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Role of MPK12 in stomata movement

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ABSTRACT

Arabidopsis thaliana Mitogen-Activated Protein Kinase 12 is an important regulator of guard cell signaling in response to high and low CO_2. In this study, the role of MPK12 kinase activity for CO_2 signaling was investigated by genetic approach. The stomatal conductance was measured in a gas-exchange phenotyping platform. The transgenic lines with two kinase hyperactive versions: MPK12(Y122C) and MPK12(DE), and two kinase inactive versions: MPK12(G53R) and MPK12(K70R) were generated via *Agrobacterium*-mediated transformation with the respective T-DNA plasmids. MPK12 deletion mutant (*mpk12-4*) was used for the transformation. The time-resolved patterns of stomatal conductance were compared with wild-type plants as a positive control and the *mpk12-4* as a negative control. Here we report that the MPK12 protein regulates stomatal CO_2-signaling in a kinase activity independent manner. However, verification of the result in the second and third generations of transgenic plants is required.

Keywords: Stomata, molecular signaling, Mitogen-Activated Protein kinase, MPK12 *Arabidopsis thaliana*

CERCS: B310 Physiology of vascular plants, B225 Plant Genetics

MPK12 roll õhulõhede regulatsioonis

Lühikokkuvõte:

Varasemad tööd on näidanud, et *Arabidopsis thaliana* mitogen-aktiveeritud proteiini kinaas 12 on oluline õhulõhede madala ja kõrge CO_2 toimelises regulatsioonis. Käesolevas töös rakendati geneetilist lähenemist, et uurida MPK12 kinaasse aktiivsuse olulisust CO_2 toimelises signalisatsioonis. Õhulõhede juhtivust ja reaktsioone uuriti gaasivahetuse fenotüpiseerimise platvormil. Konstrueeriti ja uuriti järgmiseid transgeenseid liine: kaks kinaasi aktiivsust võimendavat punktmutatsiooni MPK12(Y122C) ja MPK12(DE) ja kaks kinaasi aktiivsust allasuruvat mutatsiooni MPK12(G53R) ja MPK12(K70R). Mutantsed versioonid transformeeriti Agrobakteri vahendusel taimedesse kus MPK12 valk on deleteeritud (*mpk12-4*). Saadud transgeensete taimede õhulõhede juhtivusi võrreldi metsiktüüpi taimede kui positiivse kontrolli ja *mpk12-4* kui negatiivse kontrolliga. Saadud tulemused näitavad, et MPK12 kinaasi aktiivsus ei ole vajalik õhulõhede CO_2-toimeliste reaktsioonide reguleerimisel, kuigi katseid tuleb korrata järgmise põlvkonna liinides.

Võtmesõnad: Õhulõhed, molekulaarne signalisatsioon, mitogeen-aktiveeritud proteiini kinaas, MPK12, *Arabidopsis thaliana*

CERCS: B310 Soontaimede füsioloogia , B225 Taimegeneetika

TABLE OF CONTENT

TERMS, ABBREVIATIONS AND NOTACIONS

GHG – greenhouse gases

H^+-ATPase – acidifying plasma- and endo- membrane transporter protein in eukaryotic cell

ABA – plant hormone abscisic acid

C-terminus - carboxyl terminus of an amino acids chain

N-terminus - amino terminus of an amino acids chain

α-helix - the coiled structural arrangement of many proteins consisting of a single chain of amino acids stabilized by hydrogen bonds (*Alpha-helix,* Merriam Webster)

β-sheet - the structural arrangement of many proteins in which two or more short regions of the polypeptide chain align adjacently and are stabilized by hydrogen bonds into sheets with a pleated or accordionlike appearance (*Beta-sheet,* Merriam Webster)

MAPK - Mitogen-activated Protein Kinase

MPK12 – *Arabidopsis thaliana* Mitogen-activated Protein Kinase 12 (Tair protein locus: AT2G46070.1)

PCR - polymerase chain reaction

T-DNA -transfer DNA

RB – right border, 25-base-pair repeat sequence; the T-DNA transfer initiation sequence

LB – left border, 25-base-pair repeat sequence; the T-DNA transfer termination sequence

AMT - Agrobacterium-mediated transformation

dNTP - deoxyribonucleotide triphosphate

cDNA – complementary deoxyribonucleic acid sequence, synthesized from messenger RNA

gDNA – genomic (chromosomal) deoxyribonucleic acid sequence

gMPK12 – genomic *Arabidopsis thaliana* Mitogen-activated Protein Kinase 12 gene sequence

cMPK12 – complementory *Arabidopsis thaliana* Mitogen-activated Protein Kinase 12 gene sequence

ProMPK12 - *Arabidopsis thaliana* Mitogen-activated Protein Kinase 12 promoter sequence

TerMPK12, MPK12t - *Arabidopsis thaliana* Mitogen-activated Protein Kinase 12 terminator sequence

ATP – adenosine triphosphate

ProUBQ, pUBQ – *A. thaliana* ubiquitin 10 (Tair gene locus: AT4G05320) promoter

tNos - *A. tumefaciens* Nopaline synthase terminator

INTRODUCTION

Throughout the Earth history, the atmospheric CO_2 concentration varied in different periods. It is predicted to be more than 3000 ppm in Silurian when the vascular plants emerged and was reducing throughout the Devonian period when the large vascular plants were developing (Berner, 2006; Beerling, Osborne and Chaloner, 2001; Chaloner, 2003). The stomatal pore became a regulatory mechanism that allowed the balance between plant water loss through transpiration and the CO_2 uptake as photosynthesis substrate. This regulatory mechanism contributed to the establishment of the atmosphere composition and climate for the emergence and flourishing of vertebrate terrestrial life on the planet. (Berry, Beerling and Franks, 2010)

The stomata respond to several environmental signals including changes in CO_2 concentration. These responses are crucial for plant stress resistance and ecological plasticity. These features are important for the agricultural industry, plant biodiversity conservation, and fundamental comprehension of plant physiology, However, the exact signaling pathway underlining the physiology of CO_2 stomata response remains obscure. Recently, Mitogen-Activated Protein kinase MPK12 has been characterized to be an important regulator specifically interplaying in the CO_2 signaling pathway (Tõldsepp *et al.*, 2018; Jakobson *et al.*, 2016) MAPKs are important components of physiological regulation in eukaryotic organisms mainly due to their abilities to phosphorylate other proteins and by that activating various cellular processes. However, they can also function by distinct mechanisms which are independent of their catalytic activities. In this study, we hypothesized and investigated potential mechanisms underlying the contribution of MPK12 to the CO_2 guard cell signaling pathway.

LITERATURE REVIEW

The recent Intergovernmental Panel on Climate Change (IPCC) report has once again declared the uptrend of atmospheric greenhouse gases (GHG) concentration and average yearly temperature on the planet. Since 2011 atmospheric carbon dioxide (CO_2) concentration has risen by 19 ppm and approached the average annual concentration of 410 ppm, which is 47% (by 131.6 ± 2.9 ppm) growth since 1750 (IPCC, 2021). The American Meteorological Society's State of the Climate Report reports CO_2 concentration to be raising by an average of 2.4 ± 0.4 ppm per year between 2010 and 2019 (Blunden, 2021; IPCC, 2021).

The average annual global surface temperature has risen by 0.8-1.3℃ from the beginning of the observations. According to all future projection scenarios presented in the IPCC Report, the global surface temperature will continue increasing and may reach 1.5-2.0℃ with the worst-case prediction beyond 4.4℃ by the end of XXI century (IPCC, 2021). The temperature extremes affect agricultural and ecological regions and this will increase the evaporation rate. At the same time, the precipitation distribution pattern is altering, causing excessive and frequent rainfall in certain areas and severe drought episodes in others (IPCC, 2021).

The CO_2 emission is the main climatic-impact driver as among well-mixed GHGs it mostly contributes to the mean surface temperature growth and the ocean acidification (IPCC, 2021). Vascular plants are a major carbon sink of the planet as in contrast to other photoautotrophic organisms the plants provide long-term organic carbon storage capacity in the plant biomass (Geider *et al.*, 2001). The mitigation of GHG emissions in agriculture is possible, among others, through the improvement of water-management practices in the field (Smith *et al.*, 2007).

The vascular plants are the main contributors to the net primary production in terrestrial ecosystems and subsequently, the main initial carbon and energy source for heterotrophic organisms, including humans (Field *et al.*, 1998). Atmospheric CO_2 is a carbon source for all autotrophic organisms that is fixed via the Calvin–Bassham–Benson cycle (PubChem'PubChem Pathway Summary for Pathway CALVIN-PWY, Calvin-Benson-Bassham cycle,' 2004). This pathway plays a central role in the overall global energetics on Earth, as it is a primary metabolic energy source. The solar energy captured by the photoactive pigments is transformed into chemical energy in the carbohydrate molecules. Generally, the carbohydrates are synthesized from CO_2 and water (H_2O). Thus, CO_2 and H_2O are the main substrates for photosynthesis (Alehina, Balkonin and Gavrilenko, 2005).

The water molecules are absorbed by the root system and constantly transported through the plant body. Water is raised from the rhizosphere via xylem vessels to the leaves. Ultimately, H_2O evaporates from the leaves to the atmosphere. Water evaporation from the leaves is called transpiration. The essence of transpiration is the evaporation of liquid water through the cell wall into extracellular spaces within leaves, and the vapor diffusion through the stomatal pores to the atmosphere. The main driving force of water movement in the plant is decreasing water potential in the soil, the root system, the stems, the leaves, the atmosphere continuum. Basically, the plant is constantly losing water partly by transpiration, metabolism, and for utilizing it for cooling. The water loss is controlled by the degree of stomatal pore opening located in the leaf and stem epidermis. Outer layer of epidermal cells is covered with cuticular waxes that substantially prevent the escape of the water molecules (Johansson *et al.*, 2020; Pallardy, 2008). Simultaneously, the CO_2 molecules enter the plant body by the same path (Pallardy, 2008). Thus, the stomata maintain the equilibrium between the water loss through transpiration and CO_2 fixation in photosynthesis.

The stomatal structure

The stomata are pores in the leaf epidermal tissue, and their length is 10-80 μm (Haworth *et al.*, 2021). The pore is formed by guard cells. Their structure, shape, and number vary among plant families, species, and accessions (Hetherington and Woodward, 2003; Franks and Farquhar, 2007). General stomatal complexes resemble a kidney-like shape guard cells typical for *Arabidopsis thaliana*. However, there are stomatal pore complexes distinct in their mechanics. For example, the stomatal opening may involve the deformation of subsidiary cells surrounding the stomata in some plants (Franks and Farquhar, 2007). Guard cell's rigid cell wall determines the cell shape that ensures the aperture between guard cells widens when the cells experience increasing turgor pressure. Upon signals promoting stomatal opening, the H^+-ATPase initiates proton outflow from the guard cell and this creates membrane hyperpolarization. The change in the membrane potential, in turn, leads to an influx of charged molecules within the cell via voltage-dependent ion channels. The raised concentration of charged molecules subsequently leads to water influx by osmosis. The back wall (outer relative to the aperture) of the guard cell bulges away due to its elasticity whereas a more rigid front wall forms the stomatal aperture. (Kirkham, 2005). The process is schematically represented in Figure 1. The H^+-ATPase inactivation reduces the membrane potential and subsequently initiates the ions outflow. The reduction of cell sap salinity leads to water diffusion in the intercellular space and the guard cell shrinks (Jezek and Blatt, 2017). Light, low CO_2 concentration and elevated air humidity trigger stomatal opening. On the contrary, darkness, elevated CO_2 concentration, low air humidity, air pollutants, and hormone signaling molecules

trigger stomatal closure due to the backward membrane depolarization, loss of charged molecules followed by water diffusion to the intracellular space. Stomatal opening/closing mechanism is, thus, considered the main switch for carbon cycling in the ecosystem (Tian *et al.*, 2015).

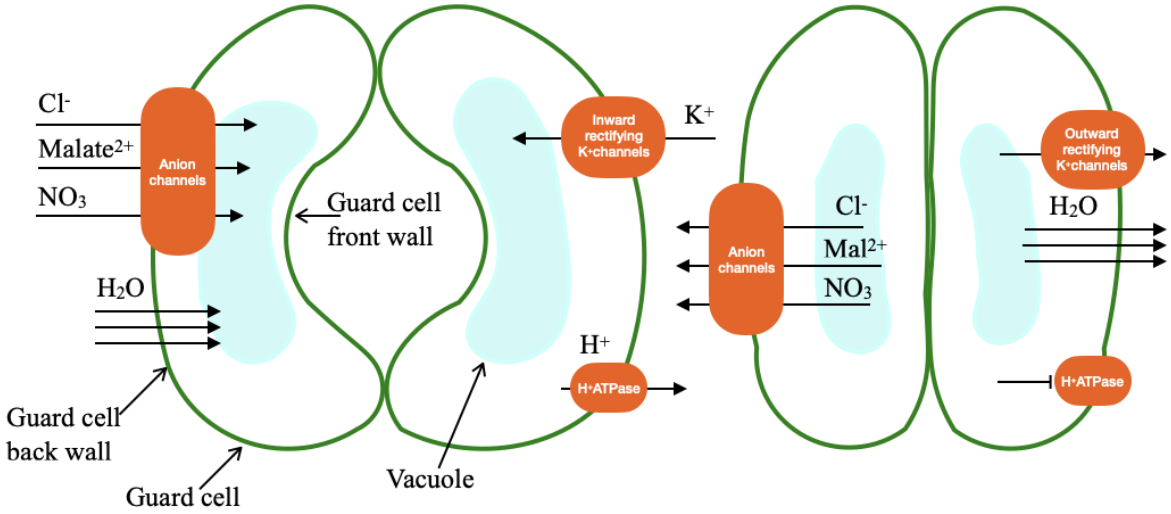

Figure 1. Scheme of stomatal opening (left) and closure(right). The activated H^+-ATPase pumps out protons from the cell, which leads to the membrane hyperpolarization and activation of inward rectifying K^+ channels and other anion channels. Incoming charged molecules increase the salinity of the intracellular space and water diffusion into the cell. The specific structure of the cell wall determines the aperture formation as the cell swells. Inactivation of H^+-ATPase leads to the plasma membrane depolarization and outward rectify activity of proton pumps. The water diffuses along the concentration gradient into the intercellular space - the guard cell s shrinks and stomata close.

The atmospheric CO_2 concentration growth creates an environmental pressure leading to physiological and developmental adaptations of various plant species. Besides the decreased stomatal opening, changes in stomatal density have also been reported (Lake and Woodward, 2008; Engineer *et al.*, 2016). The consequences of changing atmospheric air composition extend beyond CO_2. Various toxic gases can enter the guard cells forcing them to reduce the aperture. In contrast, to cope with the increasing temperatures the plants have to accelerate transpiration. Therefore, the stomata form the main gas-exchange hub and one of the key points in understanding the metabolic adaptation of the plants to the global environmental changes.

Guard cell signaling

Among other stimuli plant hormone abscisic acid (ABA) and CO_2 are important signals for stomata guard cell movement. Experimental data suggest that neither short-term shifts nor long-term exposure to increased or decreased CO_2 concentration is associated with alternation of guard cell ABA concentration. These results suggest that guard cell CO_2 signaling is ABA independent, although ABA signaling amplifies high CO_2 stomata response (Hõrak *et al.*, 2016; Hsu *et al.*, 2018).

As described above, the stomata closure is mediated by the shrinking of the guard cells due to loss of turgor. Water diffuses out through the guard cell plasma membrane due to efflux of $Malate^{2-}$, Cl^- and subsequent activation of K^+ efflux via voltage-dependent activation of K^+ efflux channels. Anion efflux is facilitated by S-type anion channels such as SLOW-ANION CHANNEL 1 (SLAC1), SLAC1 HOMOLOGUE 3 (SLAH3), and R-type anion channels such as QUICKLY ACTIVATING ANION CHANNEL 1 (QUAC1) encoded by AtALMT12 (Vahisalu *et al.*, 2008; Negi *et al.*, 2008; Meyer *et al.*, 2010; Geiger *et al.*, 2011). Loss-of-function *slac1* mutation leads to hyperaccumulation of the anions in the guard cell (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). SLAC1 is an anion-channel indispensable for the stomatal closure in response to darkness, ABA, and CO_2 signaling but it is activated via different pathways and by different mechanisms as the transmembrane domain is known to be required for CO_2 signaling whereas C- and N-termini seem to be involved in ABA signaling pathway (Hõrak *et al.*, 2016; Yamamoto *et al.*, 2016; Wang *et al.*, 2016b; Negi *et al.*, 2008; Hsu *et al.*, 2021; Hsu *et al.*, 2018). SLAC1 has been reported to be activated via phosphorylation to initiate conformational changes of the protein (Li *et al.*, 2022).

Stomatal CO_2 signaling

The entry of CO_2 molecules to the guard cells is facilitated by aquaporins PIP1 and PIP2 (Wang *et al.*, 2016b). CO_2-induced signaling pathway begins from CO_2 conversion into bicarbonate anion ($HCO3^-$) catalyzed by β-carbonic anhydrase 1 (CA1) and β-carbonic anhydrase 4 (CA4) (Wang *et al.*, 2016b; Hu *et al.*, 2010; Chen *et al.*, 2017). Moreover, experimental data suggest synergic functionality of the CA proteins as over-expression of each of the respective genes alone could rescue the double mutant phenotype *ca1ca4* to a certain extent (Engineer *et al.*, 2016; Hu *et al.*, 2015). Interestingly, CA4 is mainly localized to the plasma membrane whereas CA1 is expressed and preferentially localized in the chloroplasts (Hu *et al.*, 2015). Furthermore, intercellular bicarbonate can directly interact with SLAC1 and thus SLAC1 could function as a cellular CO_2 sensor (Zhang *et al.*, 2018). According to the current guard cell signaling model, HCO_3^- is an intermediate signaling molecule in CO_2 response initiating the following bicarbonate signaling pathway. However, the bicarbonate signal detection molecule is not yet known.

The schematic representation of CO_2 signaling pathway is represented in Figure 2.

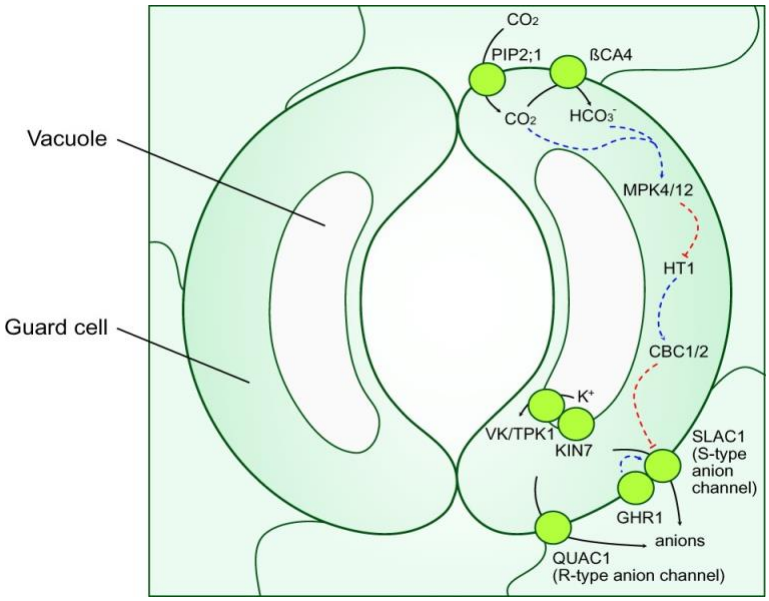

Figure 2. **Schematic representation of stomata CO_2 signaling pathway in a simplified form From (Dubeaux *et al.*, 2021)**
The entrance of CO_2 molecules into the guard cells are facilitated by aquaporins (PIP1/PIP2); in the cytosol the CO_2 is converted into $HCO3^+$ by β-CARONIC ANHYDRASE 1 and 4 (CA-1/CA-4); the following signaling cascade involves MITOGEN-ACTIVATED PROTEIN KINASE 4 and 12 (MPK4/MPK12); MPK12 interacts with HIGH LEAF TEMPERATURE 1 (HT1); CONVERGENCE OF BLUE LIGHT (BL) AND CO2 1 and 2 (CBC1/CBC2) is involved in guard cell signaling to blue light and stomata opening in response to CO_2. However, the exact pathway and intermediate molecules sensing, and convergence bicarbonate yet are to be determined. However, the exact pathway and intermediate molecules sensing, and converging bicarbonate are yet are to be determined. GUARD CELL HYDROGEN PEROXIDE-RESISTANT (GHR1) was proposed to be a scaffold protein for S-type anion channel activation (SLAC1) (Hõrak et al. 2016, Sierla et al. 2018).

Plasma membrane receptor-like pseudokinase GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1 (GHR1) is also involved in the SLAC1 activation. GHR1 is a leucine-rich repeat receptor-like kinase (LRR-RLK) that performs neither kinase activity on SLAC1 nor autophosphorylation, thus it is considered to be a pseudokinase. Nevertheless, GHR1 interaction with SLAC1 leads to the S-type anion channel activation. Possibly, GHR1 serves as a scaffold protein facilitating access of other proteins that are needed for SLAC1 regulation (Sierla *et al.*, 2018). *ghr1-3* mutant plants show a strongly impaired high CO_2 response in whole plant gas exchange experiments. Sierla et al. concluded that the role of GHR1 is more crucial to CO_2 stomatal response than OST1 (Sierla *et al.*, 2018).

HIGH LEAF TEMPERATURE 1 (HT1) is a Raf-like protein kinase playing a central role in stomatal responses to changes in CO_2 concentration. The loss-of-function mutant *ht1-2* leads to completely abolished low and high CO_2 responses and exhibits constitutively closed stomata (Hõrak *et al.*, 2016; Hashimoto *et al.*, 2006). HT1 is known to be preferentially associated with the guard cell plasma membrane. It was shown to interact with and can phosphorylate OST1

thereby inhibiting the kinase activity of OST1 on SLAC1 (Tian et al., 2015). However, these results were not confirmed in a subsequent study using various HT1 isoforms and thus inhibition of SLAC1 anion channel activation by OST1 may be via a different mechanism than direct inhibition of OST1 activity by HT1 (Tian *et al.*, 2015; Hõrak *et al.*, 2016). At the same time, HT1 inhibits SLAC1 anion channel activation by OST1 and GHR1 in the heterologous Xenopus oocyte system. HT1 was also able to phosphorylate the SLAC1 N-terminus in *in vitro* kinase assays (Hõrak *et al.*, 2016).

CONVERGENCE OF BLUE LIGHT AND CO_2 (CBC) 1 and 2 are Raf-like kinases involved in low CO_2 guard cell signaling and response to blue light. They are essential for CO_2-induced activation of S-type anion channels and are epistatic for SLAC1 and SLAH3. CBCs were reported to interact with HT1 and to be phosphorylated by HT1 *in vitro*. The single CBC1 or CBC2 mutant plants have partially impaired stomatal opening, and simultaneous disruption of these genes leads to a more severe phenotype (Hiyama *et al.*, 2017).

HT1 interacts with MITOGEN-ACTIVATED PROTEIN (MAP) KINASE4 (MPK4) and MPK12. MPK4 and MPK12 are close homologs and can function as inhibitors of HT1. Their inhibitory effect on HT1 allows SLAC1 activation by OST1 and GHR1 and subsequent stomatal closure. They both have been reported to interact with HT1 in a split-ubiquitin yeast two-hybrid assay (Y2H) and bimolecular fluorescence complementation (BiFC) analyses (Hõrak *et al.*, 2016; Jakobson *et al.*, 2016). Double mutant of *mpk12mpk4* is completely insensitive to CO_2 changes and exhibits high basal stomatal conductance, whereas *mpk4* single mutant retains normal CO_2 responses (Hsu *et al.*, 2021; Hõrak *et al.*, 2016; Tõldsepp *et al.*, 2018).

MPK4 is a ubiquitous protein localized in a variety of tissues, whereas MPK12 promoter is rather specific for guard cells suggesting MPK4 has broader function other than guard cell signaling. Indeed, MPK4 is also known to be involved in plant immunity (Jammes *et al.*, 2009; Hõrak *et al.*, 2016). MPK12 is localized to cytosol and the nucleus. It is known to be involved in calcium-dependent ABA, auxin, and H_2O_2 guard cell signaling (Jammes *et al.*, 2009). However, recent experiments showed that MPK12 mutants displayed WT-like stomatal responses to ABA and suggested a more specific role in the CO_2 signaling (Jakobson *et al.*, 2016; Tõldsepp *et al.*, 2018). GHR1 phosphorylation by HT1 is inhibited in the presence of MPK12 and MPK4 in kinase in vitro assays. However, MPK4 did not phosphorylate HT1 in the same set of experiments (Hõrak *et al.*, 2016).

Mitogen-Activated Protein Kinases

Protein kinases belong to a giant superfamily of enzymes. They are involved in the various metabolic, cell differentiation and cell cycle regulation, and signaling processes in response to stress and plant immunity. Biochemically the enzymes perform reversible phosphoryl group transfer from adenosine triphosphate (ATP) to an amino acid side chain in the substrate protein side change (Stone and Walker, 1995).

MAPKs are Threonin/Tyrosin (Thr/Tyr) phosphorylating enzymes that are usually amplifying signal transduction pathways. The MAPKs are activated upon dual phosphorylation of threonine and tyrosine residues in their conserved TXY motif and deactivated by dephosphorylation of these residues by phosphatases (Ligterink and Hirt, 2001; Turjanski, Vaqué and Gutkind, 2007; Wang *et al.*, 2016a). Most plant MAPKs are homologous to ERK type of mammalian kinases (Ligterink and Hirt, 2001). The proteins of the group 3D protein architecture are featured by a two-lobed structure with a cleft-like active site or ATP binding pocket at the interface, and the lobes are linked by the L7 linker sequence (Wang *et al.*, 2016a). The dual phosphorylation motif together with the activation loop determines the kinase substrate specificity (Ligterink and Hirt, 2001). The N-lobe is represented mainly by β-sheets and two α-helices, including a distinct αL16 helix whereas the C-lobe is formed mainly by α-helices and four β-strands. Phosphorylation of the protein leads to conformational changes in the activation loop and the flexible linker that opens the active site (Turjanski, Vaqué and Gutkind, 2007). The length of the phosphorylation loop is known to control autophosphorylation activity (Ligterink and Hirt, 2001). Some MAP kinases were reported to perform their physiological role via direct substrate-binding in a kinase-independent fashion (Rodríguez and Crespo, 2011).

The MAPKs interact with substrate and upstream activators via docking motives that determine specificity of the interaction (Turjanski, Vaqué and Gutkind, 2007). A characteristic structure of MAPKs is the MAPK insert located in the C-lobe, which was reported to be involved in the protein's localization and substrate specificity (Wang *et al.*, 2016a). Another distinct element of MAPKs is the αL16 loop sequence located in the C-terminal tail (Turjanski, Vaqué and Gutkind, 2007). It is involved in the maintenance of the kinase basal activity and activation (Wang *et al.*, 2016a).

***Arabidopsis thaliana* MPK12**

Initially, the study of natural Arabidopsis thaliana accession Cvi-0 from the Cape Verde Islands revealed a constitutively more open stomatal phenotype and reduced responses to CO_2 changes compared to the Col-0 accession (Jakobson *et al.*, 2016). These stomatal phenotypes were caused by a G53R substitution in the MPK12 gene of the Cvi-0 accession. In addition, a MPK12 deletion mutant *mpk12-4* also had more open stomata and was partly insensitive to changes in CO_2 concentrations. The stomatal phenotype of *mpk12-4* can be rescued by transformation of the wild-type MPK12 gene from the Col-0 accession (WT MPK12), but not the G53R version as in the Cvi-0 plants. At the same time, the ABA responses of both Cvi-0 plants and *mpk12-4* mutants were similar to the wild type Col-0 plants (Jakobson *et al.*, 2016). These results suggested an important role of MPK12 in the guard cell CO_2 signaling pathway.

As described above, MPK12 could inhibit HT1 kinase activity *in vitro*. Interaction between these two kinases was demonstrated in yeast and *in planta*. Nevertheless, MPK12 with a G53R point mutation disrupts the inhibitory activity of MPK12 against HT1 and interaction between the proteins (Jakobson *et al.*, 2016). The MPK12(G53R) had a reduced autophosphorylation activity, which could explain its inability to function as a HT1 inhibitor. However, another kinase-inactive form of MPK12(K70R) with a mutation at the ATP-binding site could still inhibit HT1 activity to some extent (Jakobson *et al*., 2016).

The Arabidopsis MPK12 has a kinase catalytic domain at the positions from 41 to 327 with a tyrosine-glutamate-threonine (TEY) dual phosphorylation motif in the phosphorylation lip. The 3D crystal structure of the Arabidopsis MPK6 has been determined (Wang *et al.*, 2016a), and can be used to implicate structures of other Arabidopsis MAPKs due to high sequence homology. Mutations in the ATP binding sites of MPK6 cause kinase inactivation whereas mutations in the MAPK insert and αL16 region lead to kinase activity independent of the upstream activators (Wang *et al.*, 2016a). In another study where a yeast complementation screen was employed, several constitutive active (CA) MPK6 and MPK4 were identified (Berriri *et al*., 2012a). Among them, the MPK6(Y144C) and the corresponding MPK4(Y124C) have a tyrosine to cysteine substitution close to the L7 flexible linker (Berriri *et al*., 2012a; Wang *et al*., 2016a). Two amino acid substitutions at the phosphorylation lip of both MPK6(D218G/E222A) and MPK4(D198G/E202A) also result in higher intrinsic kinase activities in the absence of upstream activators (Berriri *et al.*, 2012a). Similarly, hyperactive MPK12s can be designed by mutating corresponding amino acids as those found in the CA MPK6 and MPK4. Indeed, MPK12(Y122C)

has been shown to possess a higher autophosphorylation activity *in vitro*, and it was able to disrupt MPK12-dependent inhibition of HT1 (Jakobson *et al*., 2016).

Considering all the above described, raises a question whether kinase activity of MPK12 determines its role in stomata CO_2 signaling or is it rather regulated by its interaction with HT1?

AIM OF THE STUDY

The results of the previous research highlighted an indispensable role of the MITOGEN-ACTIVATED PROTEIN KINASE 12 (MPK12) in stomatal CO_2 signaling (Jakobson *et al.*, 2016; Tõldsepp *et al.*, 2018). Arabidopsis plants lacking the MPK12 gene (*mpk12-4*) or with a G53R point mutation in the MPK12 protein (in the Cvi-0 accession), which reduced its kinase activity, exhibited impairment in stomatal movements upon CO_2 changes. The *in vitro* kinase assay showed that MPK12 could function as an inhibitor of the HIGH LEAF TEMPERATURE 1 (HT1) kinase, suggesting a regulatory role of MPK12 in CO_2 signaling. Whether the inhibition of HT1 requires the MPK12 kinase activity or it is rather dependen on protein:protein interaction is not clear. Interestingly, the kinase-dead version MPK12(G53R) disrupted the activity of MPK12 against HT1 but another kinase-dead version MPK12(K70R) did not. At the same time, G53R but not K70R point mutation impaired MPK12-HT1 interaction in the split-ubiquitin yeast two-hybrid assays (Y2H) assay (Figure 3; Y.-S. Wang, unpublished data). This leads to a hypothesis that the interaction between HT1 and MPK12 but not the MPK12 kinase activity is crucial for guard cell CO_2 signaling.

The aim of this study was to investigate the role of MPK12 kinase activity in the stomatal CO_2 signaling and determine whether the kinase activity of the protein is required for the response. To achieve this goal, various versions of MPK12, either defected in the kinase activity or the ability to interact with HT1, were transferred to plant lines where MPK12 was deleted (*mpk12-4*). The stomatal responses to CO_2 changes in these transgenic plants were compared to the wild-type (Col-0 accession) and the *mpk12-4* plants to determine if any of these MPK12s can restore the CO_2 responses in the *mpk12-4* background.

EXPERIMENTAL PART

Five different versions **of MPK12 have been selected for the experiment**

The *Arabidopsis thaliana* (L.) Columbia-0 (Col-0) is a natural accession of the species originating from Central Europe and is commonly used as a plant model for genetic and plant physiology research. In the Arabidopsis whole genome sequencing project, the Col-0 accession was used as the reference plant, and hence the Col-0 MPK12 sequence is referred as the wild-type MPK12.

The **MPK12 (G53R)** is naturally found in the Cvi-0 accession of *A. thaliana*, with its MPK12 having arginine to glycine substitution at the position 53 compared to the Col-0 accession. This amino acid is located in the glycine-rich loop which is involved in the ATP binding. This version has already been investigated *in vitro* and *in planta* and was found to impair stomatal CO_2 signaling similar to MPK12 deletion mutant (*mpk12-4*). The mutation leads to reduced autophosphorylation activity and reduced inhibition of HT1 kinase activity in the *in vitro* kinase assay. It also showed weaker interaction with HT1 in the bimolecular fluorescence complementation (BiFC) and split-ubiquitin yeast two hybrid assay (Y2H) assays. The results of split-ubiquitin yeast two-hybrid assay performed by Yuh-Shuh Wang are represented in Figure 3. The G53R substitution leads to a protrusion of the arginine site chain on the protein's surface that may contribute to the interaction interference (Jakobson *et al.*, 2016)

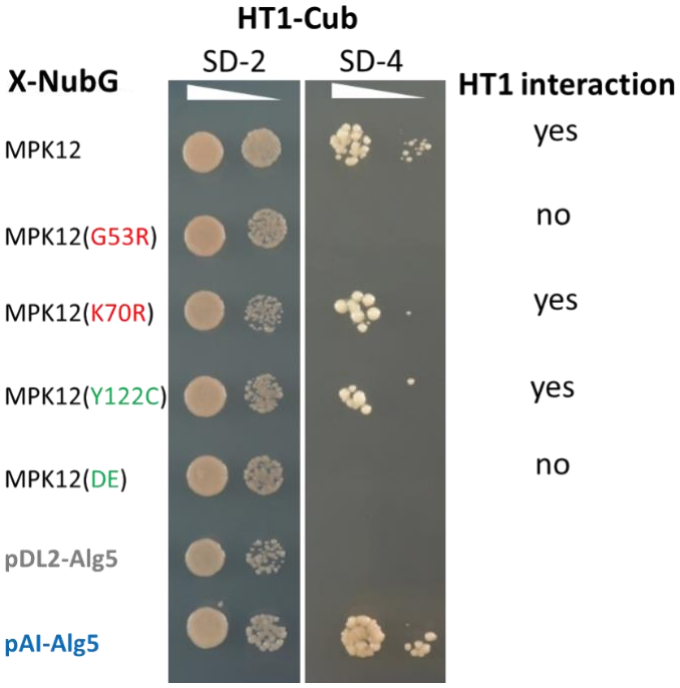

Figure 3. HT1 interaction with MPK12 variants (performed by Yuh-Shuh Wang). HT1 interacts with wild-type MPK12, MPK12 (K70R) and MPK12(Y122C) in split- ubiquitin yeast two hybrid assay,but MPK12(G53R) and MPK12(DE) do not.

The position of the point mutations in the structure of MPK12 used in the study is depicted in Figure 4.

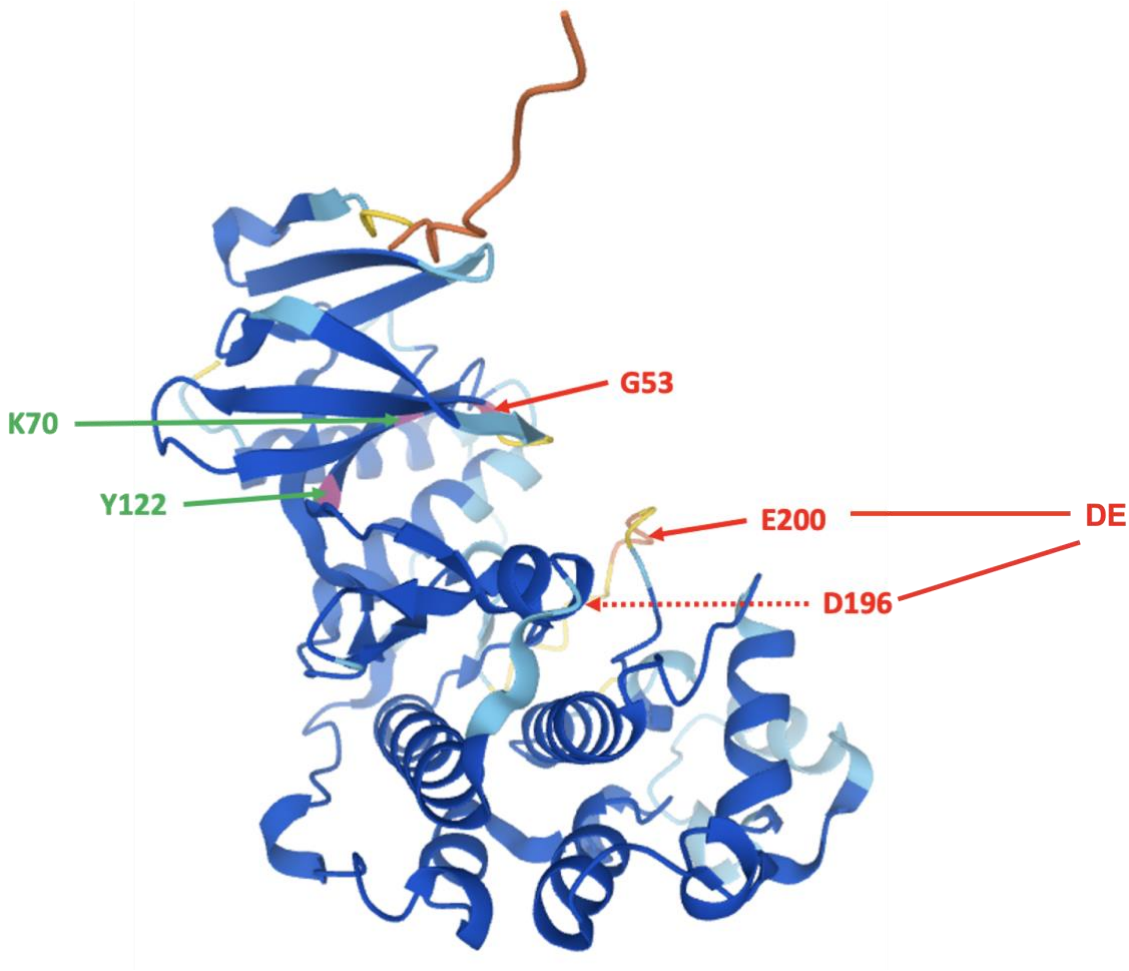

Figure 4. Position of single amino acid substitutions of MPK12 prepared with AlphaFold (Jumper *et al.*, 2021; Varadi *et al.*, 2022). Glycine at position 53 (G53) is localized in the ATP-binding pocket. Lysine at position 70 (K70) is localized in the ATP-binding pocket. Tyrosine at position 122 (Y122) is localized in the ATP-biding pocket and near the L7 flexible linker. Aspartate at position 196 and glutamate at position 200 (DE) is localized in the phosphorylation lip.

The **MPK12 (K70R)** point mutation, bearing lysine to arginine substitution at the position 70, was studied in the same research and is a kinase-inactive version according to the *in vitro* kinase assay reported in (Jakobson *et al.*, 2016). This mutant still retained the ability to interact with HT1 based on the BiFC and split-ubiquitin Y2H assays (Figure 3, Y.-S. Wang, unpublished data).

In the same study, **MPK12 (Y122C)** with a tyrosine to cysteine substitution at position 122 has been shown to be a hyperactive version with increased autophosphorylation activity. This version was able to interact with HT1 to a level similar to the wild-type MPK12 (Figure 3, Y.-S. Wang, unpublished data).

The **MPK12 (DE)** mutant has aspartate to glycine substitution at position 196 and glutamate to alanine substitution at position 200. These amino acids are located in the phosphorylation lip that

forms the catalytical domain. This mutant has been designed based on sequence homology with a hyperactive MPK6 and MPK4 (Berriri *et al.*, 2012). However, its hyperactivity must be verified by the *in vitro* kinase assay. The MPK12 (DE) had a very weak interaction to HT1 based on the BiFC and split-ubiquitin Y2H assays (Figure 3, Y.-S. Wang, unpublished data).

Thus, the above-described amino acid point mutations, along with the wild-type MPK12 from the Col-0 accession, were selected to test the hypothesis stated in the project.

MATERIALS AND METHODS

Plant material

The *Arabidopsis thaliana* MPK12 sequence Columbia-0 accession (Col-0) was used as a template for the gene amplification. The Col-0 was used as a positive control in the stomatal conductance phenotyping. The MPK12 deletion mutant plants (*mpk12-4*) that had the whole MPK12 gene deleted in the Col-0 genetic background were used for *Agrobacterium tumefaciens*-mediated plant transformation. The *mpk12-4* plants were employed as a negative control in the stomatal conductance measurement experiments.

Transgenic plants with various versions of MPK12 constructs were generated in the *mpk12-4* background. The first generation of transgenic plants (T1) were analyzed in a gas-exchange phenotyping platform and some of the T2 plants were checked for their MPK12 protein expression by fluorescence microscopy.

Plant maintenance

The seeds undergone selection were sown in plastic pots with peat-perlite (2:1) mixture after 2-4 days of cold treatment at 4℃. The plants were cultivated in Snijders Scientific Microclima and CLF Plant Climatics GmbH SE-41AR3cLED plant growth chambers. The following growth conditions were provided: short-day 8/16 hours light/darkness conditions with 23/19℃ temperature and ~60% relative air humidity and a photosynthetic photon flux density of 250 μmol photons m^{-2} s^{-1} in the growth chambers. The pots used in the experiment allow complete sealing by a glass covering the soil layer and seed germination into the around 0.5 cm in diameter hole. 2 days before the experiment, the distance between the edge of the hole and the plant is hermitized with wax.

Molecular cloning

MPK12 mutagenesis

The single-base substitutions of MPK12 were introduced by a two-step overlap extension polymerase chain reaction (PCR) from DNA of Col-0 accession. First, 5’ and 3’ of the MPK12 sequences with an overlapped region containing the point mutation were amplified separately. These two fragments were then used as the templates in the subsequent PCR round with primers flanking the whole MPK12 region. The gene sequence flanking primers introduced the *Bsa*I recognition sites followed by specific sequences that would be compatible for further Golden Gate Assembly. The overlap PCR was performed by Phusion PFU DNA Polymerase (Thermo Fisher

Scientific) with a proof-reading activity. The reaction of 20 µl total volume was assembled in 13.9 µl of water combined with 4µl 5x HF Phusion Buffer (Thermo Fisher Scientific), 0.2 µl of the enzyme, 0.4 µl of 10mM dNTPs, 0.5 µl of the genomic DNA clone, and 0.5µl of each of the 10µM primers. The primer pairs used for the amplification and all following cloning steps are shown in Table 1. The following thermocycler program was used: with an initial temperature of 95℃ for 2 minutes, 2 lower stringency of denaturation at 95℃ for 15 sec, primer annealing at 53℃ for 15 sec and DNA extension at 72℃ for 30 seconds, followed by 28 cycles of higher stringency with primer annealing temperature increased to 65℃, and a final extension at 72℃ for 3 minutes and then kept at 25℃. The PCR products were purified using FavorPrep PCR Purification Mini Kit according to the manufacturer FAVORGEN Biotech Corporation manual.

Destination vector

The PCR products with the native genomic sequence of MPK12 and its versions with the site-specific mutations were directly integrated into a destination vector by the Golden Gate methodology. The destination binary vector, PFAST-R_amilCP/HygR_P19_pYSWb1, was modified from the pHEE2E-TRI vector (Wang *et al.*, 2015) with addition of Tombuviral RNA silencing suppressor P19 (Garabagi *et al.*, 2012), a PFAST-R cassette that encodes a seed-specific red fluorescent protein (Shimada, Shimada and Hara-Nishimura, 2010)), and a dropout DNA of the purple-blue non-fluorescent chromoprotein from *Acropora millepora* (amilCP) flanked by *Bsa*I recognition sites for negative *E.coli* selection (Alieva *et al.*, 2008). All these gene cassettes, along with a hygromycin resistant gene, are located within the T-DNA region, which would be transferred to the plant cells. The binary plasmid also contains kanamycin resistance (KanR) selection marker outside of the T-DNA region for *Escherichia coli* selection. T-DNA region flanked by the border repeat sequences (RB, LB) has the below-described components. The binary system part incorporates the origin of replication from pVS1 plasmid (pVS1-ori) replication protein (pVS1-RepA) for the *Ti*-plasmid maintenance in Agrobacterium and stability protein (pVS1-staA) for the bacteria segregation stability (Heeb *et al.*, 2000). This destination vector was constructed by Yuh-Shuh Wang and the MPK12 cassettes were assembled by the Golden Gate method to replace the amilCP dropout.

Golden Gate plasmid assembly

The first set of MPK12 constructs contained the genomic MPK12 and its mutants with the native regulatory elements (ProMPK12:gMPK12:tMPK12) without any tag were PCR-amplified (see MPK12 mutagenesis) and directly immobilized to the destination vector by the Golden Gate method. The second set included a yellow fluorescent protein and a HA tag (mVenus-HA) fused to the C-terminus of MPK12. The promoter and genomic coding sequence of MPK12 (ProMPK12:gMPK12), the mVenus-HA tag, and the terminator (tMPK12) were previously cloned in the designated Level 0 vectors to create the Plant MoClo parts (Engler *et al.*, 2014) or using purified PCR products as the Level 0 parts. These parts were then assembled into the destination binary vector via Golden Gate Assembly. Similarly, another set of MPK12 constructs that had the cDNA sequence of MPK12 (cMPK12) fused to the mVenus-HA under the control of the Arabidopsis ubiquitin 10 promoter (ProUBQ10) and the *A. tumefaciens* nopaline synthase terminator (tNOS) were assembled from Level 0 parts into the destination binary vector. The specification of the Golden Gate reactions performed for the study is listed in Table 2.

The MPK12 cDNA sequence fused with fluorescence protein mVenus, and a copy of HA-tag driven by polyubiquitin 10 *A. thaliana* promoter intended to ensure detection of the goi expression in plant tissue has been cloned by a similar protocol. The Pro+5'UTR part (GGAG-AATG) with pUBQ, CDS1 non-stop codon part with MPK12 cDNA clone and its single substitution versions (AATG-TTCG), mVenus-HA-tag (TTCG-GCTT) and *A. tumefaciens* Nopaline synthase terminator (tNos) pICH41421/H11 standard 3'UTR+Ter part (GCTT-CGCT) were delivered in PFAST-R_amilCP/HygR_P19_pYSWb1 (GGAG-CGCT) destination vector.

The following thermocycler settings were used for the Golden Gate assembly reaction throughout the procedures: 8 cycles of restriction digestion at 37℃ for 5 minutes and ligation at 16℃ for 5 minutes, and kept at 37℃ until transformation into *E. coli*.

Plasmid amplification

The Golden Gate products were used for *E. coli* transformation with 10 minutes of cold incubation followed by 1.5 minutes of heat shock at 42℃ and 40-minutes incubation in 300µl of liquid LB media at 37℃. The bacteria were plated on selective solid LB plates with kanamycin for overnight growth. The above described non-fluorescent chromoprotein was used for negative colony selection.

A selected white colony from each plate was cultured overnight in liquid LB media for the plasmid amplification. The plasmid was extracted using FavorPrep Plasmid Extraction Mini Kit according to the manufacturer's manual. The plasmid DNA concentration was quantified with a NanoDrop spectrophotometer (Thermo Fisher). All the level 0 parts and selected construct plasmids were verified by PCR amplification and Sanger sequencing. The primers used for sequencing are listed in Table 3. The selection of the primers aimed to cover point mutations and the junction regions during DNA assembly.

Table 1. Primers used in genomic MPK12 molecular cloning

Sequence	Template	Primer pair	Primer sequence	Product length	Date
genomic MPK12 amplification from Arabiposis thaliana DNA extract					
gMPK12 (Col-0)	Columbia-0 DNA extraction product	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	2704 bp	
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
Oligonucleotide-directed mutagenesis genomic MPK12 by overlap PCR (I amplification)					
5' gMPK12 (G53R)	gMPK12 (Col-0)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	738 bp	02.06.2021
		X-05 reverse	ACCAGACAATACGGCAAGCGCCTCTACC		
gMPK12 (G53R) 3'	gMPK12 (Col-0)	X-06 forward	CTTGCCGTATTGTCTGGTAAGTTCTTTTGA	2020 bp	02.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
5' gMPK12 (K70R)	gMPK12 (Col-0)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	863 bp	02.06.2021
		K70R-reverse	CATTACCGATCTTCCTAATAGCCACTTTC		
gMPK12 (K70R) 3'	gMPK12 (Col-0)	K70R-forward	GAAAGTGGCTATTAGGAAGATCGGTAATG	1876 bp	02.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
5' gMPK12 (Y122C)	gMPK12 (Col-0)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	1171 bp	02.06.2021
		Y122C-reverse	GTCCATTAACTCACAGACAATGTAGAC		
gMPK12 (Y122C) 3'	gMPK12 (Col-0)	Y122C-forward	GTCTACATTGTCTGTGAGTTAATGGAC	1581 bp	02.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
5' gMPK12 (DE)	gMPK12 (Col-0)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	1488 bp	02.06.2021
		DE-reverse	CGACATATGCAGTCATGAAGCCTGTGTCG		
gMPK12 (DE) 3'	gMPK12 (Col-0)	DE-forward	CGACACAGGCTTCATGACTGCATATGTCG	1266 bp	02.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		

The *Bsa*I (*Eco31*I) recognition sites are highlighted in green, the sticky end nucleotides left after the restriction digestion are identified in red, and the single oligonucleotides substitutions are pinpointed in orange.

Table 1. Primers used in genomic MPK12 molecular cloning (continue)

Oligonucleotide-directed mutagenesis genomic MPK12 by overlap PCR (II amplification)					
gMPK12	gMPK12 (Col-0)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	2704 bp	03.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
gMPK12(G53R)	5' gMPK12 (G53R) & gMPK12 (G53R) 3'	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	2704 bp	03.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
gMPK12(K70R)	5' gMPK12 (K70R) & gMPK12 (K70R) 3'	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	2704 bp	03.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
gMPK12(Y122C)	5' gMPK12 (Y122C) & gMPK12 (Y122C) 3'	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	2704 bp	03.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
gMPK12(DE)	5' gMPK12 (DE) & gMPK12 (DE) 3'	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	2704 bp	03.06.2021
		XII-60 reverse	CGTGGTCTCAAGCGCCAATGTCTAATTTATAATC		
genomic DNA sequence with point mutation PCR amplification					
ProMPK12:gMPK12:tMPK12	P10-17 gMPK12	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	3004 bp	21.06.2021
		XII-42 reverse	ACGGTCTCACGAACCGTGGTCAGGATTGAATTTGAC		
ProMPK12:gMPK12 (G53R):tMPK12	P10-18 gMPK12(G53R)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	3004 bp	17.09.2021
		XII-42 reverse	ACGGTCTCACGAACCGTGGTCAGGATTGAATTTGAC		
ProMPK12:gMPK12 (K70R):tMPK12	P10-19 gMPK12(K70R)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	3004 bp	17.09.2021
		XII-42 reverse	ACGGTCTCACGAACCGTGGTCAGGATTGAATTTGAC		
ProMPK12:gMPK12 (Y122C):tMPK12	P10-20 gMPK12(Y122C)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	3004 bp	17.09.2021
		XII-42 reverse	ACGGTCTCACGAACCGTGGTCAGGATTGAATTTGAC		
ProMPK12:gMPK12(DE) :tMPK12	P10-21 gMPK12(DE)	XII-57 forward	TGTGGTCTCAGGAGTGAGGTAAACAAACAAGTTT	3004 bp	17.09.2021
		XII-42 reverse	ACGGTCTCACGAACCGTGGTCAGGATTGAATTTGAC		

The *Bsa*I (*Eco31*I) recognition sites are highlighted in green, and the sticky end nucleotides left after the restriction digestion are identified in red.

Table 2. Golden Gate assembly specification.

Plasmid number	Construct name	Clonning parts	Cloning methodology
P10-17	PFAST-R_gMPK12/HygR_P19_pYSWb1	PFAST-R_amilCP/HygR_P19_pYSWb1 (GGAG-CGCT) - 0,5 µl ProMPK12: gMPK12:TerMPK12 (GGAG-CGCT) - 1µl overlap PCR product	Total volume 10µl: 1 µl - 10x Ligase buffer 0,2 µl - T4 DNA ligase 0,2 µl -FD *Eco31*I (*Bsa*I) 7,1 µl - H_2O Initial temperature 37°C 8 cycles: 37°C - 5 min/ 16°C - 5 min Keep temperature 37°C
P10-18	PFAST-R_gMPK12(G53R)/HygR_P19_pYSWb1		
P10-19	PFAST-R_gMPK12(K70R)/HygR_P19_pYSWb1		
P10-20	PFAST-R_gMPK12(Y122C)/HygR_P19_pYSWb1		
P10-22	PFAST-R_gMPK12(DE)/HygR_P19_pYSWb1		
P11-17	PFAST-R_gMPK12-mVenus-HA/HygR_P19_pYSWb1	PFAST-R_amilCP/HygR_P19_pYSWb1 (GGAG-CGCT) - 0,5 µl Native Pro + 5UTR + gMPK12: gMPK12 (GGAG-TTCG) - 1µl PCR product mVenus + HA-tag/F8_seq(TTCG-GCTT) 0,5 µl Native ter: MPK12t/plCH441432 (GCTT-CGCT) - 0,5 µl	Total volume 10µl: 1 µl - 10x Ligase buffer 0,2 µl - T4 DNA ligase 0,2 µl -FD *Eco31*I (*Bsa*I) 6,1 µl - H_2O Initial temperature 37°C 8 cycles: 37°C - 5 min/ 16°C - 5 min Keep temperature 37°C
P11-18	PFAST-R_gMPK12(G53R)-mVenus-HA/HygR_P19_pYSWb1		
P11-19	PFAST-R_gMPK12(K70R)-mVenus-HA/HygR_P19_pYSWb1		
P11-20	PFAST-R_gMPK12(Y122C)-mVenus-HA/HygR_P19_pYSWb1		
P11-21	PFAST-R_gMPK12(DE)-mVenus-HA/HygR_P19_pYSWb1		
P11-22	PFAST-R_pUBQ:cMPK12:tNos-mVenus-HA/HygR_P19_pYSWb1	PFAST-R_amilCP/HygR_P19_pYSWb1 (GGAG-CGCT) - 0,5 µl Pro + 5UTR: ProUBQ10 /plCH51277 (GGAG-AATG) - 0,5 µl cMPK12/A11 (AATG-TTCG) - 0,5 µl mVenus + HA-tag/MoCLoF8 (TTCG-GCTT) - 0,5 µl 3UTR + TerNos: pICH41421-MoCloH11 (GCTT-CGCT) - 1 µl	Total volume 10µl: 1 µl - 10x Ligase buffer 0,2 µl - T4 DNA ligase 0,2 µl -FD *Eco31*I (*Bsa*I) 5,6 µl - H_2O Initial temperature 37°C 8 cycles: 37°C - 5 min/ 16°C - 5 min Keep temperature 37°C
P11-23	PFAST-R_pUBQ:cMPK12(G53R):tNos-mVenus-HA/HygR_P19_pYSWb2		
P11-24	PFAST-R_pUBQ:cMPK12(K70R):tNos-mVenus-HA/HygR_P19_pYSWb1		
P11-25	PFAST-R_pUBQ:cMPK12(Y122C):tNos-mVenus-HA/HygR_P19_pYSWb1		
P11-26	PFAST-R_pUBQ:cMPK12(DE):tNos-mVenus-HA/HygR_P19_pYSWb1		

Arabidopsis thaliana transformation

Arabidopsis thaliana MPK12 deletion (*mpk12-4*) mutant plants were transformed by direct flower dip into *Agrobacterium tumefaciens* culture. The *A. tumefaciens* GV3101 strain competent cells harboring virulence helper system (*vir*) with a gentamycin resistance marker were transformed with the binary plasmids described in the "Molecular cloning" section. T About 1µg of plasmids were added into 20µl of the frozen Agrobacterial competent cells. The Agrobacteria were incubated for 10 minutes under heat shock conditions at 37℃ followed by incubation in 300 µl LB media at 28℃ for 20-40 minutes and plated on solid media LB media containing kanamycin and gentamycin antibiotics.

A colony was selected after 3 days of growth on the plate. The selected colony was cultured overnight in 4 ml of LB liquid media with 4µl gentamicin and kanamycin at 28°C and 250rpm. The overnight culture was transferred to a 200 ml Erlenmeyer flask with 40 ml LB media and continued to culture for 4 hours. The culture was poured into a 50 ml canonical tube and centrifuged for 10 minutes at 4000 rpm.

50 ml of floral dip solution containing 5% sucrose and 0.05% Silwet-L77 was prepared on a magnetic mixer. The Agrobacteria pellet was resuspended in the solution. The flower was dipped into the suspension for the entire length of the pedicel. The plants were maintained under long-day conditions (16/8 h light/darkness) until the harvesting of the seeds. The transgenic seeds were manually selected under a fluorescence stereomicroscope by the PFAST-R marker.

Gas exchange measurement

The plants were analyzed in a multi-chamber gas-exchange measurements system that determines stomatal conductance from the difference of CO_2 and water vapor concentration within the chamber where the A. thaliana plant is located and the air with reference concentrations is flushed through the chamber. Such instrument allows undisruptive phenotyping of plant gas exchange phenotypes (Kollist *et al.*, 2007). Stomata conductance of 3-4 weeks old plants were analyzed in ambient (400 ppm), high (800 ppm), and low (100 ppm) CO_2 conditions. The analyses started after stabilizing the stomata conductance after 1,5-2 hours. Each CO_2 treatment lasted 56 minutes.

The MPK12 deletion Arabidopsis thaliana mutant (*mpk12-4*) was used as a transformation background for analyzed transgenic lines. Thus, *mpk12-4* plants served as a negative control, whereas Columbia accession Col-0 was used as a wild-type positive control. The phenotyping was performed with first generation of transgenic plants (T1) for the set with intact genomic sequence

(PFAST-R_gMPK12/HygR_P19_pYSWb1) in two replications and for the sets with mVenus fusion in two replicates.

The analysis of ProMPK12:gMPK12/*mpk12-4* transgenic plants has been repeated twice and overall, from 1 (for ProMPK12:gMPK12(G53R)/*mpk12-4* mutant only) to 5 independent lines were analyzed. The ProMPK12:gMPK12-mVenus-HA/*mpk12-4* plants were analyzed in 2-5 repetitions. Four to six independent lines of ProUBQ:cMPK12-mVenus-HA/*mpk12-4* were phenotyped.

Plant genotyping

The presence of the transgene in the selected transgenic plants was verified after the phenotyping by PCR and Sanger sequencing. The plant DNA was extracted from a leaf sample. The lysis was performed in 200 µl 1x Edwards solution (20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 25 mM EDTA, and 0.05% SDS) by physical disruption (Edwards, Johnstone and Thompson, 1991). The samples were centrifuged for 10 minutes, and 1 µl was used for PCR reaction by DreamTaq polymerase. Additionally, selected PCR products were sequenced. The primers used for genotyping are listed in Table 4.

Confocal microscopy

Expression of the MPK12-mVenus-HA fusion protein was verified in some transgenic plants transformed with plasmids bearing genomic sequence of MPK12 (or its mutated versions) fused with mVenus-HA set. The imaging was performed using a Zeiss LSM710 confocal microscope with 20x magnification objective and 1 Airy Unit (AU) pinhole diameter. The mVenus signals were excited by a 514 nm laser and the emission was collected in the 518-564 nm wave-length range.

Table 3. Sequence verification by plasmid sequencing and colony PCR

Transgene	Primer pair	Primer sequence	Product length	Date	Notes
PFAST-R gMPK12 /HygR_P19_pYSWb1 set GG plasmid sequencing					
gMPK12 gMPK12(G53R) gMPK12(K70R) gMPK12(Y122C) gMPK12(DE)	XII-51 forward	ACGAGCTAAAGATTGGTGATTTTG		21.06.2021	MPK12 IV exon
	XII-50 reverse	AAACGTCACTGAGTTCACCGCAG			MPK12 II exon
	VI-64 forward	TCCCTACTGTCTGGAGAATCAAGCTCT			MPK12 I exon
	II-60 forward	GTAAAACGACGGCCAGT			vector
PFAST-R_gMPK12-mVenus-HA /HygR_P19_pYSWb1 set GG plasmid sequencing					
gMPK12-mVenus-HA gMPK12(G53R)-mVenus-HA gMPK12(K70R)-mVenus-HA gMPK12(Y122C)-mVenus-HA gMPK12(DE)-mVenus-HA	XIII-36 reverse	CCTCGCCGGACACGCTG		23.09.2021- 01.10.2021	mVenus
	XII-51 forward	ACGAGCTAAAGATTGGTGATTTTG			MPK12 IV exon
	XII-50 reverse	AAACGTCACTGAGTTCACCGCAG			MPK12 II exon
	XII-67 reverse	CTCTGTTATAAGCCTAAGCTGATG			MPK12 IV exon
	XV-29 reverse	CCTCTTCGCTATTACGCCAG			pYSWb1 - vector
PFAST-R_pUBQ:cMPK12:tNos-mVenus-HA /HygR_P19_pYSWb1 set GG plasmid sequencing					
cMPK12-mVenus-HA cMPK12(G53R)-mVenus-HA cMPK12(K70R)-mVenus-HA cMPK12(Y122C)-mVenus-HA cMPK12(DE)-mVenus-HA	XIII-36 reverse	CCTCGCCGGACACGCTG		23.09.2021- 01.10.2021	mVenus
	XII-51 forward	ACGAGCTAAAGATTGGTGATTTTG			MPK12 IV exon
	XII-50 reverse	AAACGTCACTGAGTTCACCGCAG			MPK12 II exon
	XV-29 reverse	CCTCTTCGCTATTACGCCAG			pYSWb1 - vector
PFAST-R_pUBQ:cMPK12:tNos-mVenus-HA /HygR_P19_pYSWb1 *E. coli* colony PCR					
cMPK12-mVenus-HA cMPK12(G53R)-mVenus-HA cMPK12(K70R)-mVenus-HA cMPK12(Y122C)-mVenus-HA cMPK12(DE)-mVenus-HA	III-15 forward	TCGGATCCGAGTCAGTAATAAACGG	1834 bp	01.10.2021	pUBQ
	III-24 reverse	TACGTCGCCGTCCAGCTC			mVenus
	III-25	CCAACGAGAAGCGCGATC	529 bp		mVenus
	XV-29 reverse	CCTCTTCGCTATTACGCCAG			pYSWb1 - vector

Table 4. Primers employed for plant genotyping

Transgene	Primer pair	Primer sequence	Product length	Date	Notes
gMPK12_pFAST-R/HygR_P19_pYSWb1					
ProMPK12:gMPK12 /*mpk12-4* transgenic plants genotyping by PCR					
gMPK12 gMPK12(G53R) gMPK12(K70R) gMPK12(Y122C) gMPK12(DE)	VI-64 forward	TCCCTACTGTCTGGAGAATCAAGCTCT	1102 bp	15.12.2021	Start-codon
	XII-67 reverse	CTCTGTTATAAGCCTAAGCTGATG		23.12.2021	MPK12 IV exon
	XII-51 forward	ACGAGCTAAAGATTGGTGATTTTG	778 bp	15.12.2021	MPK12 IV exon
	VI-65 referse	ACTACGGTGGTCAGGATTGAATTTGAC		23.12.2021	MPK12 VI exon
pFAST-R_gMPK12/HygR_P19_pYSWb1 transgenic plants genotyping by sequencing					
ProMPK12:gMPK12-mVenus-HA /*mpk12-4* transgenic plants genotyping by sequencing					
gMPK12	XII-51 forward	ACGAGCTAAAGATTGGTGATTTTG		29.12.2021	MPK12 IV exon
	XII-67 reverse	CTCTGTTATAAGCCTAAGCTGATG			MPK12 IV exon
gMPK12(K70R) gMPK12(Y122C)	VI-64 forward	TCCCTACTGTCTGGAGAATCAAGCTCT		29.12.2021	Start-codon
gMPK12(DE)	VI-65 referse	ACTACGGTGGTCAGGATTGAATTTGAC		29.12.2021	MPK12 VI exon
p-FAST-R_gMPK12-mVenus-HA /HygR_P19_pYSWb1					
ProMPK12:gMPK12-mVenus-HA /*mpk12-4* transgenic plants genotyping by PCR					
gMPK12 gMPK12(G53R) gMPK12(K70R)	XII-47 forward	CATCAAAGTTGTACCGACACACGG	870 bp	24.01.2022	MPK12 I exon
	XII-69 reverse	GCTTGCAAGAACAACTTCCGA			MPK12 IV exon
gMPK12(DE)	XII-68 forward	GACCAGTGATCAATGCCGT	985 bp	14.01.2022	MPK12 III exon
	VI-65 reverse	ACTACGGTGGTCAGGATTGAATTTGAC			MPK12 VI exon
p-FAST-R_cMPK12-mVenus-HA /HygR_P19_pYSWb1					
ProUBQ:cMPK12-mVenus-HA /*mpk12-4* transgenic plants genotyping by PCR					
cMPK12 cMPK12(G53R) cMPK12(K70R)	XII-47 forward	CATCAAAGTTGTACCGACACACGG	543 bp	14.01.2022	MPK12 I exon
	XII-69 reverse	GCTTGCAAGAACAACTTCCGA			MPK12 IV exon
cMPK12(DE)	XII-68 forward	GACCAGTGATCAATGCCGT	706 bp	14.01.2022	MPK12 III exon
	VI-65 reverse	ACTACGGTGGTCAGGATTGAATTTGAC			MPK12 VI exon

RESULTS AND DISCUSSION

The MPK12 deletion plants of *A. thaliana* Col-0 accession (*mpk12-4*) were used for transformation with T-DNA constructs harboring various MPK12 versions. Three sets of plasmids were assembled with T-DNA region bearing five different MPK12 versions. The T-DNA maps are depicted in Figure 5. The first set incorporates genomic sequence of MPK12 and four mutated versions. The choice of the native sequence was dictated by the goal to minimize possible interference of protein expression and post-translational modifications. However, agrotransformation often results in variability in transgene protein expression levels, which also contributes to variations in the phenotype among different transgenic lines (Gelvin, 2003).

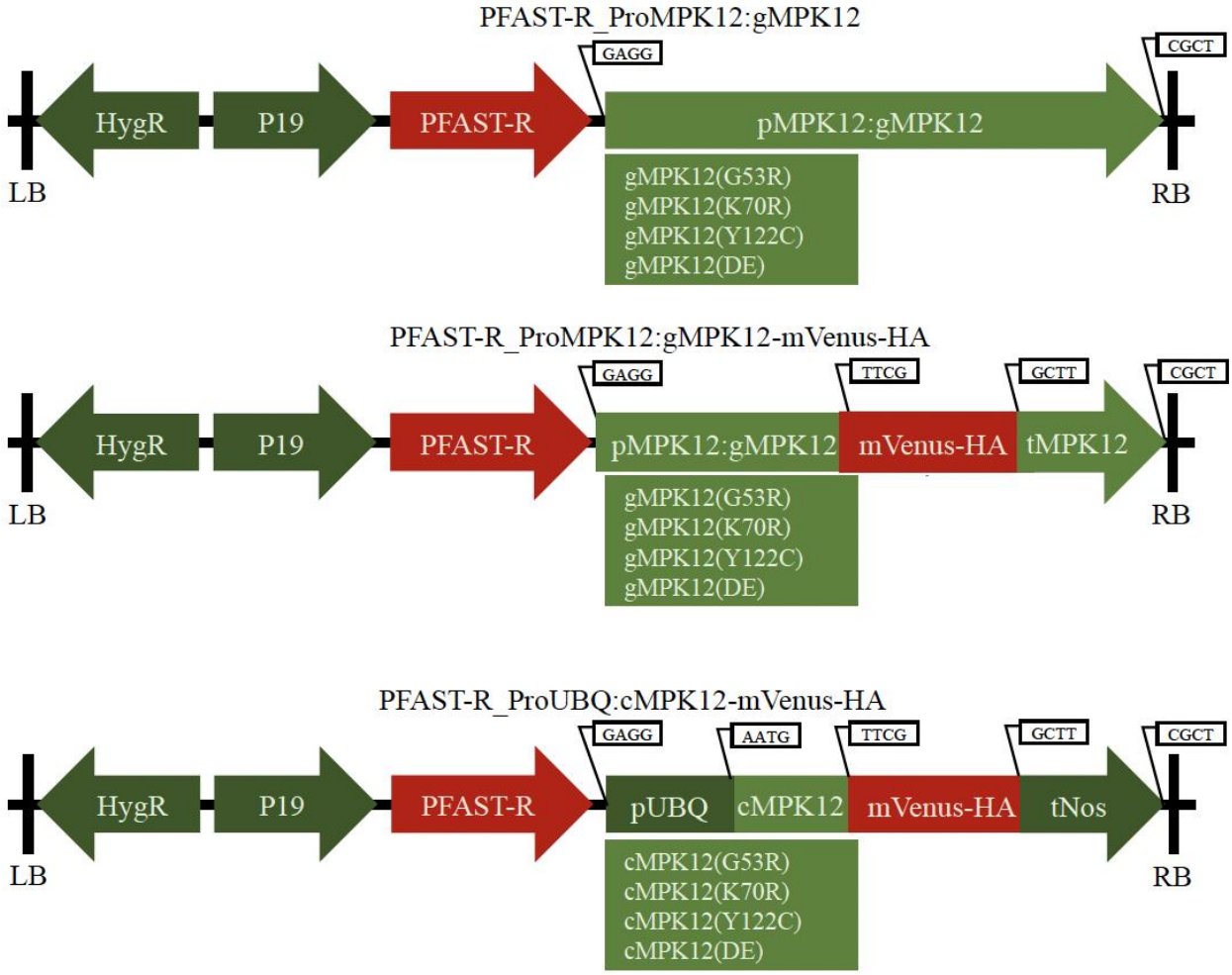

Figure 5. T-DNA constructs maps. LB- left border T-DNA sequence, RB- right border T-DNA sequence; HygR- Hygromycin B resistance marker gene, P19 – gene silencing suppressor; PFAST-R - Oleosin *A. thaliana* protein gene sequence fused with red fluorescence protein; pMPK12 (ProMPK12) – promoter MPK12; gMPK12 – genomic MPK12 sequence; pUBQ – plant ubiquitin 10 promoter; cMPK12 – coding MPK12 sequence, mVenus – yellow fluorescent protein gene; HA - Human Influenza Hemagglutinin tag; tNos - *A. tumefaciens* Nopaline synthase terminator. The overhangs after *BsaI* restriction digestion of the Golden Gate parts and the PCR products are depicted above the respective parts.

To verify the protein expression level, two sets of transgenic lines where the *MPK12* is fused with a yellow fluorescent protein gene (mVenus) followed by a copy of Human Influenza Hemagglutinin tag (HA-tag, YPYDVPDYA) have been prepared (Kremers *et al.*, 2006). These allow verification of protein expression levels by fluorescence microscopy and by western blot analyses, respectively. One of these sets is based on the genomic sequence of MPK12 that includes its native promoter and terminator.

However, the native MPK12 promoter (ProMPK12) is preferentially functional in guard cells that may complicate fluorescence detection by microscopy (Jammes *et al.*, 2009). Therefore, another set of constructs where the complementary DNA (cDNA) *MPK12* versions fused to the mVenus-HA and expressed under a ubiquitous promoter (pUBQ10) was designed to insure the detection of the protein expression in most plant cells. In all three sets of constructs, the T-DNA cassette bears two plant selection markers: a hygromycin resistance gene (HygR) and a PFAST-R system where a red fluorescence protein was expressed in oil bodies of the seeds. In addition, a gene silencing suppressor (P19) was included to minimize transgene silencing.

An example of visual verification of transgenic MPK12 expression with confocal microscopy is presented in Figure 6. The preferential expression of MPK12 driven by its native promoter could be seen in the guard cells where the fluorescence is detected in the cytosol and the nucleus. This construct set allows confirmation of the protein expression after plant phenotyping. This is especially valuable to ensure MPK12 expression in transgenic plants harboring MPK12 mutants which could not complement stomatal CO_2 responses in the *mpk12-4* background.

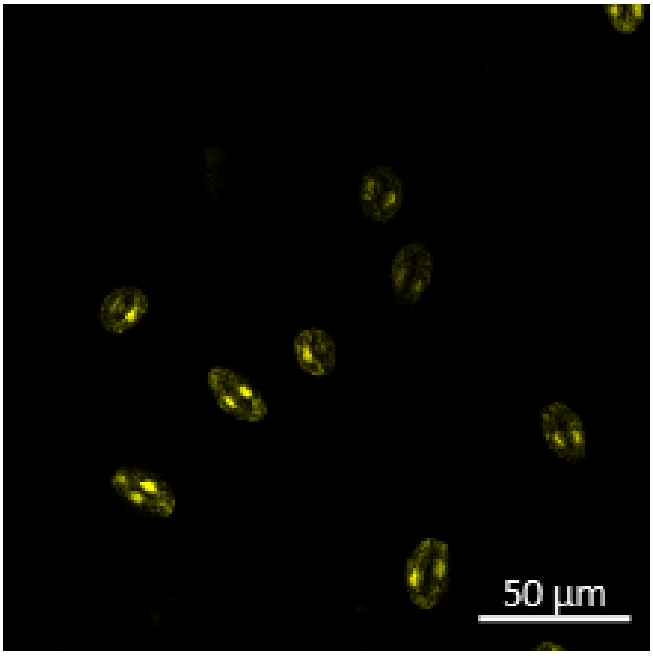

Figure 6. **Confocal microscope image of PFAST-R_gMPK12-mVenus-HA/mpk12-4 plant guard cell.** The chimeric MPK12 fused with mVenus protein expressed under native MPK12 promoter preferentially expressed in guard cells.

Transgenic plants phenotyping results

The degree of the stomatal opening is measured as the stomatal conductance. The stomatal conductance is calculated as a rate of H_2O evaporation through stomatal pores under a constant airflow rate in mmol/m^2/s. At the beginning of each experiment, the gas-exchange rate was stabilized at 400ppm CO_2, which is the ambient condition. Basal stomata conductance is the rate of water vapor evaporation at the ambient CO_2 concentration that corresponds to the beginning of the experiment.

The gas-exchange experiment was performed with the first generation of transgenic plants (T1). The steady-state stomatal conductance was calculated as an average for 3 consecutive measurements before application of elevated CO_2. The basal stomata conductance at the beginning of the experiment for all tested independent transgenic lines is presented in Figure 7. The basal stomatal conductance of *mpk12-4* plants transformed with wild-type MPK12 (WT MPK12) were similar to the Col-0 positive controls. Transformation of MPK12(K70R) and MPK12(Y122C) into *mpk12-4* also brought stomatal conductance to the values similar to Col-0 for the three tested sets of constructs. MPK12 deletion mutant (*mpk12-4*) exhibited more opened stomata compared to Col-0 as reported previously (Jakobson *et al*., 2016). Surprisingly, MPK12(DE) mutant, which is hyperactive in theory, displayed higher basal stomatal conductance that is similar to *mpk12-4*. The number of MPK12(G53R) transgenic plants was too small in the experimental sets to draw a firm conclusion. Nevertheless, the plants exhibited a more opened stomata phenotype compared to Col-0 and was more similar to the *mpk12-4* plants (Jakobson *et al.*, 2016). Overall, WT MPK12, MPK12(K70R), and MPK12(Y122C) transformant had basal stomata conductance similar to Col-0, whereas MPK12(G53R) and MPK(DE) did not.

To analyze stomatal response to CO_2 treatment an average stomatal conductance for 3 consecutive measurements after 40 minutes of the treatment was calculated for each independent line, and the difference between the results and the basal stomatal conductance was calculated. The results are presented in Figure 8. The high CO_2 (800ppm) response was always impaired in *mpk12-4* and the kinase-dead MPK12(G53R) plants, which coincides with the previous research (Jakobson *et al.*, 2016). Similar to the *mpk12-4* response to low CO_2 treatment, plants carrying a G53R or DE mutations had a weaker opening response compared to the Col-0. The average stomatal conductance of kinase hyperactive MPK12(DE) plants under high CO_2 treatment was similar to *mpk12-4.*

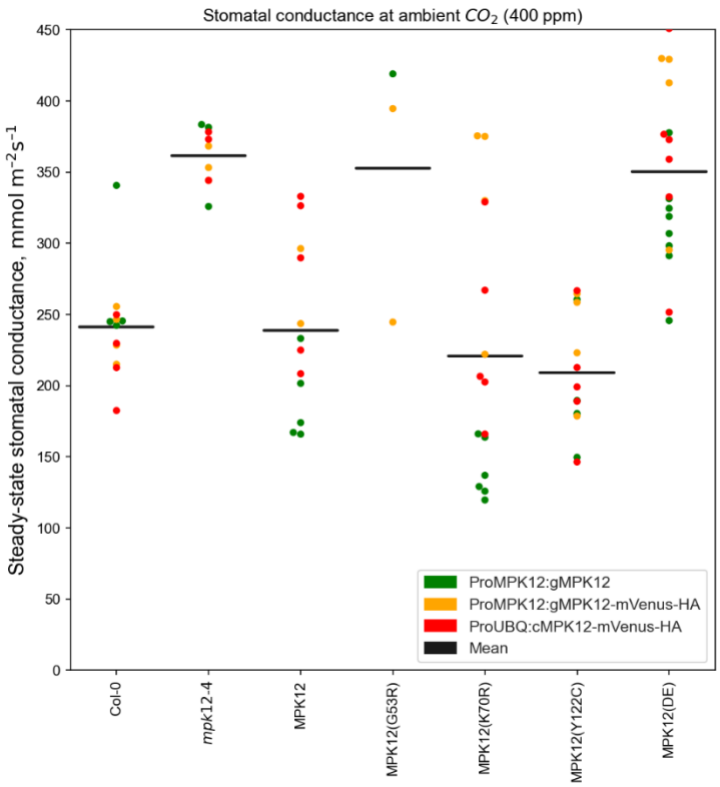

Figure 7. Steady-state stomatal conductances of studied plant lines. Steady state stomatal conductance values at standard conditions for each phenotyped plant; the mean values (shown as horizontal line) *were* calculated for Columbia-0, mpk12-4 mutant and plants transformed with each type of MPK12 into mpk12-4 among 3 different construct sets. The Col-0 and *mpk12-4* plants tested together with different sets of constructs are highlighted in respective colors.

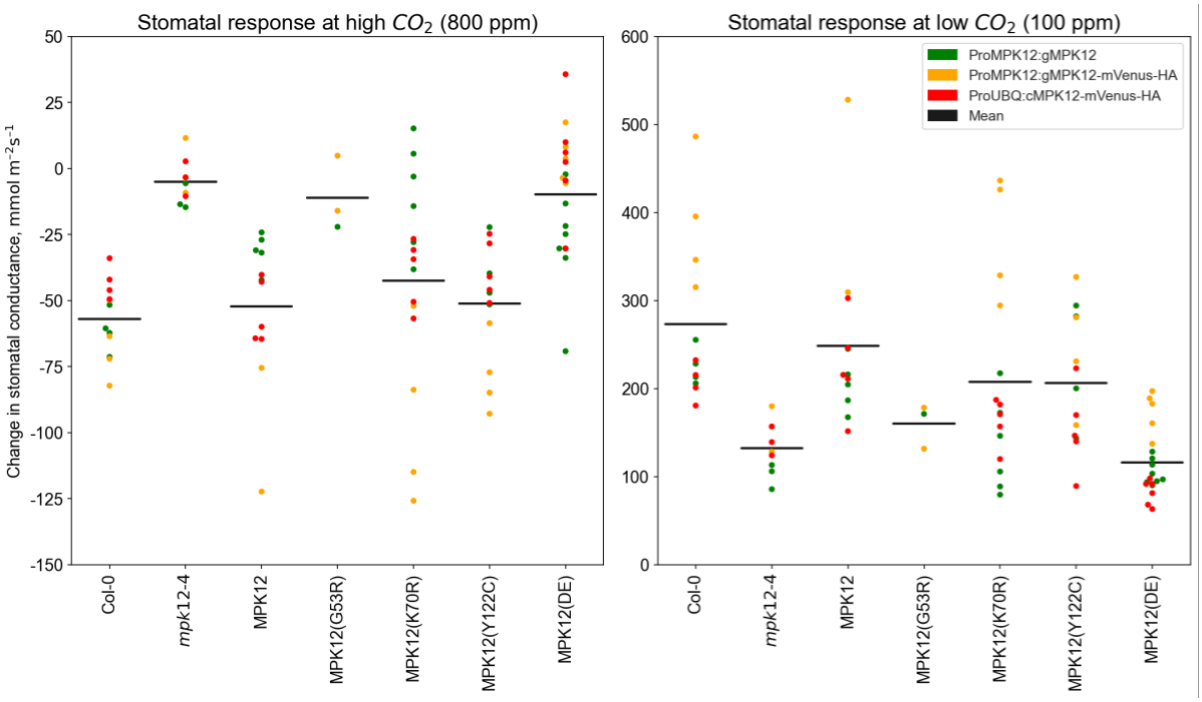

Figure 8. Stomatal responses to high and low CO_2 treatment. Stomatal conductance response to high and low CO_2 treatment for independent experimental lines under investigation. Values are calculated as a difference between average value of 3 consecutive measurements after 40 minutes of the treatment and the steady-state value at standard conditions before application of treatment. The Col-0 and *mpk12-4* plants tested together with different sets of constructs are highlighted in respective colors.

The results of stomatal conductance measurements for all studied independent lines are shown in the Supplementary Figures 1, 3 and 5. These figures allow tracing the response throughout the experiment for each line independently. The ProMPK12:gMPK12(DE) #4-line's low CO_2 response was *mpk12-4*-like.

The *mpk12-4* plants transformed with WT MPK12 closed stomata to a similar degree as Col-0 as expected. The stomatal opening in response to low CO_2 treatment of these plants was also similar to Col-0. Thus, WT MPK12 T-DNA restored stomatal response in MPK12 deletion mutant *mpk12-4* (after such statement reader would like to know where to look, thus include Fig nr here).

The kinase-dead MPK12(K70R) and kinase hyper-active versions MPK12(Y122C) demonstrated high CO_2 response rather similar to Col-0 and WT MPK12. There were several outliers among MPK12(K70R) plants belonging to the ProMPK12:gMPK12 set (ProMPK12:gMPK12 MPK12(K70R)#2 and ProMPK12:gMPK12 MPK12(K70R)#6, see Supplementary Figure 1 and 2), the presence of the transgene in these plants was verified by genotyping. These plants responded to low CO_2 treatment although the response was weakened compared to other lines carrying the same transgene. However, response of these plants was rather similar to Col-0, which is easier to observe in Supplementary Figure 2 where the kinetics of the results were shown in relative units. The difference was among individual plants could be attributed to variation in the protein expression level and analysis in the future generations is needed.

The plants transformed with cDNA of wild-type MPK12 fused with mVenus expressed under ubiquitous promoter (ProUBQ:cMPK12-mVenus-HA) exhibited a higher degree of low and high CO_2 stomatal response. The *A. thaliana* ubiquitin 10 promoter is moderately strong, and may enhance MPK12 gene transcription compared to its native promoter (Grefen *et al.*, 2010).

The variation of the low CO_2 response is better represented on the response kinetics curves plots in Supplementary Figures 1, 3, 5. The differences in basal stomata conductance could be explained by the protein expression level that is often observed in T1 plants. The kinetics of stomatal response could be compared in relative units. They are calculated as a ratio of the observed value to the steady-state stomatal conductance before the onset of CO_2 change. The results of gas-exchange experiments in relative units for independent lines were consistent for all three sets of constructs (Supplementary Figures 2, 4, and 6). Although the high CO_2 treatment response was not clear for some of the independent lines of ProMPK12:gMPK12 set (Supplementary Figure 1 and 2), this may be attributed to variation in the protein expression level as the lines with weak

high CO_2 response demonstrated reduced low CO_2 response compared to Col-0 WT. Nevertheless, in two other sets of transgenic constructs (ProMPK12:gMPK12-mVenus-HA and pUBQ:cMPK12-mVenus-HA, Supplementary Figures 3-6) both CO_2-induced stomata closure and stomata opening are clearly identified for K70R and Y122C mutant plants but not in G53R, DE and *mpk12-4*.

Strictly speaking, averaging the phenotyping results in independent lines of T1 plants is not the best practice as Agrobacterium-mediated transformation does not result in the generation of genetically identical plants and used T-DNA cassette sequences were not similar either. However, it was performed for visualization purposes and on the bases of a clearly tracible tendency in the CO_2 treatment responses for the independent lines.

To visualize the low CO_2 response average values for experimental variants and controls were calculated for all three sets together. The *mpk12-4* demonstrated impaired low CO_2 response (Figure 9). The kinase-dead MPK12(G53R) and kinase hyperactive MPK12(DE) complementation lines low CO_2 response was similar to the MPK12 deletion mutant. At the same time, kinase-dead MPK12(K70R) and kinase-hyperactive MPK12(Y122C) stomatal conductance responses at low CO_2 were similar to Col-0 wild type plants.

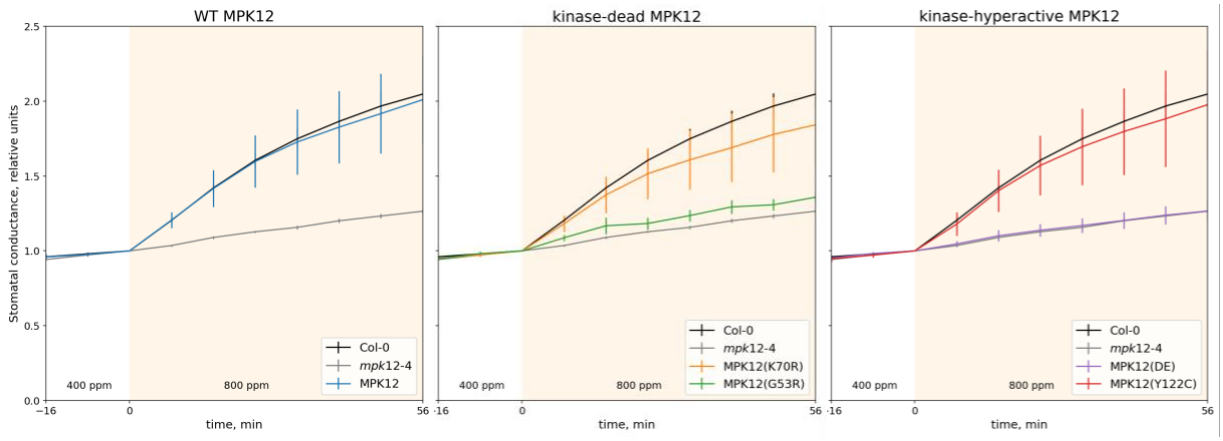

Figure 9. The kinetic curves of stomatal conductance response to low CO_2 treatment (100ppm). The beginning of the treatment is 0 minutes. The result is the averaged for all independent lines among 3 sets of constructs transformed to *mpk12-4*. The relative stomatal conductance was calculated as a ratio of the measured stomatal conductance at a 0-time point, i.e. to the basal stomatal conductance at ambient CO_2 (400 ppm) of the respective independent line.

Similarly, averaged high CO_2 treatment response kinetics curves among all the MPK12 variants among the 3 sets of transgenic lines in relative were generated. Overall, the standard error was large only for MPK12(K70R) lines, the possible reason was previously described. The number of the experimental lines in kinase-dead MPK12(G53R) is rather small to draw a final conclusion on

the bases of averaged results, however, their high CO_2 response was suppressed as observed for *mpk12-4*.

The same tendency in the guard cell signaling was traced for kinase hyperactive versions. They did not respond similarly, the response of MPK12(DE) was similar to MPK12 deletion mutant *mpk12-4*. MPK12(Y122C) responded similarly to Col-0.

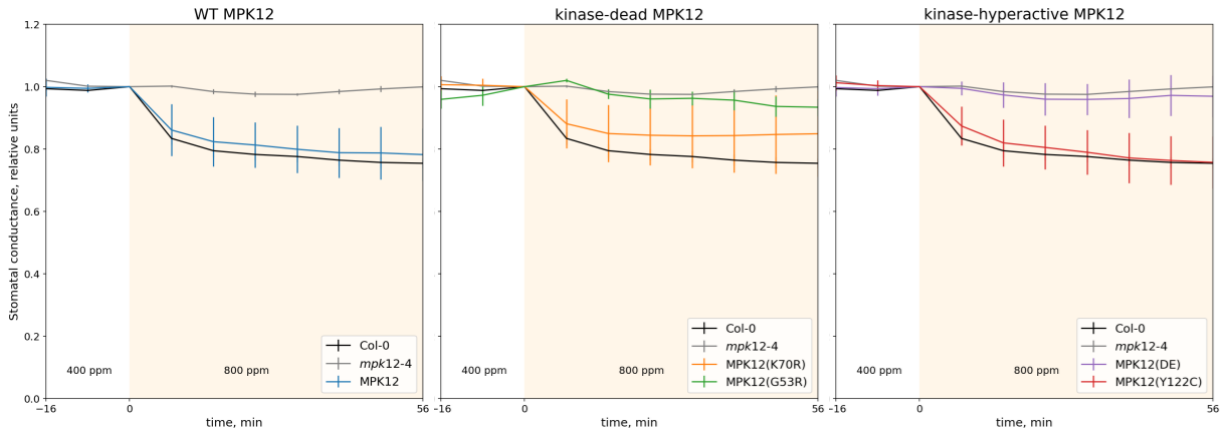

Figure 10. The kinetic curves of stomatal conductance response to high CO_2 treatment (800ppm). The beginning of the treatment is 0 minutes. The result is the average for all independent lines among 3 construct sets in relative units. The relative units are calculated as a ratio of the measured stomatal conductance at a time point 0 at ambient CO_2 (before the treatment) of the respective independent line.

Taking together, the low and high CO_2 response was disrupted in the MPK12 deletion control (*mpk12-4*) and their stomata were constitutively opened. Transformation of the WT MPK12 restored the CO_2-response phenotype, the plants displayed responses similar to *A. thaliana* Columbia ascension (Col-0). The MPK12 with G53R point mutation did not restore CO_2 response; two independent lines of ProMPK12:MPK12(G53R)-mVenus-HA/*mpk12-4* displayed variation of basal stomatal conductance of 186 mmol m^{-2} s^{-1}. Nevertheless, the high/low CO_2 treatment response was similar to *mpk12-4*. These results concede with those obtained by L. Jakobson et al. (2016) showing that G53R substitution in MPK12 impairs its interaction by HT1 and thus this version of MPK12 cannot inhibit HT1 which is essentially and important step in launching stomatal closure in response to elevated CO_2. However, kinase inactive version of MPK12(K70R) restored CO_2 response similar to Col-0 and as this version was shown to interact well with HT1 (Figure 3, Y. S. Wang unpublished data) it can be concluded that HT1:MPK12 interaction does not depend on MPK12 kinase activity.

Among kinase hyperactive versions MPK12 with Y122C mutation complemented the MPK12 deletion phenotype, however constitutively active kinase with DE mutation displayed a disrupted response to CO_2. The transgene presence was verified for all MPK12 lines with DE point mutations by PCR or second-generation transgenic seeds screening.

Overall, these results demonstrate that the kinase activity of MPK12 is not involved in stomatal CO_2 signaling. However, phenotyping results in the first generation of transgenic plants often result in variation in protein expression levels due to the nature of Agrobacterium-mediated transformation (AMT). The studies of the AMT mechanism report preferential T-DNA region targeting to host's transcriptionally active promoters. However, among others Kim & Gelvin reported frequent integration of T-DNA in heterochromatic regions in the experiments without selection pressure (Kim and Gelvin, 2007). An epigenetic goi silencing in the host may be involved in the variability of the expression level (Schubert *et al.*, 2004; Meyer, 1995). Moreover, multiple T-DNA copies integration is a well-known phenomenon (Neve *et al.*, 1997; Schubert *et al.*, 2004; Betts *et al.*, 2019). Nevertheless, the transgenic plant independent lines of the first generation are so-called sister transformants. The sister transformants carry the same transgene cassette otherwise having the same genome. Strictly speaking, the sister transformants' sets of genes are not identical and should not be considered as replicates. Thus, statistical treatment of the generated data is not relevant. Usually, analysis of second and third generations of transgenic plants are needed in order to reduce noise caused by the above-described reasons and perform statistical analysis of the results. Due to time-constraints these experiments are just under way and could not be included into the current thesis. The phenotyping has to be reproduced for several independent lines. Additionally, phenotyping of heterozygous plants obtained by back-crossing to the initial accession is performed (Col-0 in this case), this excludes possible interference of an essential gene by the transgene.

Considering the results of split-ubiquitin yeast two hybrid assay (Figure 3, Y. S. Wang unpuplished data), it is tempting to speculate that protein-protein interaction between HT1 and MPK12, but not kinase activity of MPK12, is required for guard cell CO_2 signaling. Although further investigation of the topic and confirmation of the gas-exchange results in future transgenic plant generations are needed.

Some protein pseudokinases are known to retain their biological functionality via alternative mechanisms that do not require the catalytical activity (Sheetz and Lemmon, 2022). Moreover, human MAPK SIGNAL-REGULATED KINASES 2 (ERK2) has been reported to perform their functionality independent of the phosphorylation activity (Rodríguez and Crespo, 2011). Thus, this study does not exclude the possible involvement of MPK12 kinase activity in other molecular signaling pathways.

SUMMARY

The role of MPK12 and its kinase activity in stomatal CO_2 signaling was explored. To test the hypothesis, transgenic lines carrying following MPK12 versions with amino acids substitutions were investigated: kinase inactive – MPK12(G53R) and MPK12(K70R); and kinase hyperactive MPK12(Y122C) and MPK12(DE). Three T-DNA sets were generated, and transgenic plant lines were produced: first set carries genomic sequence of MPK12 or its mutated versions, two other sets included tags for verification of the protein expression under stereomicroscope or by protein immunoprecipitation.

The phenotyping of the first generation of transgenic plants was performed in gas-exchange phenotyping platform. The stomatal conductance of the plants was measured and analyzed. According to the results, the role of MPK12 is pivotal in stomata CO_2 signaling. However, MPK12 kinase activity is not involved in the CO_2 signaling, instead its interaction with HT1 kinase is required. This is in line with the results of split-ubiquitin yeast two-hybrid Y2H assay performed by Y. S. Wang where MPK12(Y122C) and MPK12(K70R) mutant interacted with HT1 and MPK12(DE) and MPK12(G53R) did not exhibit the interaction. Taking together these results suggest that interaction between MPK12 and HT1 is more important in the CO_2-induced signaling pathway than the MPK12 kinase activity.

However, due to time-constraints mutant plant screening were performed in first-generation transgenic plants. Due to the nature of Agrobacterium-mediated transformation, the results have to be verified in the third generation of transgenic plants and statistical analysis for these lines has to be done.

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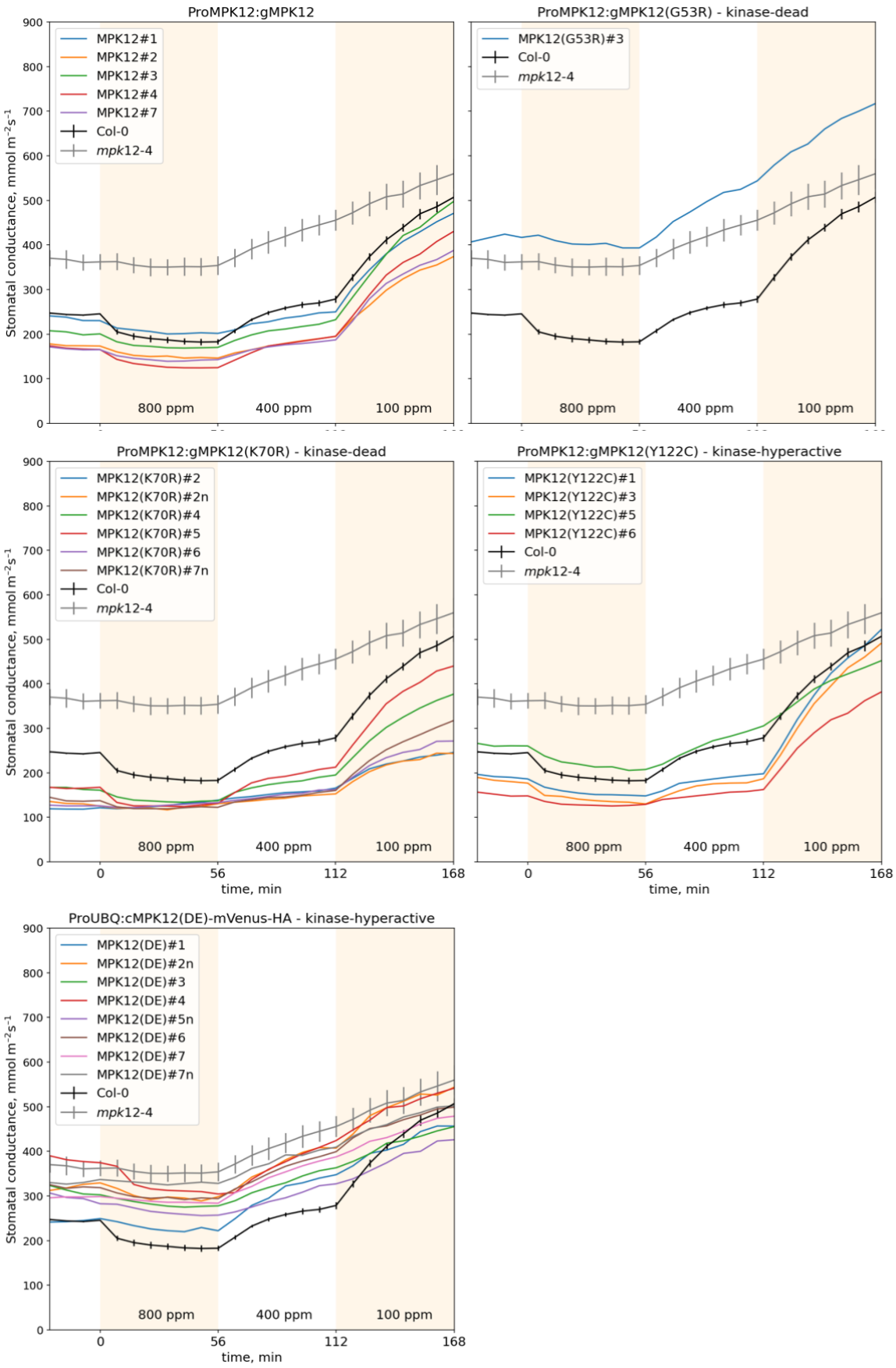
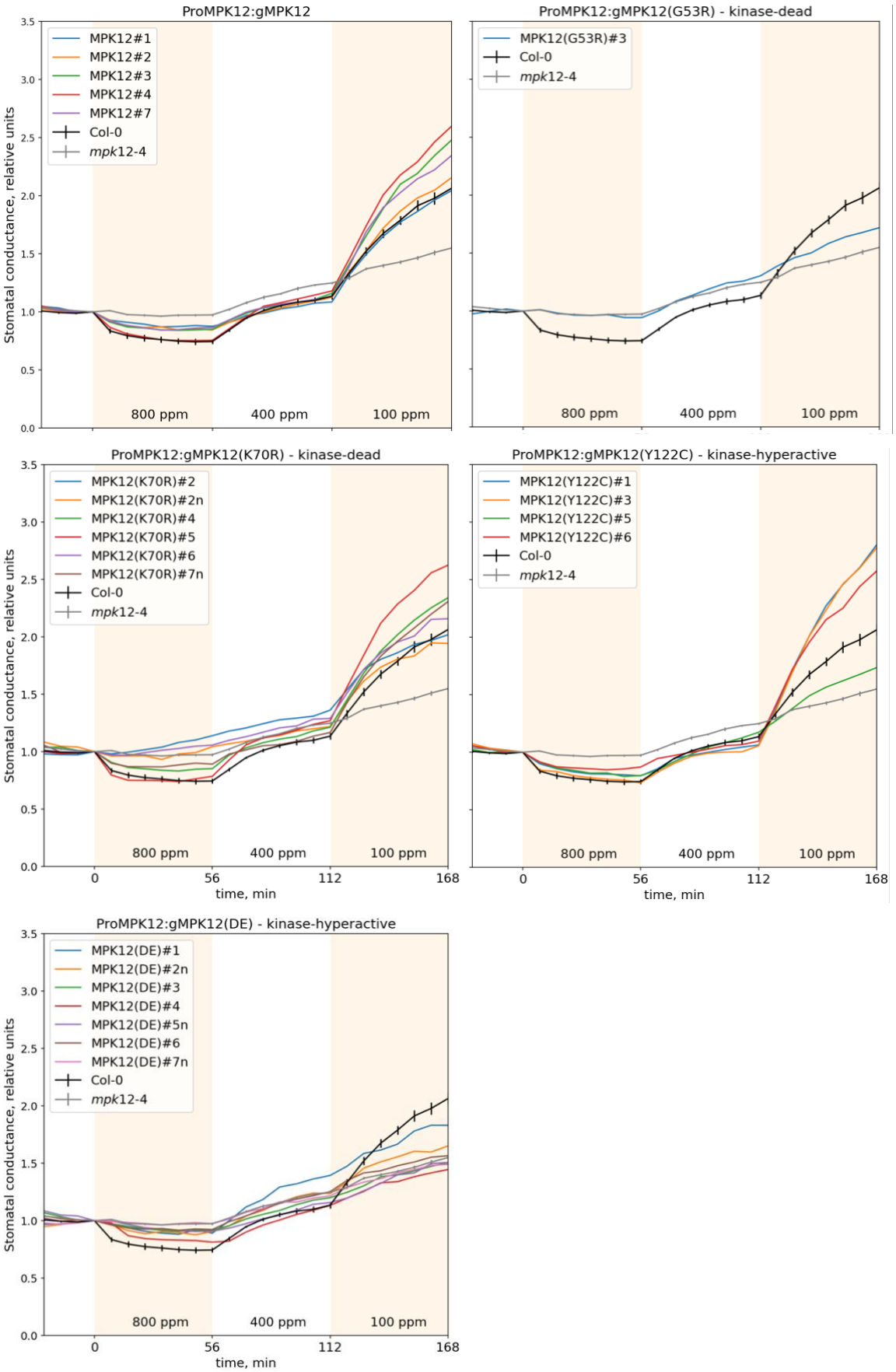
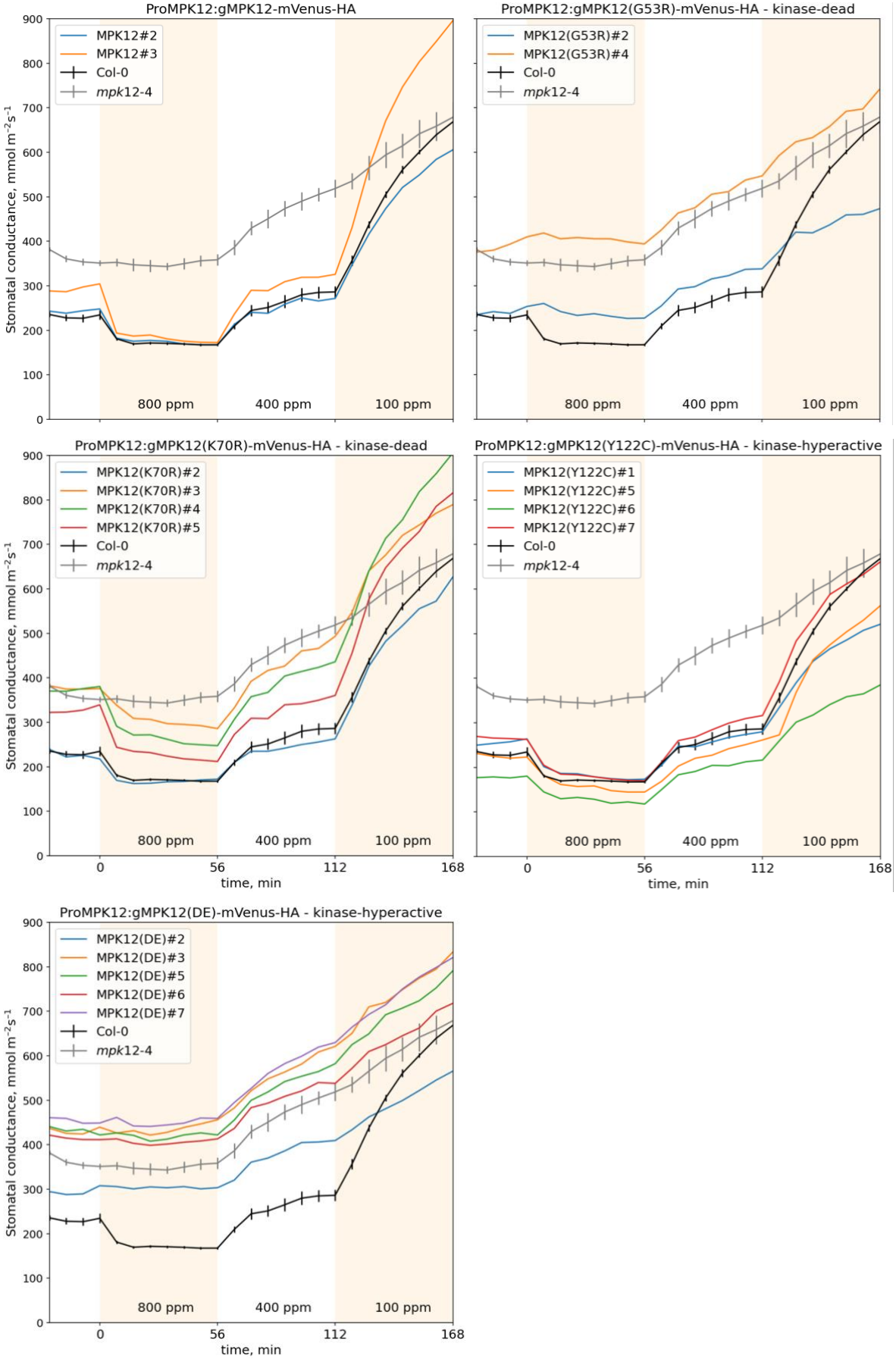
SUPPLEMENTARY MATERIALS



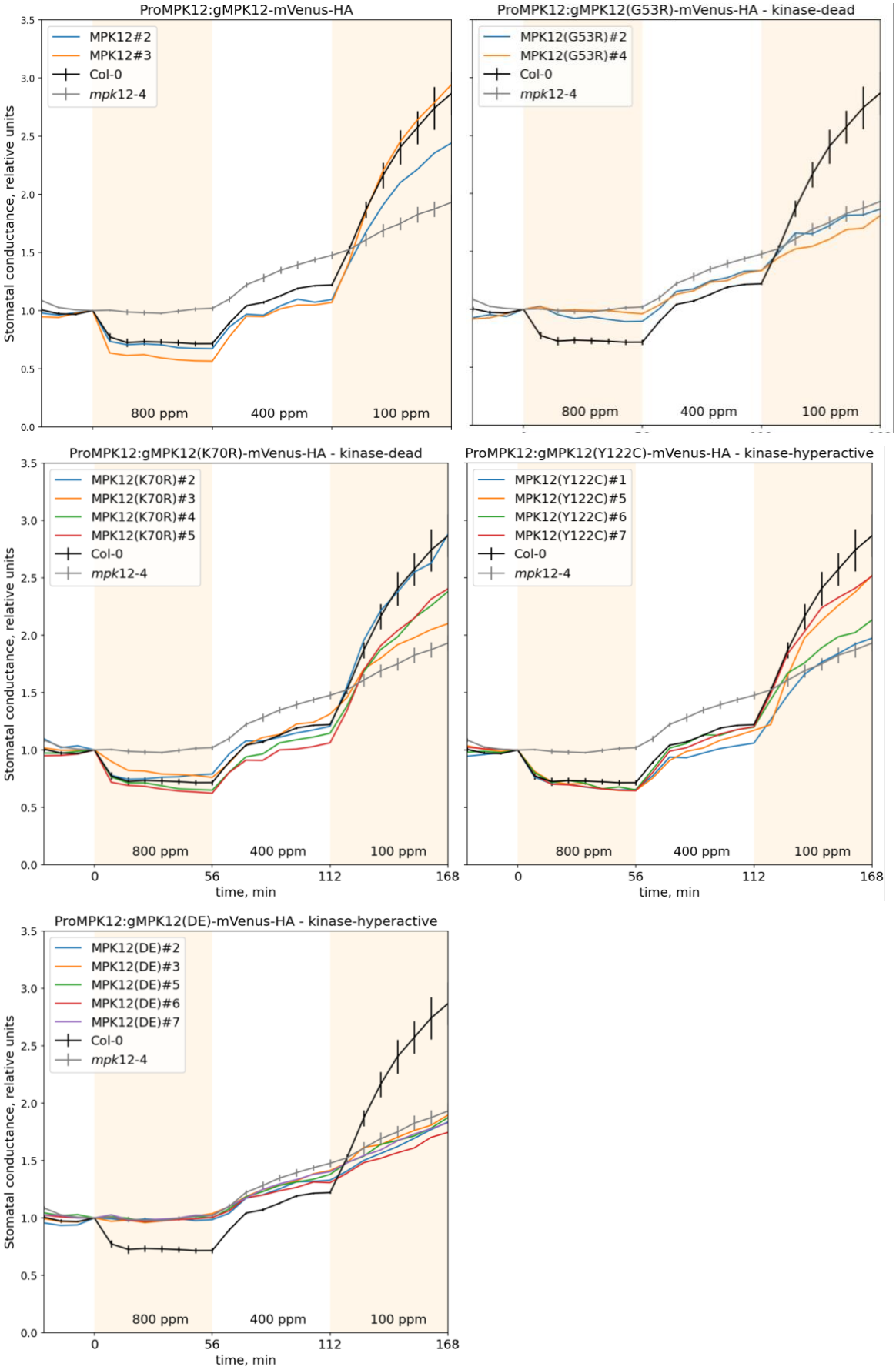
Supplementary Figure 1. Plant gas exchange phenotyping results proMPK12:MPK12 transformed to MPK12 deletion background *mpk12-4*. Independent lines time-resolved patterns of stomatal conductance during CO_2 shifts in absolute units is shown.



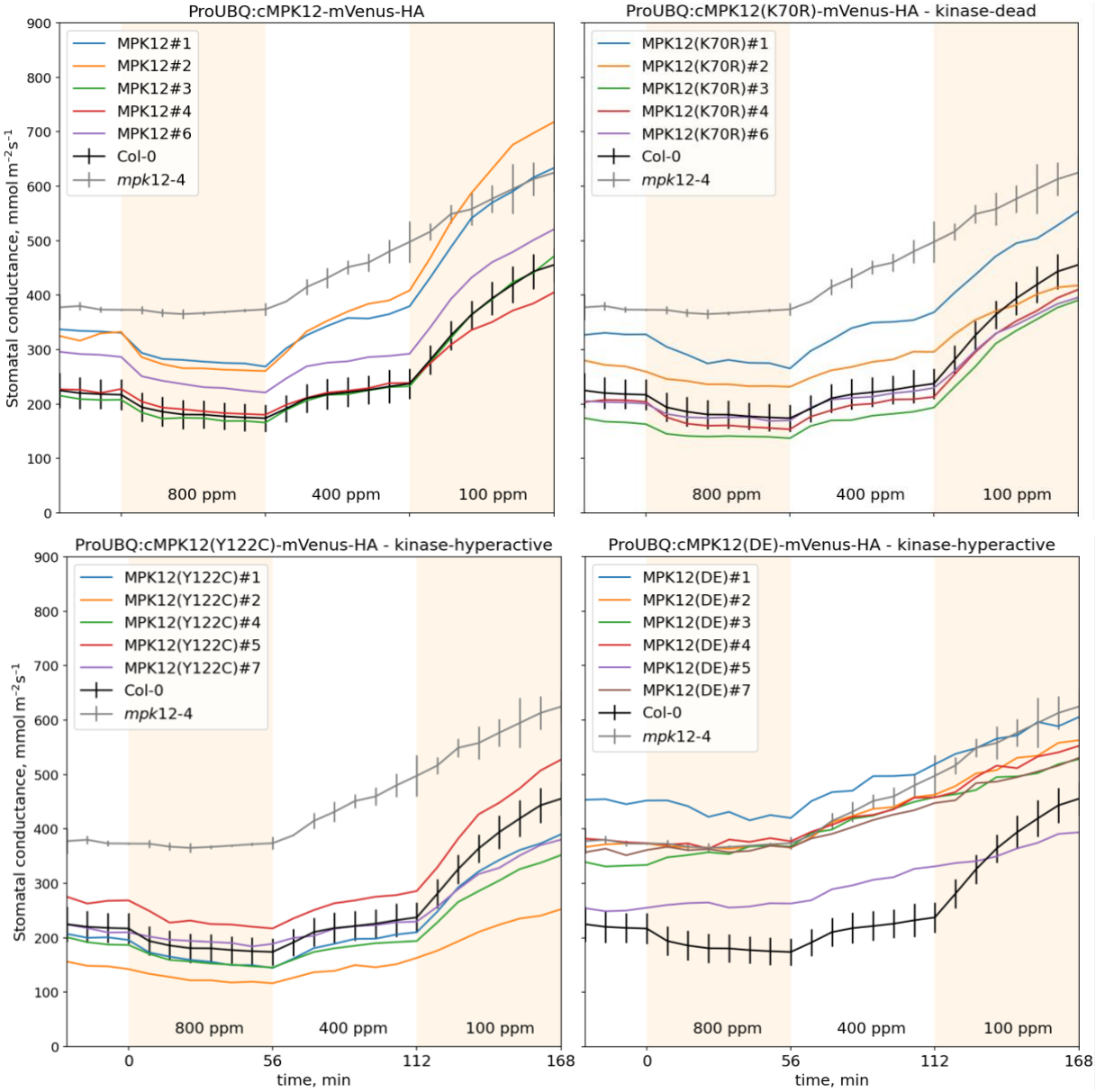
Supplementary Figure 2. Plant gas exchange phenotyping results proMPK12:MPK12 transformed to MPK12 deletion background *mpk12-4*. Independent lines time-resolved patterns of stomatal conductance during CO_2 shifts in absolute units is shown in relative units.



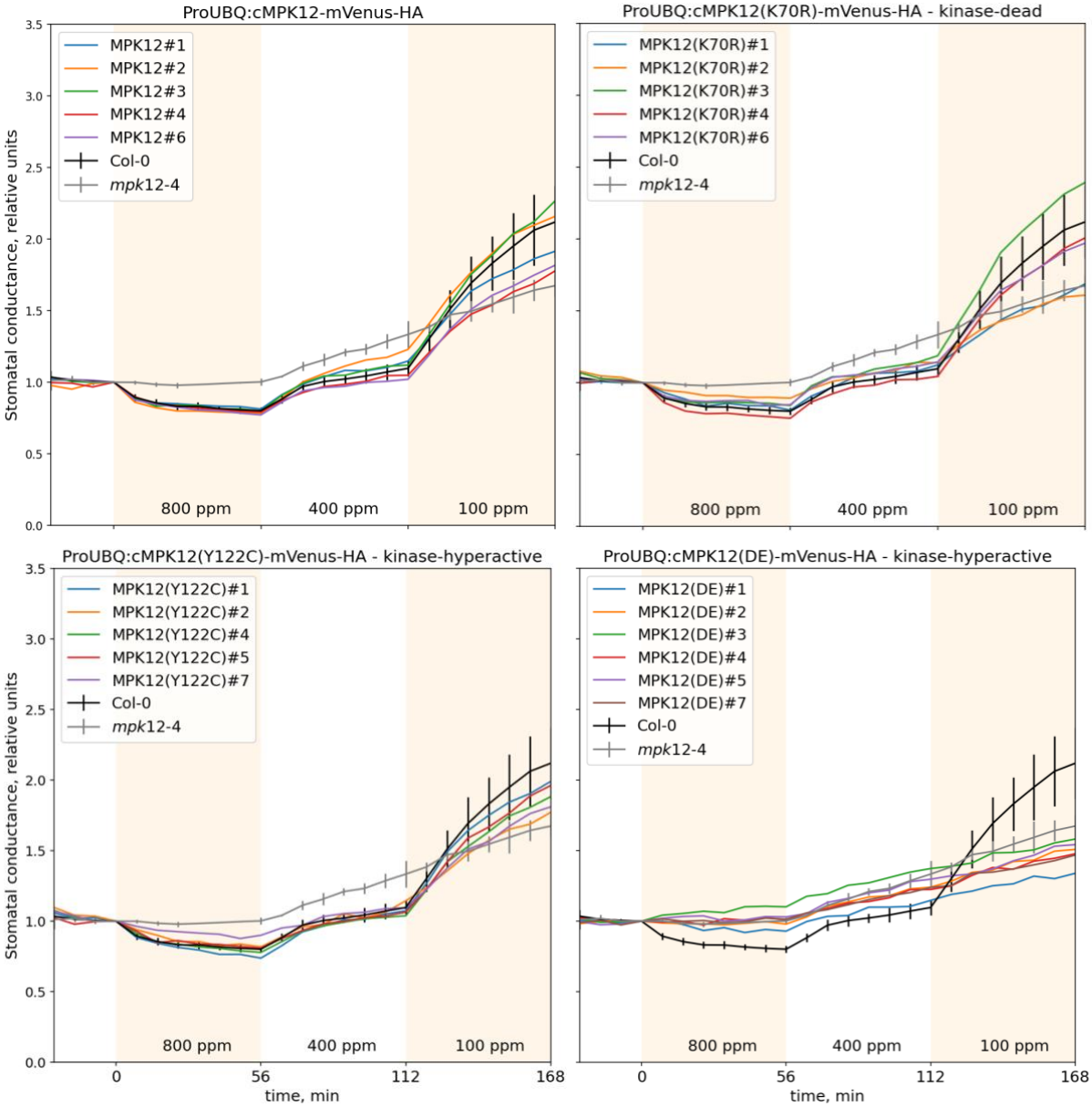
Supplementary Figure 3. Plant gas exchange phenotyping results proMPK12:gMPK12 with mVenus and HA-tag transformed to MPK12 deletion background *mpk12-4*. Independent lines time-resolved patterns of stomatal conductance during CO_2 shifts in absolute units is shown in absolute units.



Supplementary Figure 4. Plant gas exchange phenotyping results proMPK12:gMPK12 with mVenus and HA-tag transformed to MPK12 deletion background *mpk12-4*. Independent lines time-resolved patterns of stomatal conductance during CO_2 shifts in absolute units is shown in relative units.



Supplementary Figure 5. Plant gas exchange phenotyping results proUBQ10:cMPK12 with mVenus and HA-tag transformed to MPK12 deletion background *mpk12-4*. Independent lines time-resolved patterns of stomatal conductance during CO_2 shifts in absolute units is shown in absolute units.



Supplementary Figure 6. Plant gas exchange phenotyping results proUBQ:gMPK12 with mVenus and HA-tag transformed to MPK12 deletion background *mpk12-4*. Independent lines time-resolved patterns of stomatal conductance during CO_2 shifts in absolute units is shown in relative units.

Tumor inducing (*Ti*) plasmid sequence sequence PFAST-R_gMPK12/HygR_P19_pYSWb1 Kanamycin resistance gene Left border Cauliflower Mosaic Viral promoter:Hygromycin resistance gene: Cauliflower Mosaic Viral terminator MAS promoter:P19 gene silencing suppressor PFAST-R MPK12 promoter:genomic MPK12:MPK12 terminator Right border

G53R (guanine - > cytosine)

K70R (adenine - > guanine)

Y122C (adenine - > guanine)

D196 (adenine - > guanine)

E200 (adenine - > cytosine)

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T-DNA region sequence of PFAST-R_ProMPK12:gMPK12-mVenus-HA /HygR_P19_pYSWb1

Left border Cauliflower Mosaic Viral promoter:Hygromycin resistance gene: Cauliflower Mosaic Viral terminator MAS promoter:P19 gene silencing suppressor PFAST-R MPK12 promoter:genomic MPK12:MPK12 terminator mVenus HA-tag Right border

G53R (guanine - > cytosine)

K70R (adenine - > guanine)

Y122C (adenine - > guanine)

D196 (adenine - > guanine)

E200 (adenine - > cytosine)

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T-DNA region sequence of PFAST-R_ProUBQ:cMPK12-mVenus-HA /HygR_P19_pYSWb1

Left border Cauliflower Mosaic Viral promoter:Hygromycin resistance gene: Cauliflower Mosaic Viral terminator MAS promoter:P19 gene silencing suppressor PFAST-R Ubiquitin 10 promoter:complementory MPK12:MPK12 terminator mVenus HA-tag:Nopaline synthetase terminator Right border

G53R (guanine - > cytosine)

K70R (adenine - > guanine)

Y122C (adenine - > guanine)

D196 (adenine - > guanine)

E200 (adenine - > cytosine)

tggcaggatatattgtggtgtaaacaaattgacgcttagacaacttaataacacattgcggacgtttttaatgtactgaattaacgccgaattaattcggggg atctggattttagtactggattttggttttaggaattagaaat
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