase, the elevation of CO_2 fails to induce a robust interaction between these HT1 isoforms and MPK4 and MPK12. The absence of interaction allows these HT1 isoforms to remain active even under elevated CO_2 conditions, leading to sustained CBC1 kinase activity and consequently a constitutively open stomatal phenotype. The previously unrecognized mutations, HT1-G89R and HT1-R173Q, may exert similar, though not identical, effects, with the HT1-R173Q effect appearing to be partial, and the HT1-G89R potentially influencing kinase activity itself.

- Taken together, the recognition of the CO_2 sensor involving HT1-MPK4/MPK12 in guard cells and the understanding of the molecular mechanisms governing stomatal conductance within the MPK4/12-HT1-CBC1 and CBC2 CO_2 signaling core in this study may pave the way for future precise engineering of plant water use efficiency and carbon assimilation, particularly in the light of the ongoing rise in atmospheric CO_2 concentrations.

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SUMMARY IN ESTONIAN

MPK ja HT1 kinaaside iseloomustamine CO_2-toimelises õhulõhede liikumistes

Taimed omastavad atmosfääri süsinikdioksiidi (CO_2) fotosünteesi käigus ja varustavad seeläbi toidu ja kiudainetega peaaegu kõiki mitteautotroofseid organisme. Lisaks mängib CO_2 olulist rolli signaalimolekulina, mida kasutavad erinevad organismid erinevates olukordades.

Õhulõhed on mikroskoopilised, kahest sulgrakust moodustuvad poorid taimelehtede pinnal. Sulgrakud tajuvad muudatusi nii taime kasvukeskkonnas kui ka taime sees ja rakendavad saadud teavet õhulõhede avatuse reguleerimiseks. Fotosünteesis assimileeritav atmosfääri CO_2 pääseb taime läbi avatud õhulõhede, samas väljub fotosünteesi käigus vabanev hapnik ja toimub transpiratsioon e. vee aurumine atmosfääri taimede pinnalt. Pidevalt muutuvates keskkonnatingimustes peavad taime sulgrakkude sensorsüsteemid leidma tasakaalu CO_2 sissepääsu ja transpiratsioonilise vee kaotamise vahel. Sulgrakud on võimelised avanema ja sulguma sõltuvalt CO_2 kontsentratsiooni muutumisest, sõltuvalt valgustingimustest, õhu niiskuse sisaldusest ja reaktsioonina patogeenidele. Kliimamuutuste tõttu on atmosfääri CO_2 tase jätkuvalt kasvav ja see viib õhulõhede osalise sulgumiseni, mis võib vähendada taimede vee kaotamist transpiratsiooni teel, aga ka pidurdada fotosünteesi ja taimede kasvu. Seetõttu on oluline selgitada õhulõhede CO_2 tajumise mehhanismi, kuna see reguleerib nii taime veekasutuse efektiivsust kui ka süsiniku ringlust ökosüsteemides.

Sulgrakkude CO_2 tajumise peamine mehhanism ei olnud käesoleva projekti alustamisel selge. Korduvalt on näidatud, et valkude fosforüülimisel on keskne roll sulgrakkude molekulaarsete mehhanismise käivitamisel ja õhulõhede avanemise ja sulgemise kontrollimisel. Meie ja teiste varasemad uuringud näitasid, et mitogeeni poolt aktiveeritava kinaasi MPK12 ja Raf-tüüpi kinaasi HT1 vaheline interaktsioon on oluline õhulõhede avatuse reguleerimisel vastusena CO_2 kontsentratsiooni muutustele.

Käesolevas töös kasutasime geneetilisi, biokeemilisi ja valgu struktuuri modelleerimise meetodeid, et uurida MPK12 katalüütilist ja mittekatalüütilist funktsiooni sulgrakkude CO_2-toimelises signaaliülekandes, mis hõlmab HT1 kinaasi aktiivsuse inhibeerimist. Demonstreerisime, et kõrgem CO_2 tase kutsub esile MAP kinaaside MPK4/MPK12 ja HT1 interaktsiooni, mis viib HT1 kinaasi aktiivsuse pärssimiseni. Madala CO_2 tingimustes fosforüülib ja aktiveerib HT1 teist Raf-kinaasi CBC1, mis toimib sulgumisel negatiivse regulaatorina. Tuvastasime füsioloogiliselt olulised amino happed CBC1 valgus, mida fosforüleerib HT1.

Geneetilise sõeluuringu käigus leidsime mitmeid dominantse iseloomuga mutatsioone HT1 valgus, mis kutsuvad esile sulgrakkude CO_2 tajumise kadumise ja oluliselt avatumad õhulõhed. Näitasime, et dominantsed HT1 mutatsioonid takistavad CO_2/vesinikbikarbonaadi poolt indutseeritud MPK4/12 ja HT1 vahelist

interaktsiooni ja HT1 inhibeerimist. Selgitasime HT1:MPK12 interaktsiooni kasutades antud valgukompleksi struktuuri modelleerimist AlphaFold2 abil.

Meie tulemused näitasid, et MPK12 kinaasi aktiivsus ei ole vajalik CO_2 sensori toimimiseks ega ka HT1 pärssimiseks ja sellele järgnevaks CO_2 poolt põhjustatud õhulõhede sulgumiseks. Esitatud tulemused näitavad, et MPK4/12 ja HT1 vaheline interaktsioon toimib peamise CO_2 sensorina sulgrakkudes.

Sulgrakkude CO_2 sensori kirjeldamine ja MPK4/12-HT1-CBC1 CO_2 signaaliülekande mehhanismi selgitamine sillutab teed sordiaretuslikule tööle, mille eesmärk on suurendada taimede veekasutuse efektiivsust ja süsiniku omastamist muutuvas kliimas.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisors Hannes Kollist and Yuh-Shuh Wang for their support and guidance during my phd study. I learnt a lot during the study from both of you.

I would like to thank all the past and present members of the Plant Signal Research Group. Many thanks to Hanna Hõrak and Ingmar Tulva for teaching me statistics.

I am also thankful to my father and mother for always supporting me, and to my dear sisters for taking care of our family.

PUBLICATIONS

CURRICULUM VITAE

Name: Chung-Yueh Yeh
Date of birth: 24.05.1989
Citizenship: Taiwan
Contact: Nooruse 1, Tartu 50411, Estonia
chung.yueh.yeh@ut.ee

Education

2016–…	doctoral studies in Engineering and Technology, University of Tartu
2013–2016	Master's studies in Plant Biology, National Taiwan University
2007–2011	Bachelor's studies in Biotechnology and Food Science, National Taiwan Ocean University
2004–2007	Ping-Bei senior high school

Professional employment

2020–…	Junior research fellow of plant biology, University of Tartu
2011–2012	Soldier, Taiwanese military Police

Scientific publications

Chung-Yueh Yeh, Yuh-Shuh Wang, Yohei Takahashi, Katarina Kuusk, Karnelia Paul, Triinu Arjus, Oleksii Yadlos, Julian I Schroeder, Ivar Ilves, Alfonso T Garcia-Sosa, Hannes Kollist (2023). MPK12 in stomatal CO_2 signaling: function beyond its kinase activity. New Phytoloist, 239(1): 146–158. doi: 10.1111/nph.18913.

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ELULOOKIRJELDUS

Nimi: Chung-Yueh Yeh
Sünniaeg: 24.05.1989.
Kodakondsus: Taiwan
Kontakt: Nooruse 1, Tartu 50411, Estonia
chung.yueh.yeh@ut.ee

Hariduskäik

2016–…	Tartu Ülikool, tehnika ja tehnoloogia doktoriõpingud
2013–2016	Magistriõpe, Taimbioloogia, Taiwani Rahvusülikool
2007–2011	Bakalaureuseõpe, Biotehnoloogia ja Toiduteadus, Taiwani Rahvuslik Ookeaniülikool
2004–2007	Ping-Bei Gymnasium

Erialane teenistuskäik

2020–…	Taimebioloogia nooremteadur, Tartu Ülikool
2011–2012	Sõdur, Taiwani sõjaväepolitsei

Teaduspublikatsioonid

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