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Author's contribution

I. I performed some of the experiments, analysed the data, visualized the results, and participated in the writing and editing of the manuscript.

II. I elaborated part of the methodology, performed some of the experiments, analysed and compiled the data, visualized the results, and participated in the writing and editing of the manuscript.

III. I participated in planning the screening method and the research, conducted the screen and most of the experiments, analysed the data, visualized the results, and wrote the manuscript.

ABBREVIATIONS

ABA	abscisic acid; a plant hormone associated with drought response
ABA1/ABA3	ABA DEFICIENT 1/ABA DEFICIENT 3; proteins involved in ABA biosynthesis
ABI1/ABI2	ABA INSENSITIVE 1/ABA INSENSITIVE 2; type 2C protein phosphatases involved in response to ABA
ALMT12/QUAC1	ALUMINUM-ACTIVATED MALATE TRANSPORTER 12/QUICK-ACTIVATING ANION CHANNEL 1; anion channel responsible for rapid type anion efflux from the guard cells
BDG	BODYGUARD; a protein involved in cuticle formation
cb	*cool breath*; a mutant library isolated from the new screen
$[CO_2]$	CO_2 concentration
C_i	intercellular $[CO_2]$; CO_2 concentration in the space between the cells
CPK21/CPK23	CALCIUM-DEPENDENT PROTEIN KINASE 21/ CALCIUM-DEPENDENT PROTEIN KINASE 23; proteins involved in SLAC1 activation
CYP707a1	CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 1; a protein responsible for ABA catabolism in guard cells
CYP707a3	CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 3; a protein responsible for ABA catabolism in vascular tissues
GFP	GREEN FLUORESCENT PROTEIN; reporter protein used to visualize the intracellular location of other proteins attached to it
GUS	β-glucuronidase; reporter enzyme used to visualize the intracellular location of other proteins attached to it
g_{st}	stomatal conductance; shows the amount of H_2O in moles that exits the plant through the stomata per one m^2 of leaf area per second
HAB1	HYPERSENSITIVE TO ABA 1; type 2C protein phosphatase involved in ABA response
HT1	HIGH LEAF TEMPERATURE 1; protein kinase that regulates stomatal responses to CO_2
MPK	MITOGEN-ACTIVATED PROTEIN KINASE
NIL	near-isogenic line; plant line obtained through repeated backcrossing between genetically distinct parental lines
OST1/SnRK2e	OPEN STOMATA 1/SUCROSE NONFERMENTING 1 (SNF1)-RELATED PROTEIN KINASE 2E; ABA-activated protein kinase

OST2/AHA1	OPEN STOMATA 2/ARABIDOPSIS H^+-ATPase; plasma membrane proton pump involved in stomatal opening
PP2C	type 2C protein phosphatase; protein phosphatases that function as negative regulators of ABA signalling
PYR/RCAR	PYRABACTIN RESISTANCE/REGULATORY COMPONENT OF ABA RECEPTOR; ABA receptor family
QTL	quantitative trait locus; a section of DNA that correlates with variation in a phenotype
RIL	recombinant inbred line; homozygous plant line obtained by repeated selfing of an F2 plant derived from a cross of two different parents
SLAC1	SLOW TYPE ANION CHANNEL 1; anion channel responsible for slow type efflux of NO_3^- and Cl^- from the guard cells
SLAH3	SLAC1 HOMOLOG 3; voltage-dependent nitrate-activated S-type anion channel responsible for efflux of NO_3^- and Cl^- from guard cells
WT	wild type
WUE	water use efficiency; ratio of assimilated CO_2 in µmoles to the water transpired in mmoles

INTRODUCTION

Land plants are crucial components of all terrestrial ecosystems on Earth. Thus, the colonization of land by plants was an essential event for the contemporary ecosystems. This was accompanied by a sequence of evolutionary adaptations necessary for plants to survive in a dry environment. The pathways of stomatal regulation and cuticle biosynthesis evolved during the same era in the early land plants, approximately 450 million years ago (Kenrick and Crane, 1997; Cui et al., 2016). These adaptations are also important nowadays for the survival of land plants and the maintenance of agricultural crop yields.

Stomata are microscopic pores surrounded by specialized cells called guard cells located in the aboveground epidermis of land plants. Accurate modification of the width of the stomatal aperture is the key for plant survival in response to stressors and environmental signals, such as drought, temperature, light, and pathogens. This delicate and precise regulation is achieved through different molecular pathways in the guard cells that involve various proteins, plant hormones such as abscisic acid (ABA) and secondary messengers. Molecular signals are eventually converted to changes in the volume of guard cells, which regulate the opening and closing of stomatal pores. The volume of guard cell can change in a few tens of minutes by rearranging the size of the vacuole. Hence, understanding the signalling pathways in the guard cells activated in response to different stimuli would allow us to design appropriate molecular targets that can be used to breed crops with improved water management.

The cuticle is a lipid-based water-repellent biopolymer, which covers all aboveground parts of plants. At night, when the stomata are closed, the cuticle is the main regulator of the plant gas exchange; during daytime, the cuticle restricts gas exchange to the stomata. The cuticle is also essential in other agricultural aspects, such as resistance to pathogens or fruit cracking. Cuticular permeability can be adjusted in response to different environmental factors. The major constituent of the cuticle is the cutin, which accounts for 40%–80% of the weight of the cuticle. Thus, understanding the mechanisms of biosynthesis and the assembly of cuticle would benefit crop breeding for increased drought tolerance or pathogen resistance.

The studies that form the basis of the current thesis cover research on both stomatal signalling and cuticle formation. The ABA receptors, together with the signalling proteins were already discovered a few years ago, but their role in determining whole-plant steady-state stomatal conductance, and regulating stomatal closure elicited by environmental factors was still unclear. Here the results of an extensive analysis of ABA-related mutants subjected to four different abiotic stimuli are presented. Moreover, this work investigates in depth the signalling pathway leading to high CO_2-induced stomatal closure and proposes a new model for CO_2 signalling in the guard cells involving MPK12. The cuticle-related BODYGUARD (BDG) belongs to the α/β-hydrolase fold protein superfamily, but its role in the cuticle formation was speculative. The current thesis adds new information related to the function of the BDG and its role in the biosynthesis of cutin monomers.

1. REVIEW OF THE LITERATURE

1.1. Transpiration and water use efficiency of plants

Coordination and regulation of gas exchange is of high importance to the survival of land plants and agricultural crop yields, because CO_2 must be enabled to penetrate the leaf to supply photosynthesis, yet water loss and transpiration should be minimized to avoid desiccation, drought stress, and death. Transpiration is at the same time necessary for the transport of water and nutrients from roots to shoots, and for cooling the plant. Plant water status and transpiration rate are largely dependent on the width of the stomatal aperture, but in drought conditions, when stomata are already closed, water permeability of the whole epidermal layer—covered by the lipid layer called the cuticle—becomes important. A number of other morphological traits also influences transpiration rate, such as the density and distribution of stomata, and leaf epidermal structure and internal organization. Changing stomatal apertures is a unique way of regulating plant water loss and is a dynamic and quick process.

Food security and increasing crop yield are currently one of the greatest global challenges. The major limitation of crop yield is water availability (Araus et al., 2002). In order to ensure sustainable agriculture, a reduction in crop water use is required, or in other words, we need to produce 'more crop per drop' (Morison et al., 2008). At leaf level, the ratio of CO_2 uptake to water loss (A/E) through stomata determines plant water use efficiency (WUE) (Lawson et al., 2014). Stomatal guard cells face challenges to find the balance between ensuring CO_2 uptake for Calvin cycle activity with the production of organic compounds, and guaranteeing the supply of water to the plant. Thus, one of the challenges of future scientific research is to improve plant WUE and survival in reduced water conditions, which among other things also requires fundamental research on stomatal regulation (Lawson et al., 2014).

1.2. Regulation of the stomatal aperture in plants

1.2.1. Ion movements during stomatal closure

Stomata close in response to darkness, reduced air humidity, pathogen attack, high [CO_2], and air pollutants such as ozone. Stomata open in response to light, elevated air humidity, and low [CO_2]. The size of a stomatal pore is directly dependent on the turgor pressure and volume of the guard cell vacuole (Fischer, 1973). This is controlled by regulating ion fluxes through different types of channels and transporters in the vacuolar tonoplast and the guard cell plasma membrane (figure 1). Regulation of ion fluxes is possible owing to the lack of functional plasmodesmata in mature guard cells (Oparka and Roberts, 2001).

When open, ion channels facilitate rapid ion diffusion down electrical and concentration gradients. They are either inactive or activated through a conformational change. Ion transporters—including pumps—however, move ions

against the gradient by consuming ATP energy (Gadsby, 2009). Compared to one-gated channels, ion pumps need two gates, one at either side of the membrane, which function alternatingly like the water-locks on the canals of Amsterdam (Gadsby, 2009). Consequently, ion pumps transport ions several orders of magnitude slower than channels (Gadsby, 2009).

Stomatal closure is mainly accompanied by different stimulus-activated one-gated channels. Among them, plasma membrane anion channels have an essential role in stomatal closure, as several closing signals were shown to alter the activity of these anion channels (Roelfsema et al., 2012). There are rapid- (R-) and slow- (S-) type anion efflux channels that are differentiated by their kinetic properties (Schroeder and Keller, 1992). R-type channels are activated within milliseconds by depolarization, they deactivate rapidly at hyperpolarized potentials and inactivate over time (within minutes) (Keller et al., 1989; Hedrich et al., 1990; Schroeder and Keller, 1992). The activation of S-type channels occurs from seconds up to a minute, and they lack time-dependent inactivation (Linder and Raschke, 1992; Schroeder and Keller, 1992). R-type anion channels are permeable to NO_3^-, Cl^-, SO_4^{2-}, and malate (Keller et al., 1989; Hedrich and Marten, 1993; Frachisse et al., 1999; Pei et al., 2000; Roberts, 2006). S-type anion channels are also permeable to several anions: NO_3^- permeates 20 times quicker than Cl^- and malate permeates four times slower than Cl^- (Schmidt and Schroeder, 1994).

Stomatal closure requires anion efflux by S-type anion channels, such as SLAC1 and SLAH3 (Negi et al., 2008; Vahisalu et al., 2008), and R-type anion channels such as QUAC1/AtALMT12 (Meyer et al., 2010), which depolarize the guard cell plasma membrane. Change in polarization leads to the activation of outward-rectifying K^+ channels, such as GORK1 (Schroeder et al., 1987; Ache et al., 2000; Hosy et al., 2003). The flow of cations and anions out of guard cells leads to a decrease in osmotic potential and drives water out of the cell through PIP-type aquaporins (Groszmann et al., 2016). Shrinkage of the guard cells results in closure of the stomatal pore (figure 1).

S-type anion channels provide a central molecular mechanism to control stomatal closing via long-term anion efflux and depolarization (Schroeder and Keller, 1992). The S-type anion channel SLAC1 has an essential role in stomatal closure in response to environmental and endogenous signals (Negi et al., 2008; Vahisalu et al., 2008). SLAH3 is another S-type anion channel expressed in guard cells (Geiger et al., 2011). Both SLAC1 and SLAH3 are 8–20 times more permeable to NO_3^- than to Cl^- (Geiger et al., 2009; Lee et al., 2009b; Geiger et al., 2011). However, due to the higher permeability to NO_3^- of SLAH3 than SLAC1, and the reduced anion currents of *slah3-1* in nitrate-based media compared to WT-like currents in *slac1-3* (Geiger et al., 2011), SLAH3 is especially important for the efflux of NO_3^-, whereas SLAC1 is considered to be primarily important for the release of Cl^- (Kollist et al., 2014). The efflux of $malate^{2-}$ via slow-type anion channels is still unclear.

The rapid-type channel QUAC1 is located in the plasma membrane of guard cells and transports malate and sulphate anions out of them (Meyer et al., 2010;

Sasaki et al., 2010). QUAC1 channel activity is malate-sensitive and voltage-dependent (Meyer et al., 2010). In *Vicia faba*, the R-type currents were also shown to activate in response to extracellular malate (Hedrich and Marten, 1993). The remaining R-type currents in the *quac1* mutant may also indicate that other members of the ALMT family encode additional QUACs (Meyer et al., 2010; Hedrich, 2012). The molecular mechanism of R-type nitrate and chloride efflux remains unrevealed.

Potassium transport across the plasma membrane depends on the K^+ concentration gradient and on the electrical potential of the plasma membrane. In *Arabidopsis thaliana*, it is facilitated by outward-rectifying K^+ channels such as GORK1, which belongs to the Shaker-like protein family (Ache et al., 2000; Hosy et al., 2003). However, it was recently suggested that KUP6 and KUP8 from the HAK/KUP potassium transporter family could provide an additional mechanism for potassium efflux from the guard cells, regulated by ABA signalling via OST1 (Osakabe et al., 2013). Based on the phenotype of the *gork/kup6/kup8* triple mutant, it is likely that KUP6, KUP8, and GORK1 act in parallel to mediate K^+ efflux from the guard cells.

As the plasma membrane is rather impermeable to water, and since mature guard cells have non-functional plasmodesmata (Oparka and Roberts, 2001), aquaporins provide the channel through which most water moves (Groszmann et al., 2016). PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1) was recently identified as the key aquaporin that mediates water efflux during ABA-induced stomatal closure (Grondin et al., 2015). However, Wang and colleagues showed that mutating PIP2;1 alone was not sufficient to impair ABA-induced stomatal closure (Wang et al., 2016b). As some PIPs are hetero-tetrameric and there are 13 PIPs in the Arabidopsis genome (Quigley et al., 2001; Groszmann et al., 2016), it is plausible that there is functional redundancy among PIPs, and several genes would need to be knocked-out for a detectable phenotype.

Oscillations of cytosolic free Ca^{2+} are also accompanied by stomatal closure induced by various stimuli (Schroeder and Hagiwara, 1990; Grabov and Blatt, 1998; Pei et al., 2000; Roelfsema and Hedrich, 2010). The generation of $[Ca^{2+}]_{cyt}$ oscillations or so-called Ca^{2+}-induced Ca^{2+} release is a result of both Ca^{2+} influx through the plasma membrane Ca^{2+}-permeable channels, and the release of Ca^{2+} from endomembrane compartments, such as the endoplasmic reticulum (ER), vacuole, and plastids (Hetherington and Brownlee, 2004; Roelfsema and Hedrich, 2010). $[Ca^{2+}]_{cyt}$ oscillations affect stomatal closure in two ways: (1) the 'calcium reactive' way, which causes rapid, but short-term closure, followed by re-opening in response to the initial elevation of $[Ca^{2+}]_{cyt}$; (2) the long-term 'calcium programmed' way, which decreases the steady-state aperture depending on the frequency, duration, and amplitude of the $[Ca^{2+}]_{cyt}$ oscillations (Allen et al., 2001). Unfortunately, despite extensive mutant screenings and other studies, no hyperpolarization-activated calcium channel, and therefore the exact mechanisms, have to date been identified.

In addition, the activity of the plasma membrane H^+-ATPases is inhibited during ABA-induced stomatal closure, presumably to suppress the stomatal

opening pathway (Roelfsema et al., 1998). During stomatal closure, the inward-rectifying K^+ channels are inhibited by plasma membrane depolarization, and Ca^{2+} increases (Grabov and Blatt, 1999). For example, activity of the inward-rectifying potassium channel KAT1 is reduced by depolarization of the plasma membrane, and actively inhibited even more via phosphorylation by OST1 (Sato et al., 2009).

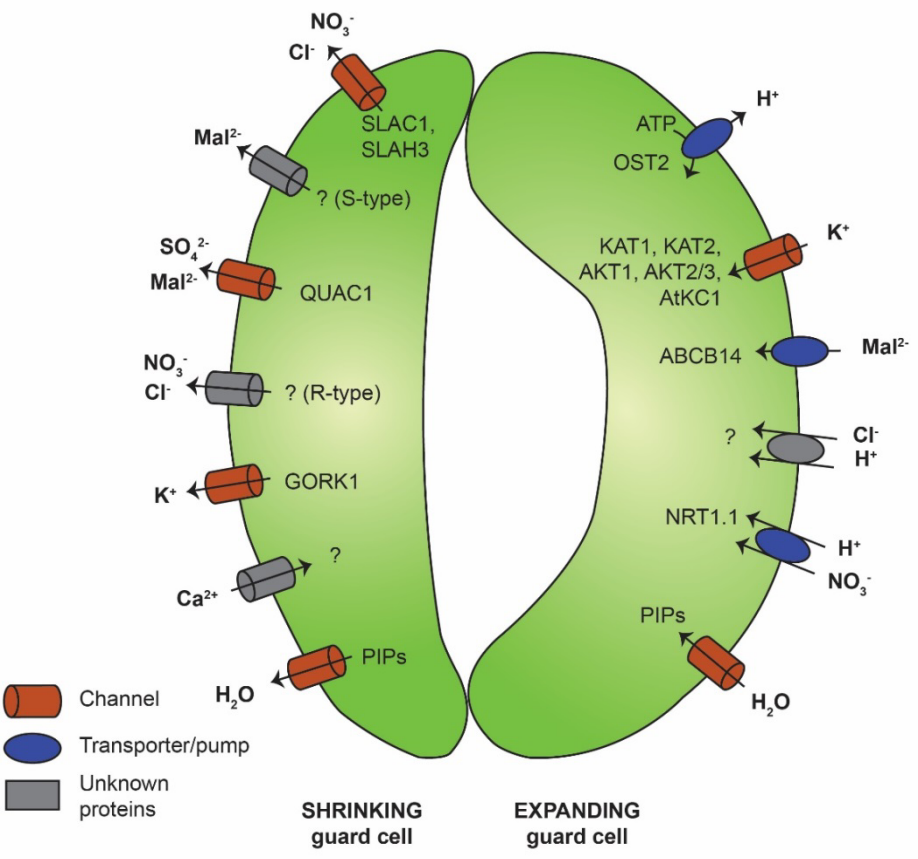


Figure 1. Overview of transport proteins identified or physiologically proposed to function in the guard cell plasma membrane during stomatal closing (left) or opening (right). (Left) Slow-type anion channels SLAC1 and SLAH3 allow the exit of chloride and nitrate. The voltage-dependent rapid-type channel QUAC1 mediates malate and sulphate efflux. The outward-rectifying K^+ channel GORK1 is sensitive to cytosolic alkalization and enables the efflux of potassium. A decrease in osmotic potential leads to the efflux of water through PIP-type aquaporins. (Right) When H^+-ATPase AHA1/OST2 is activated, the efflux of H^+ causes hyperpolarization of the guard cell membrane, which in turn activates hetero-tetrameric inward-rectifying K^+ channels. Potassium influx is counterbalanced by anion influx of malate^{2-} (ABCB14), NO_3^- (NRT1.1), and Cl^- transporters.

1.2.2. Ion movements during stomatal opening

During stomatal opening, ions move into guard cells against the concentration gradient, thus mostly requiring the activity of pumps and other transporters (figure 1). Stomatal opening is launched by the hyperpolarization of guard cell membrane potential generated by the active H^+-ATPases, and hence the extrusion of H^+ ions from the guard cells. The proton pump OST2 is activated by phosphorylation (Kinoshita and Shimazaki, 1999). Hyperpolarization of the plasma membrane activates voltage-regulated inward-rectifying potassium channels (Schroeder et al., 1987; Roelfsema et al., 2001), which are hetero-tetrameric channels composed of different members of the Shaker family (Wang and Wu, 2013).

K^+ ions are counterbalanced with anions, such as malate^{2-}, NO_3^-, and Cl^- (figure 1). Malate^{2-} is the major anionic species that accumulates during stomatal opening, either as a product of starch breakdown in the guard cells (Pandey et al., 2007), or in response to influx of malate^{2-} from the apoplast via the malate transporter ABCB14 (Lee et al., 2008). Uptake of NO_3^- mediated by the channel AtNRT1.1 (CHL1) (Guo et al., 2003), and uptake of Cl^- by a yet unknown protein, likewise contribute to the increase in intracellular solute content. The flow of cations and anions into the guard cells leads to an increase in osmotic potential and thus induces water flow into the cell through PIP-type aquaporins (Groszmann et al., 2016). Swelling of the guard cells results in opening of the stomatal pore.

Generally high K^+ and Ca^{2+} concentrations act antagonistically, thus if stomatal opening is stimulated by K^+, it should be inhibited by Ca^{2+} (Roelfsema and Hedrich, 2005). The molecular regulators of intracellular Ca^{2+} during stomatal opening remain unrevealed.

1.2.3. Stomatal signalling induced by ABA

ABA is an organic molecule found in organisms from all kingdoms of life except Archaea (Hartung, 2010; Hauser et al., 2011). In plants it is a hormone related to signalling of stress responses—for example of drought—in humans of inflammation, and in some parasitic protists for the exit from the host cell (Bruzzone et al., 2007; Nagamune et al., 2008; Hauser et al., 2011). The hormone also regulates stomatal movements in response to abiotic and biotic stimuli. Although a vast amount of research has already been conducted on ABA, and during the last decades many molecular components involved in regulating stomatal movements have been identified (Assmann and Jegla, 2016), the interconnection between ABA and different stressors in stomatal signalling remains vague.

Cellular ABA levels fluctuate constantly, enabling plants to adjust to changing environmental conditions and physiological needs. For example, under stress conditions ABA levels increase in several tissues of higher plants. The primary source of ABA is *de novo* synthesis (Nambara and Marion-Poll,

2005). In addition, leaves are supported with ABA via long-distance transport from roots in the form of free ABA, as well as in the physiologically inactive form of ABA as ABA-glucose esters (Sauter and Hartung, 2000; Hartung et al., 2002). However, in guard cells, local production of ABA is possible and might be sufficient to facilitate stomatal movements (Bauer et al., 2013). The level of biologically active ABA can be decreased via two catabolic pathways: degradative hydroxylation (three different pathways); and conjugation with glucose (Nambara and Marion-Poll, 2005; Okamoto et al., 2009). Conjugated ABA could be reactivated again by hydrolysing ABA-glucose esters and thereby contributing to the active ABA pool (Lee et al., 2006).

In the guard cells, the ABA molecules are first recognised by the ABA receptors PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) (figure 2; Ma et al., 2009; Park et al., 2009). Thereafter a complex of three molecules is formed – ABA, PYR/RCAR and TYPE 2C PROTEIN PHOSPHATASES (PP2C), which leads to inhibition of PP2C phosphatase activity (Ma et al., 2009; Park et al., 2009), and enables activation of positive regulators such as OPEN STOMATA 1 (OST1), Ca^{2+}-dependent kinases CPKs, and Ca^{2+} sensor-kinase modules CALCINEURIN B–LIKE PROTEIN/CBL INTERACTING PROTEIN KINASE (CBL/CIPK) (Geiger et al., 2009; Lee et al., 2009b; Umezawa et al., 2009; Geiger et al., 2010; Maierhofer et al., 2014). Activated OST1, CPKs, and CBL/CIPKs in turn target further downstream components, among them being the S-type anion channels SLAC1 and SLAH3, and the R-type anion channel QUAC1 (Geiger et al., 2009; Lee et al., 2009b; Imes et al., 2013; Edel and Kudla, 2016).

There are 14 members in the PYR/RCAR protein family in Arabidopsis. Depending on the oligomeric state there are at least two subclasses of PYR/RCARs: dimers (PYR1, PYL1, PYL2, and possibly PYL3) and monomers (e.g. PYL4, PYL5, PYL6, PYL8, PYL9, PYL10) (Nishimura et al., 2009; Yin et al., 2009; Hao et al., 2011). At least six (PYR1, PYL1/2/3/4/12) of the 14 PYR/RCARs interacted with PP2Cs in the ABA-dependent manner in yeast (Park et al., 2009). Additionally, PYL1/5/8/10 were also shown to block both ABI1 and ABI2 activity *in vitro* in an ABA-dependent manner (Ma et al., 2009). However, the *in vitro* experiments of Hao and colleagues showed that out of 10 tested PYR/RCARs, five (PYR1, PYL1/2/3/4) inhibited PP2C phosphatase activity in ABA-dependent manner, whereas the rest (PYL5/6/8/9/10) inhibited phosphatase activity to certain extent also in the absence of ABA (Hao et al., 2011). Recently it was shown that the ABA-independent activity might have been induced by BSA (bovine serum albumin) in the reaction mixture and that elimination of BSA from the reaction mixture elicited ABA-dependent inhibition of PP2C activity also by PYL10 (Li et al., 2015). As the single mutants do not show any phenotypes, it is likely that there is large functional redundancy among PYR/RCAR proteins (Park et al., 2009). Accordingly, the highest steady-state stomatal conductance and strongest ABA insensitivity was observed in the mutant with six PYR/RCARs knocked out (Gonzalez-Guzman et al., 2012).

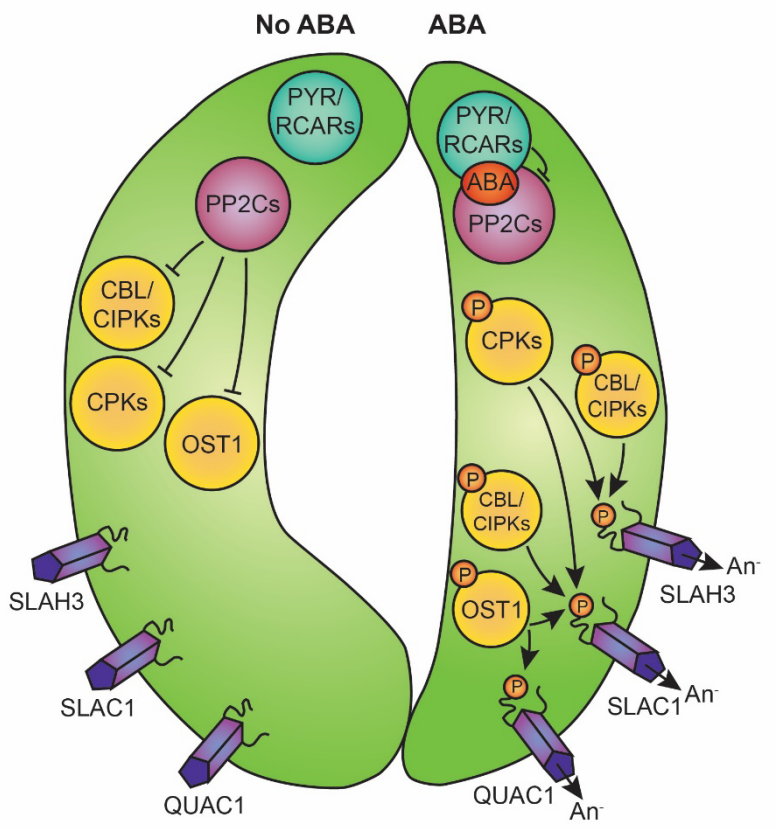


Figure 2. Functioning of the ABA signalosome in guard cells in the absence (left guard cell) and presence (right guard cell) of ABA. (Left) In the absence of ABA, ABA receptors PYR/RCARs and PP2Cs do not interact (Ma et al., 2009; Park et al., 2009; Yin et al., 2009). PP2Cs are active and deactivate their downstream targets OST1, CPKs, and CIPKs, which in turn also keeps the anion channels SLAC1, SLAH3, and QUAC1 unphosphorylated and closed. (Right) ABA molecules are first perceived by the ABA receptors PYR/RCARs (Ma et al., 2009; Park et al., 2009). The formation of a complex between ABA, PYR/RCARs, and PP2Cs leads to the inhibition of PP2C phosphatase activity. As a consequence, the downstream targets of PP2Cs, such as OST1, CPKs, and CIPKs can be activated. The phosphorylation targets of OST1 are SLAC1 (Geiger et al., 2009; Lee et al., 2009b; Umezawa et al., 2009) and QUAC1 (Imes et al., 2013). CPK21 phosphorylates both SLAC1 and SLAH3 (Geiger et al., 2010; Geiger et al., 2011). CBL/CIPK complexes are also able to activate both SLAC1 and SLAH3 (Maierhofer et al., 2014). Phosphorylated anion channels SLAC1, SLAH3, and QUAC1 drive the efflux of anions out of the guard cell and trigger stomatal closure.

PP2Cs are the type 2C protein phosphatases. Six out of nine proteins in the group A of PP2C family (ABI1, ABI2, HAB1/AtP2C-HA, HAB2, AHG1, PP2CA/AHG3) function as negative regulators of ABA signalling (Gosti et al., 1999; Merlot et al., 2001; Saez et al., 2004; Kuhn et al., 2006; Yoshida et al.,

2006; Nishimura et al., 2007). The ABA binding to the PYR/RCAR receptors creates a surface that enables the receptors to interact with and inhibit the PP2C active site (Melcher et al., 2009; Yin et al., 2009). The single knock out mutants of ABI1 and ABI2 showed hardly any difference from WT in ABA-induced stomatal closure, however, the double mutant exhibited considerably more closed stomata in response to ABA (Merlot et al., 2001). On the other hand, enhanced ABA-induced stomatal closure could be observed already in the *hab1* single mutant (Saez et al., 2006). The mutants of PP2CA showed hypersensitivity to ABA-induced stomatal closure only at low ABA concentrations, whereas overexpression of PP2CA caused strong ABA insensitivity (Kuhn et al., 2006). The triple mutant *hab1-1 abi1-2 abi2-2* showed extreme hypersensitivity to exogenous ABA in guard cells (Rubio et al., 2009).

The protein kinases of the SNF1-RELATED PROTEIN KINASE 2 (SnRK2)-type family are only found in plants and there are 10 such kinases encoded in Arabidopsis (Hrabak et al., 2003). ABA induces strong activation of SnRK2.2/SRK2D, SnRK2.3/SRK2I and SnRK2.6/SRK2E/OST1, and slight activation of SnRK2.7 and SnRK2.8 was also observed (Boudsocq et al., 2004). ABA-mediated activation of SnRK2 kinases was shown to take place through autophosphorylation (Belin et al., 2006; Ng et al., 2011). Recently it was revealed that OST1 is able to homo- and heteromerize with itself as well as with SnRK2.2, SnRK2.3, and SnRK2.8 (Waadt et al., 2015). There are several substrates and downstream targets for SnRK2-s; to name some, anion channels SLAC1 (Geiger et al., 2009; Lee et al., 2009b), QUAC1 (Imes et al., 2013) and KAT1 (Sato et al., 2009), K^+ uptake transporter KUP6 (Osakabe et al., 2013), PP2As (Waadt et al., 2015) and PIP2;1 (Grondin et al., 2015).

One of the targets of OST1 is SLOW ANION CHANNEL1 (SLAC1). SLAC1 is opened by phosphorylation, which induces an efflux of anions from the guard cells (Geiger et al., 2009; Lee et al., 2009b; Vahisalu et al., 2010). The stomata of *slac1* were insensitive to ABA and activation of S-type anion currents by ABA was very weak in the mutant (Vahisalu et al., 2008). Together with SLAC1, another S-type anion channel SLAH3 is also responsible for anion efflux during stomatal closure. SLAH3 is activated by nitrate ions and by protein kinase CPK21, but not by OST1 as shown by the heterologous expression system in *Xenopus laevis* oocytes (Geiger et al., 2011).

Externally applied ABA induces Ca^{2+} oscillations in the guard cells of several plant species, such as *V. faba* (Schroeder and Hagiwara, 1990), *Nicotiana tabacum* (Marten et al., 2007), and Arabidopsis (Allen et al., 1999; Allen et al., 2001). Arabidopsis guard cells impaired in $[Ca^{2+}]_{cyt}$ oscillations induced by Ca^{2+}-chelator BAPTA exhibit only 30% of the normal ABA-induced stomatal closure (Siegel et al., 2009). These results indicate the importance of $[Ca^{2+}]_{cyt}$ in ABA-induced stomatal closure. A rise of $[Ca^{2+}]_{cyt}$ from resting to elevated levels alone triggers neither full activation of ion channels nor stomatal response (Siegel et al., 2009). However, when ABA is present, guard cells are sensitive to changes in their internal concentration of Ca^{2+} ions, so that they can activate SLAC1 by stimulating e.g. calcium-dependent protein kinases (CDPKs

or CPKs) (Brandt et al., 2015). CPKs combine a kinase domain with a Ca^{2+} sensor domain in one protein. An additional class of Ca^{2+}-regulated kinases—CBLs—are Ca^{2+} sensors that can specifically interact with and activate a family of kinases termed CIPKs. The interdependence of ABA and Ca^{2+} signalling is supported by the fact that proteins involved in either pathway share common targets, e.g. SLAH3 is activated by CPK21 and CBL1/9+CIPK23, but de-activated by ABI1 (Geiger et al., 2011; Maierhofer et al., 2014), or SLAC1 is activated by CPK5, CPK6, CPK21, and CPK23, but deactivated by ABI1, ABI2, and PP2CA (Brandt et al., 2015).

Heterologous expression of SLAC1 and any of the Ca^{2+}-regulated kinases in *X. laevis* oocytes showed that SLAC1 can be activated independently by CPK3 (Scherzer et al., 2012), CPK5 (Brandt et al., 2015), CPK6 (Brandt et al., 2012; Scherzer et al., 2012), CPK21 (Geiger et al., 2010), CPK23 (Geiger et al., 2010), and by the CBL1/9+CIPK23 complexes (Maierhofer et al., 2014). Additionally, CPK11 is highly expressed in guard cells and involved in ABA responses (Zhu et al., 2007; Geiger et al., 2010). However, the SLAC1 activation mechanism *in planta* appears to be more complex, for instance, the quadruple mutant of CPKs *cpk5/6/11/23* exhibited no SLAC1 activity, suggesting that the activation of the anion channel *in planta* requires both functional SnRKs as well as functional CPKs (Brandt et al., 2015). Accordingly, it was shown that for the full activation of SLAC1, phosphorylation of the site S59 by CPKs and S120 by OST1 are both needed (Brandt et al., 2015).

Another kinase, the GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1) was shown to be involved in stomatal ABA signalling (Hua et al., 2012). The *ghr1* mutant exhibited insensitivity even to 20 μM ABA, indicating the essentiality of this receptor-like kinase in ABA-induced stomatal closure (Hua et al., 2012). GHR1 was shown to interact with, to phosphorylate and to activate SLAC1 (Hua et al., 2012). However, the *in vitro* phosphorylation of SLAC1 N-terminus by GHR1 was independent of exogenously added ABA, suggesting that other partners are needed for ABA activation of GHR1 or that *in planta* GHR1 is inhibited by a yet unknown mechanism in the absence of ABA (Hua et al., 2012).

Coexpression of PIP2;1 and OST1 in the *X. laevis* oocyte enhanced water transport activity, suggesting ABA-dependent regulation of the aquaporin (Grondin et al., 2015). Surprisingly, expression of the phosphomimetic PIP2;1 S121D in *pip2;1-2* mutant exhibited elevated basal water permeability, yet ABA-induced stomatal closure was restored to the level of WT (Grondin et al., 2015).

As extracellular ABA triggers efflux of malate from the epidermal strips and thereafter stomatal closure (Van Kirk and Raschke, 1978), the role of apoplastic malate in ABA-induced stomatal closure cannot be underestimated. The mechanisms of malate transport will be discussed later in the chapters on CO_2-induced stomatal closure and opening.

1.2.4. High CO_2-induced stomatal signalling

High CO_2 concentration inside the leaf is an indication of excess of photosynthetic substrate and therefore induces stomata to close (Raschke, 1979). ABA and higher than ambient [CO_2] provoke similar ion transport mechanisms, however, the extent to which the signalling pathways of ABA and high CO_2 overlap is still under debate (Assmann and Jegla, 2016). To date several CO_2-specific regulators involved in stomatal movements of Arabidopsis have already been identified, however, the full signalling pathway is still to be elucidated.

There are different hypotheses regarding how CO_2 is sensed and signalled to modulate stomatal aperture: (1) directly mediated by CO_2 sensing in the guard cells, (2) indirectly mediated by mesophyll cells, e.g. via a signalling molecule such as malate, sucrose or ABA, or via regulating C_i (Mott, 2009; Lawson et al., 2014; Engineer et al., 2016). The direct role of guard cells in CO_2 sensing is supported by the results, where stomatal CO_2 responses were observed in isolated epidermises (Young et al., 2006). However, other studies implicate mesophyll cells also playing an important role in CO_2 sensing, mainly on CO_2 response of isolated epidermises not being as pronounced as in intact leaves (Mott, 2009). Most probably the two pathways converge at some point. The current thesis will concentrate on the direct regulation of guard cells by CO_2.

Although CO_2 should be able to penetrate lipid bilayers by diffusion, it is expected that due to the high protein and sterol content of the plasma membrane, specific channels for CO_2 might be required (Boron et al., 2011). CO_2 enters the guard cells presumably through the PIP2;1 aquaporin (Wang et al., 2016b). Some Arabidopsis PIPs—including PIP2;1—are permeable for both water and CO_2; this is achieved in a way that CO_2 follows three specific routes to cross the tetrameric aquaporin complex (Wang et al., 2007), whereas water moves only through the central pores of the monomers (Groszmann et al., 2016; Zhao et al., 2016).

The conversion of CO_2 and water to bicarbonate HCO_3^- and hydrogen ions is catalysed by carbonic anhydrases βCA4 and βCA1 (Hu et al., 2010; Hu et al., 2015). Recently it was shown that the plasma membrane-localized βCA4 can interact with PIP2;1 aquaporin (Wang et al., 2016b), which might be needed to enhance CO_2 transport by quickly assimilating it and creating a concentration gradient immediately adjacent to the aquaporin (Groszmann et al., 2016). The role of βCA1 in guard cell chloroplasts with regard to CO_2 response and photosynthesis is currently unclear.

Bicarbonate is most probably the intracellular signalling molecule that reflects the changes in the intercellular CO_2 concentration for the guard cells. Tian and co-workers have proposed that RESISTANT TO HIGH CO_2 1 (RHC1) could function as the bicarbonate-sensing component, which is able to interact with HIGH LEAF TEMPERATURE1 (HT1) (Tian et al., 2015). The protein kinase HT1 has a central role in guard cell CO_2 responses as a negative regulator (Hashimoto et al., 2006). Mutant analysis of *ht1* showed that the

kinase is important for stomatal regulation in response to CO_2, but less to ABA and light (Hashimoto et al., 2006).

The mutants of OST1 and SLAC1 both also have strongly impaired stomatal response to high CO_2, indicating the importance of OST1 and SLAC1 in CO_2-induced stomatal closure (Vahisalu et al., 2008; Xue et al., 2011). The rapid-type anion channel QUAC1 is activated by OST1 and malate (Meyer et al., 2010; Imes et al., 2013), and its mutants were defective in high CO_2-induced stomatal closure, as well as in ABA and darkness responses (Meyer et al., 2010; Sasaki et al., 2010). This suggests that QUAC1 is involved in both CO_2 and ABA signalling in the guard cells. However, the coordination of anion efflux via SLAC1 and QUAC1 in response to high CO_2 requires further clarification.

The mutants of the malate importer ABCB14 showed accelerated stomatal closure induced by high CO_2 (Lee et al., 2008). It was concluded that ABCB14 is involved in high CO_2-induced stomatal closure through recycling of malate and negatively affects stomatal closure rate (Lee et al., 2008). Thus, the absence of functional ABCB14 could presumably promote higher accumulation of malate in the apoplast, which in turn induces the accelerated stomatal closure. Further research is needed though to elucidate the range of transported substrates and the activating mechanisms of ABCB14.

Similarly to ABA, CO_2 induces an increase in cytosolic Ca^{2+} concentration (Webb et al., 1996). CO_2-induced stomatal closure was strongly inhibited in the presence of Ca^{2+}-chelators BAPTA or EGTA, indicating that stomatal closure in response to high CO_2 is strongly Ca^{2+}-dependent (Webb et al., 1996; Hubbard et al., 2012). However, Young and colleagues showed that high CO_2 partly suppressed or slowed Ca^{2+} oscillations, and suggested a model that CO_2 concentration primes the Ca^{2+} sensors by switching them from an inactivated state to an enhanced Ca^{2+}-responsive 'primed' state (Young et al., 2006). Unexpectedly, high CO_2-induced dampening of the Ca^{2+} oscillation rate was not as clear in Arabidopsis Columbia-0 accession compared to the Landsberg accession (Engineer et al., 2016). This was explained by weaker stomatal closing in response to high CO_2 in Col-0 guard cells relative to L*er* accession (Engineer et al., 2016).

1.2.5. Low CO_2-induced stomatal signalling

Reduction of CO_2 inside the leaf is a signal of a lack of substrate for photosynthesis and activates stomatal opening to let more atmospheric CO_2 into the leaf (Raschke, 1979). Whether low CO_2-induced stomatal signalling follows the same pathway to some extent as light-induced stomatal opening is currently not known.

Photosynthesis in the mesophyll reduces intercellular CO_2 concentration and it has been debated whether stomatal opening induced by red light is actually a CO_2 response rather than a light response (Lawson et al., 2014). However, stomatal response to red light was still observed in *N. tabacum* when C_i was held constant (Lawson et al., 2008). Recently, it was shown that the stomatal

opening kinetics of the Arabidopsis *ht1* mutants and the double mutant of the carbonic anhydrases βCA1 and βCA4 are partly impaired in red light and strongly affected in low CO_2 (Matrosova et al., 2015). These results support a model of a C_i-dependent and a C_i-independent component of red light induced stomatal opening in plants.

Similar to high CO_2-induced stomatal closure, carbonic anhydrases βCA1 and βCA4 seem to be required during low CO_2-induced stomatal opening, as the double mutant *ca1 ca4* was impaired in these responses (Hu et al., 2010). Since HT1 is the central component of stomatal CO_2 signalling, it is not surprising that the *ht1-2* mutant lacks the ability to open in response to low [CO_2] (Hashimoto et al., 2006).

Another mutant with impaired low CO_2-induced stomatal opening is *patrol1*, which is defective in the Munc13-like protein PATROL1 (Hashimoto-Sugimoto et al., 2013). PATROL1 is located in the endosomes, it affects the plasma membrane targeting of H^+-ATPase OST2, and it might be involved in intracellular membrane trafficking. However, the *ost2-1D* point mutant displayed clear stomatal opening in response to CO_2-free air bubbled through a solution of epidermal strips (Merlot et al., 2007).

The amount of nitrate anions in the guard cells increases threefold during stomatal opening in Arabidopsis (Guo et al., 2003). Plants lacking CHLORINA 1/NITRATE TRANSPORTER 1.1 (CHL1/NRT1.1) showed reduced nitrate accumulation in the guard cells, 40% narrower day-time stomatal apertures, and decreased stomatal opening in response to CO_2-free air in darkness (Guo et al., 2003). Interestingly, CIPK23 involved in stomatal Ca^{2+} signalling is able to phosphorylate NRT1.1, which results in a change in affinity of the transporter towards nitrate (Ho et al., 2009). Additionally, NRT1.1 is involved in root nitrate uptake (Tsay et al., 1993). Thus, NRT1.1 seems to be an important nitrate importer during low CO_2-induced stomatal opening, but the exact signalling cascade resulting in NRT1.1 activity is yet to be elucidated.

Low CO_2 also induces rapid repetitive Ca^{2+} oscillations in guard cells during stomatal opening, which was suggested to produce a molecular signal that does not permit strong Ca^{2+} reactive stomatal closure to be initiated (Young et al., 2006).

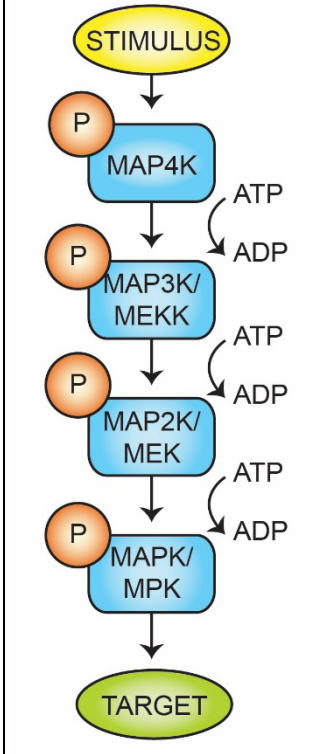
Malate also accumulates in the guard cells during stomatal opening. It was suggested that besides negatively regulating CO_2-induced stomatal closure, where ABCB14 is transporting malate from the apoplast into guard cells and thereby increasing osmotic pressure, it could also participate in facilitating stomatal opening (Lee et al., 2008).

1.2.6. Involvement of the MAPK cascade in stomatal signalling

Mitogen-activated protein kinase (MAPK) cascades can be found in all eukaryotes. In plants MAPK cascades play crucial roles, among others, in developmental processes, pathogen signalling, and hormonal regulation (Lee et al., 2016). It is estimated that about 10% of all plant kinases are involved in MAPK

pathways (Colcombet and Hirt, 2008). MAPK cascades are composed of at least three kinases: MAPK kinase kinases (MAP3Ks), MAPK kinases (MAP2Ks or MKKs), and MAPKs (or MPKs). Occasionally the MAPK module could be expanded by including specific activators of MAP3Ks called MAPKKKKs or MAP4Ks (Champion et al., 2004). These proteins are highly conserved in all eukaryotes. Canonically the signal perceived by receptors is first transduced to MAP3Ks, which subsequently leads to phosphorylation events. MAP3Ks are Ser/Thr kinases and phosphorylate MKKs on two Ser/Thr residues (S/T-XXXXX-S/T). MKKs are dual-specificity protein kinases, which can phosphorylate MPKs on Thr and/or Tyr residues in the TXY motif. Phosphorylated MPKs forward the signals to various effector molecules, such as transcription factors and other kinases. As depicted in table 1, there are 10 putative MAP4Ks, 80–85 putative MAP3Ks, 10 MKKs, and 20 MPKs in the Arabidopsis genome (MAPK Group, 2002; Champion et al., 2004; Menges et al., 2008; Andreasson and Ellis, 2010; Zulawski et al., 2014; Lee et al., 2016). The involvement of MAPK signalling cascades in stomatal movements will be discussed hereafter.

Table 1. Schematic model of the MAPK signalling cascade, together with the respective pathway components in Arabidopsis. Guard cell related genes were chosen based on the ATH1 GeneChip microarray data (Yang et al., 2008).



Pathway component	No. of genes in Arabidopsis	Guard cell-related genes
MAP4K or MAPKKKK	10	MAP4K10
MAP3K or MAPKKK or MEKK	80–85	RAF27, HT1, RAF17, RAF33, RAF38, RAF6, RAF22
MAP2K or MAPKK or MKK or MEK	10	None
MAPK or MPK	20	MPK12, MPK4, MPK9, MPK11, MPK17, MPK19

Out of 20 MPKs, at least six are highly expressed in guard cells (table 1). It was shown by Jammes and colleagues that the double mutant with point mutated *MPK9* and *MPK12* lacked ABA-induced stomatal closure, and exhibited reduced ABA inhibition of light-induced stomatal opening and enhanced transpirational water loss from the leaves (Jammes et al., 2009). Moreover, *mpk9-1 mpk12-1* displayed impaired activation of anion channels by ABA and Ca^{2+} (Jammes et al., 2009). MPK12 kinase activity was enhanced by ABA both *in vitro* and *in vivo* (Jammes et al., 2009).

In addition to MPK9 and MPK12, the regulators of innate immune response MPK3, MPK4, and MPK6 are also important in stomatal defence. In guard cell specific *MPK3* RNA interference lines, ABA inhibition of light-induced stomatal opening was impaired, but ABA-induced stomatal closure was intact (Gudesblat et al., 2007). MPK4 is strongly expressed in guard cells and was suggested to mediate stomatal defence against bacterial attack (Petersen et al., 2000; Qiu et al., 2008; Berriri et al., 2012). MPK4 is also involved in CO_2-induced stomatal closure, but not in ABA-induced stomatal closure in *N. tabacum* (Marten et al., 2008).

Moreover, MPK1 and MPK2 are activated by ABA in a SnRK2-dependent manner (Ortiz-Masia et al., 2007; Umezawa et al., 2013), and were additionally identified as a part of the ABA-activated MAPK cascade MAP3K17/18-MKK3-MPK1/2/7/14 (Danquah et al., 2015). However, the role of MPK1 and MPK2 in the guard cell ABA signalling and stomatal responses is yet to be elucidated.

There are ten proteins annotated as MKKs, but none of them are highly expressed in guard cells (table 1). The *mek1* mutants were shown to lack ABA-induced stomatal closure and to be more drought-sensitive (Xing et al., 2007).

There are 80 to 85 proteins annotated as MAP3Ks, which are divided into STE-like and Raf-like kinases (Champion et al., 2004; Zulawski et al., 2014). However, since the family was compiled based merely on phylogenetic similarity, not all of the MAP3Ks may function in the MAPK module (Champion et al., 2004). This could be the case for the Raf-like MAP3K subfamily, where no protein thus far has been found to phosphorylate MKK (Zulawski et al., 2014). Among the 80 proteins annotated to be MAP3K according to The Arabidopsis Information Resource (TAIR), there are seven with high guard cell expression, all belonging to the Raf-like subfamily (table 1). One of the Raf-like MAP3Ks is the HT1 kinase important in stomatal CO_2 signalling (MAPK Group, 2002). Moreover, recently it was shown that MAP3K18 is expressed in the guard cells, interacts with OST1 and is inhibited by ABI1, suggesting a role in stomatal ABA signalling (Mitula et al., 2015; Tajdel et al., 2016). MAP3K18 was shown to be part of an ABA-activated MAPK cascade MAP3K17/18-MKK3-MPK1/2/7/14 (Danquah et al., 2015).

MAPK cascades can be controlled and shut down via dephosphorylation by mitogen-activated protein kinase phosphatases (MKPs), which can dephosphorylate both Ser/Thr and Tyr residues, and act as negative regulators of MPKs. The Arabidopsis genome encodes five potential MKPs: MKP1, MKP2, DsPTP1, PHS1, and IBR5 (Monroe-Augustus et al., 2003). The phosphatase IBR5 (IBA

RESPONSE 5) inactivates MPK12 by dephosphorylation and functions both in ABA and auxin signalling (Lee et al., 2009a). Tobacco plants overexpressing IBR5 from rice exhibit impaired stomatal closure in response to drought and ABA (Li et al., 2012). Recently it was shown that IBR5 could possibly regulate the ubiquitination and degradation of some R proteins (CHS3) involved in defence responses, e.g. in response to low temperature (Liu et al., 2015). In addition to MKPs, other phosphatases such as PP2Cs can also target phosphorylated MPKs as substrates, e.g. ABI1 interacts with MPK6 *in planta* (Leung et al., 2006).

1.3. The cuticle as a water barrier for plants

1.3.1. Function, structure and composition of the plant cuticle

The cuticle is a lipid-based biopolymer, which covers all aboveground plant parts, except for the stems of woody plants, and it is most abundant on fleshy fruits. When stomata are closed, which on average is the case during night and under drought conditions, the cuticle mainly limits the loss and uptake of gases and vapours such as water vapour, CO_2, O_2, air pollutants, and volatile organic compounds (Goodwin and Jenks, 2005; Burghardt and Riederer, 2006; Riederer, 2006; Kosma et al., 2009). However, it has also become clear that the function of the cuticle extends well beyond its primary role as a transpiration barrier. As such, the cuticle has obtained the following functions: reflection barrier of UV radiation (Riederer, 2006), protection from pathogens (Heredia, 2003; Reina-Pinto and Yephremov, 2009; Voisin et al., 2009), maintenance of structural rigidity of the plant (Bird and Gray, 2003), and reservoir for hydrophobic compounds (Schreiber, 2005). The cuticle also determines epidermal cell differentiation into trichomes and guard cells in Arabidopsis (Gray et al., 2000; Bird and Gray, 2003; Li et al., 2007), and plays a role in pollen-stigma contacts and in prevention of post-genital organ fusions (Samuels et al., 2008). Moreover, the plant is able to adjust its cuticular permeability in order to resist severe water loss (Kosma et al., 2009).

Over half of the fatty acids made by epidermal cells of the rapidly expanding Arabidopsis stem are estimated to be channelled into the cuticular lipids of cutin and wax (Suh et al., 2005). The highest cuticle polyester loads are observed in the youngest part of the stem. This implies that the major cutin synthesis occurs in young tissues (Suh et al., 2005). During the early stages of epidermal development in shoot apices and leaf primordia, cells are covered with a 20-nm thick, amorphous procuticle (Bird and Gray, 2003; Domínguez et al., 2010). The lamellar structure of the cuticle forms and matures together with leaf maturation (Bird and Gray, 2003; Franke et al., 2005; Jeffree, 2006; Voisin et al., 2009). The thickness of Arabidopsis' mature cuticle differs among organs, being 20–25 nm on leaves and 50–80 nm on stems (Nawrath, 2002). The mature cuticle is composed of cutin, intracuticular waxes, and epicuticular wax crystals (figure 3). Some polysaccharides from the cell wall can also be present in the cuticular layer (figure 3).

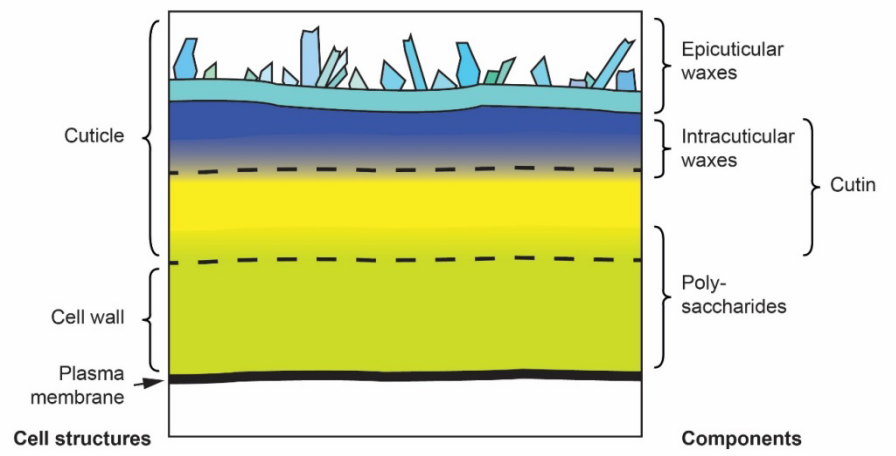


Figure 3. Cross-section of the cuticle on the leaf of Arabidopsis. The cuticular layer is composed of polysaccharides, cutin, intracuticular waxes, and epicuticular waxes. On the left side of the figure cell structures, such as the cuticle, cell wall, and plasma membrane are indicated. On the right side of the figure components of the cuticle, such as polysaccharides, cutin, and waxes are indicated.

Based on the experimental approach, the cuticle is divided into waxes, cutin, and cutan. Waxes are easily extractable crystals of hydrophobic monomeric very long chain fatty acids (VLCFA; carbon chain lengths ranging from C20 to C40), and thus most well studied. Cutin as the major constituent (40%–80% of the weight) of the cuticle, is insoluble and consists of oxygenated C16 and C18 fatty acids cross-linked with ester bonds via both primary (ω-) and secondary (midchain-) hydroxyl groups (Kolattukudy, 2001; Heredia, 2003). The cutin framework is embedded with waxes, but it is also covered by an epicuticular wax film (figure 3). On its lower side, cutin interacts with cell wall polysaccharides in order to anchor the cuticle to the epidermal cell surface (Fich et al., 2016). Cutan on the other hand, is a highly depolymerisation-resistant part of the cuticle, but it has been found in relatively few plant species (Gupta et al., 2006). Little is still known about the macromolecular and spatial structure of the cuticle.

Arabidopsis has a specific monomeric composition of the cuticle, which is suggested to enable survival with a very thin cuticle compared to other species (Franke et al., 2005). In Arabidopsis α,ω-dicarboxylic fatty acids (40%) and 2-hydroxy fatty acids (14%) are the major depolymerisation products of the cutin (Bonaventure et al., 2004; Franke et al., 2005; Kurdyukov et al., 2006). However, the flowers of Arabidopsis have a classical cutin composition (Beisson et al., 2007).

Cuticular ledges on guard cells extend over the stomatal aperture and are a conserved feature of almost all dicotyledones (Li et al., 2007). Waxes covering guard cells in Arabidopsis are different from those covering the rest of the epi-

dermal surface, they are thicker and have a higher concentration of wax-bound phenolics (Karabourniotis et al., 2001).

Another related biopolymer in plants is suberin, which accumulates in the apoplastic regions of many non-cutinized boundary cell layers, such as the periderm, endodermis, and root exodermis (Vishwanath et al., 2015). The most abundant suberin deposition can be found in, e.g. roots, potato (*Solanum tuberosum*) tubers, wounds of aerial plant parts, and in the cork of the cork oak tree (*Quercus suber*) (Vishwanath et al., 2015). The cutin of Arabidopsis exhibits suberin-like features, e.g. high content of diacids, glycerol, and phenolic content (Franke et al., 2005). Moreover, it was hypothesized that the compositional distinction between suberin and cutin might be quite artificial, and that they could share common biosynthetic and regulatory genes (Fich et al., 2016).

1.3.2. Development and biosynthesis of the *A. thaliana* cuticle

The cuticle is not a simple homogenous layer deposited on the external surface of epidermal cells, on the contrary, the cuticle changes and matures as the leaf matures (Bird and Gray, 2003). The major part of cuticle synthesis occurs in young tissues (Suh et al., 2005). The cells are covered with an amorphous, osmiophilic, water-repellent 20-nm thick procuticle during the early stages of epidermal development of shoot apices and leaf primordia. The procuticle is solely made of amorphous nonlamellate waxes (Bird and Gray, 2003; Jeffree, 2006). The lamellar structure of the cuticle and the reticulate fibrillar pattern of the cutinized portion of the cell wall become distinguishable as the cuticle forms and matures alongside the leaf (Bird and Gray, 2003; Franke et al., 2005; Jeffree, 2006; Voisin et al., 2009).

Cuticular lipid formation begins with synthesis of C16 and C18 fatty acids in leucoplasts, the small non-photosynthetic plastids in epidermal cells (figure 4; Samuels et al., 2008). Cutin lipid monomers are then modified in the ER into variously oxygenated monoacylglycerols (Fich et al., 2016). Modifications include conjugation to coenzyme A (CoA), oxidizing terminal and/or midchain carbons, and transferring the fatty acid from CoA to glycerol. The proteins involved in these modifications include among others long-chain acyl-CoA synthetases (LACS1, LACS2), cytochrome P450-dependent enzymes (CYP86A family, CYP77A family), and glycerol-3-phosphate acyltransferase (GPAT family) (figure 4).

CoA-conjugated precursors for wax biosynthesis first go through a process called fatty acid elongation (FAE), where carbons are added to achieve VLCFA (very long chain fatty acid) molecules (Kunst and Samuels, 2009). This is followed by several other modifications before very long chain alcohols, aldehydes, alkanes, and ketones are produced.

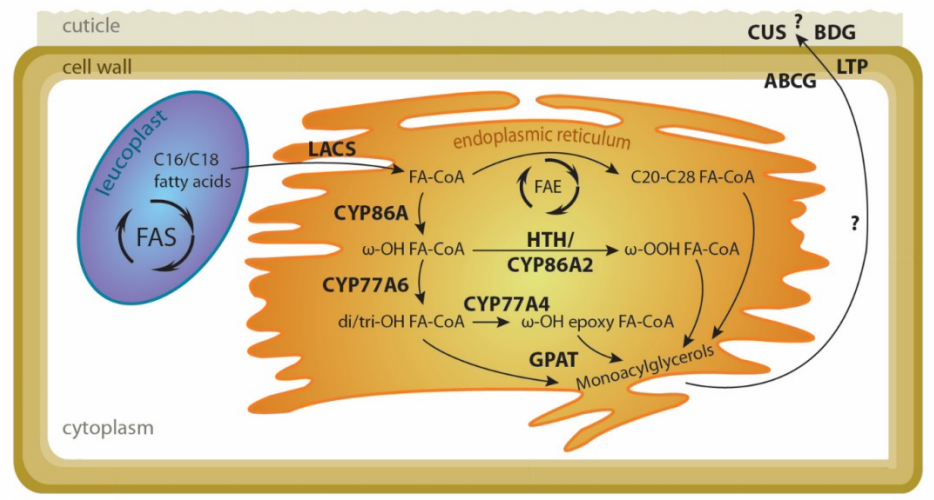


Figure 4. Biosynthesis of cuticle monomers in an epidermal cell. Lipid formation starts with the synthesis of C16 and C18 fatty acids in leucoplasts. The lipid monomers are further modified in the ER by various enzymes. Thereafter, the cuticular lipids are transported from the ER through the plasma membrane and cell wall to be polymerized into cuticular layers.

Thereafter, the highly hydrophobic cuticular lipids must be transported from the ER through the plasma membrane and cell wall. The exact mechanisms and proteins required for the transport have largely remained unknown. Intracellular movement of cuticular lipids could occur at the membrane contact sites by non-vesicular movement. Non-vesicular traffic would mean the extraction and transfer of individual lipid molecules from the bilayer of ER to the target bilayer of the plasma membrane (termed membrane contact sites), usually with the assistance of non-canonical lipid transfer proteins (Samuels and McFarlane, 2011). Transport through the plasma membrane appears to be carried out by a specific G subfamily of ATP-binding cassette (ABC) transporters (Bird et al., 2007; Panikashvili et al., 2007; Bessire et al., 2011; Panikashvili et al., 2011). Lipid-transfer proteins (LTPs) were proposed as carriers of hydrophobic lipid precursors through the hydrophilic polysaccharide cell wall (Yeats and Rose, 2008; Debono et al., 2009; Lee et al., 2009a). Alternatively, self-aggregation and phase separation as energetically less expensive mechanisms were suggested to be important in extracellular monomer trafficking (Kunst and Samuels, 2009; Fich et al., 2016).

Which enzymes are involved in the polymerization and how cutin synthesis is coordinated with the deposition of waxes is still largely unknown and a major challenge in the study of plant epidermis. Recently the GDSL-motif lipase/esterase (GDSL) superfamily was described to function as a cutin synthase (CUS) and shown to possess acyltransferase activity on the tomato fruit (Girard et al., 2012; Yeats et al., 2012). Genes orthologous to tomato *CUS1*

have so far been found from all the land plants studied to date (Yeats et al., 2014). In addition, BODYGUARD (BDG) was proposed to contribute to cutin polymerization (Kurdyukov et al., 2006), but biochemical evidence has not yet been provided.

The coding region of *BODYGUARD* (*BDG*) contains four exons and encodes a 469-amino-acid long protein belonging to the α/β-hydrolase fold protein superfamily. The knockout mutants of *BDG* were described first by Kurdyukov et al. (2006). The *bdg* mutants were clearly distinguishable from WT plants by reduced growth, abnormal leaf morphology, organ fusions, and leaf permeability to the toluidine blue dye and also to chlorophyll (Kurdyukov et al., 2006). Interestingly, *bdg* was found to be more resistant to *Botrytis cinerea* than WT plants (Voisin et al., 2009). The *bdg* mutants were reported to be hypersensitive to osmotic stress and surprisingly, under these conditions, to accumulate lower levels of ABA (Wang et al., 2011). Kurdyukov *et al.* (2006) proposed that BDG either functions as an extracellular synthase in cuticle formation or controls the proliferation/differentiation status of the epidermis via an unknown mechanism. Alternatively, in the hydrophobic environment of the cuticle, BDG may act as a transacylase, as was described for other α/β-fold proteins (Lenfant et al., 2013).

2. AIMS

The main aim of the thesis was to contribute to the characterization of the molecular targets and signalling pathways involved in plant transpiration, more specifically in stomatal regulation and cuticle formation.

ABA induces an efflux of anions from the guard cells and stomatal closure via the canonical ABA signalosome consisting of PYR/RCAR receptors, PP2C phosphatases, and OST1 kinase. Several other proteins have been shown to interact with the components of the ABA signalosome. However, little is known about their role in response to other environmental stomatal closure-inducing stimuli.

Recently, data has emerged about the involvement of proteins belonging to the MAPK cascade in guard cell signalling. But the substrates of MPK9 and MPK12 were unrevealed. The exact signal transduction mechanisms involved in guard cell signalling in response to abiotic and biotic stimuli was also uncovered.

Screening for mutants defective in transpiration has been previously used to find new regulators in guard cell signalling. However, it is expected that there are several more components involved in controlling plant transpiration, e.g. proteins mediating environmental signals in the guard cells or enzymes involved in the cuticle formation. Hence, new approaches for identifying mutants with altered transpiration are welcome.

The outcome of the new transpiration screen was a mutant allele of *BODYGUARD (BDG)*. It was suggested that BDG could control cuticle development extracellularly either during the formation of the polymer or during the proliferation of the epidermis (Kurdyukov et al., 2006). However, there is scarce information regarding its role in regulating plant transpiration, as well as how BDG functions in cuticle biosynthesis.

The objectives of this thesis were:

- To study the impact of proteins involved in stomatal ABA signalling on steady-state stomatal conductance and on stomatal responses to several abiotic stimuli such as a reduction in air humidity, an elevation in CO_2 concentration, darkness, and an elevation in O_3 concentration.
- To map the CO_2-related phenotypes of Cvi-0 accession and *cis* mutant.
- To study the molecular mechanisms of CO_2 signalling in guard cells, especially the role of MPK12.
- To develop a method for finding Arabidopsis mutants defective in daytime transpiration.
- To study the role of BODYGUARD protein in plant transpiration and cuticle formation.

3. MATERIALS AND METHODS

The details of ABA signalosome mutants, plant growth conditions, whole-rosette gas exchange measurements, and statistical analysis regarding publication I are explained in its Materials and Methods section. Mapping strategies, stomatal assays, kinase activity tests of MPKs, and other experiments are described in the Materials and Methods section of the publication II. Methodological aspects of the novel screening method, cuticular assays and expression pattern studies of BDG are provided in publication III. Methods used to obtain the unpublished data are provided below.

Transient expression in *Nicotiana benthamiana*

For transient expression experiments in *Nicotiana benthamiana*, the starting vector pCambia1390 (Snapgene) was used and two different constructs were created: *35S::BDGcDNA::sGFP(S65T)*, *35S::sGFP(S65T)::BDGcDNA*. *BDG* cDNA was amplified from WT Col-0 cDNA with cloning primers BDG_F1+EcoRI (5'- TGC TGA ATT CAT GGG ATT CTC ACG GTC G -3') and BDG_R1+NcoI (5'- TGC TCC ATG GTT AAT TTA TTG AAT GAA GTT GAG GAG -3') for the N-terminal fusion constructs and with primers BDG_F1+SalI (5'- TGC TGT CGA CAT GGG ATT CTC ACG GTC G -3') and BDG_R1+EcoRI (5'- TGC TGA ATT CAT TTA TTG AAT GAA GTT GAG GAG -3') for the C-terminal fusion constructs. Fluorescent sGFP(S65T) cDNA (Chiu et al., 1996) was amplified with cloning primers GFP_F1+SalI (5'- TGC TGT CGA CAT GGT GAG CAA GGG CGA GGA -3'9 and GFP_R1+EcoRI (5'- TGC TGA ATT CCT TGT ACA GCT CGT CCA T -3') for the N-terminal fusion constructs and with primers GFP_F1+EcoRI (5'- TGC TGA ATT CAT GGT GAG CAA GGG CGA GGA -3') and GFP_R1+NcoI (5'- TGC TCC ATG GTT ACT TGT ACA GCT CGT CCA T -3') for C-terminal fusion constructs.

For transient expression in *N. benthamiana*, transformed overnight culture (in LB+100 µM acetosyringone + antibiotics) of *A. tumefaciens* strain C58GV3101 (pMP90) was centrifuged and resuspended in the infiltration buffer (10 mM MES, pH 5.6; 10 mM $MgCl_2$; 150 µM acetosyringone) so that the final optical density (OD 600) of each suspension was ~1.0. Each construct was mixed in equal volumes with the *A. tumefaciens* line carrying a plasmid with the silencing inhibitor *35S::TSBV p19* (Voinnet et al., 2003) and let stand at room temperature for 1–3 hours. Then the abaxial leaf sides of four to six weeks old *N. benthamiana* plants were infiltrated with Agrobacterium mixtures by using a syringe without a needle. The fluorescence images were taken 2–6 days after transformation (DAT) with a Zeiss LSM 710 confocal laser scanning microscope (excitation 458 nm, emission 493–571 nm). All constructs were analysed in four to six repeats.

4. RESULTS AND DISCUSSION

4.1. Stomatal regulation in the ABA signalosome mutants

4.1.1. Stomatal conductance of the ABA signalosome mutants

To get an insight into the role of ABA in whole-plant steady-state stomatal conductance and in stomatal closure induced by four different abiotic stimuli (darkness, low air humidity, high CO_2, short-term O_3 increase), an extensive mutant analysis was carried out to study their stomatal responses to these stimuli. The selected mutants were affected in different components of ABA biosynthesis, ABA degradation, and ABA-induced signalling (figure 5).

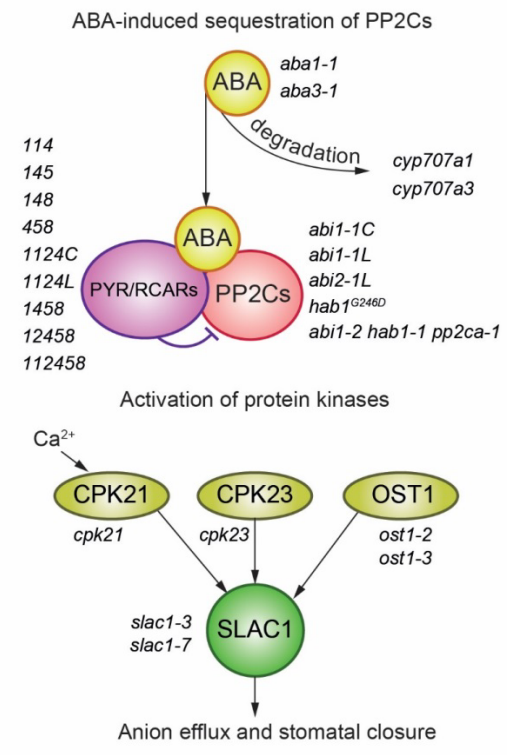


Figure 5. Schematic overview of the mutants used in the study and their role either in the ABA-induced signalling pathway or in ABA metabolism (copied from ref I). Mutants used in this study are depicted next to each molecular component. ABA biosynthesis is defective in *aba1-1* and *aba3-1*, which therefore exhibit ABA deficiency (Rock and Zeevaart, 1991; Léon-Kloosterziel et al., 1996). ABA catabolism is defective in *cyp707a1* and *cyp707a3*, which results in ABA hypersensitivity in *cyp707a1*, but not in *cyp707a3* (Okamoto et al., 2009). Three to six ABA receptors are knocked out in different PYR/RCAR mutants, which results in varying levels of reduction in ABA sensitivity (Park et al., 2009; Gonzalez-Guzman et al., 2012). The mutations in *hab1*^{G246D}, *abi1-1L* and *abi2-1L* are analogous, and all cause resistance to inhibition of

PP2C phosphatases by PYR/RCAR receptors, leading to strong ABA insensitivity (Leung et al., 1997; Robert et al., 2006; Umezawa et al., 2009). The original *abi1-1* mutant was in the L*er* accession (Leung et al., 1997), but here the corresponding mutation in the Col-0 background was also used (Umezawa et al., 2009). The mutations in *abi1-2 hab1-1 pp2ca-1* are SALK T-DNA insertions leading to full knockout of these PP2Cs and to constitutive ABA-response (Rubio et al., 2009). ABA-activated kinases are knocked out in *ost1-3*, *cpk21*, and *cpk23* mutants (Mustilli et al., 2002; Yoshida et al., 2002; Ma and Wu, 2007). The S-type anion channel SLAC1 is absent in *slac1-3* and point mutated in *slac1-7* (Vahisalu et al., 2008; Vahisalu et al., 2010).

Stomatal conductance shows the amount of H_2O in moles that exits the plant through the stomata per one m^2 of leaf area per second. In this thesis stomatal conductance was measured with fully closed whole-rosette cuvettes, where the air is constantly flowing through the cuvette and the composition of the air is detected both before and after the cuvette (Kollist et al., 2007). Here the steady-state stomatal conductance (g_{st}) reflects the average rate of stomatal conductance during the day-time period.

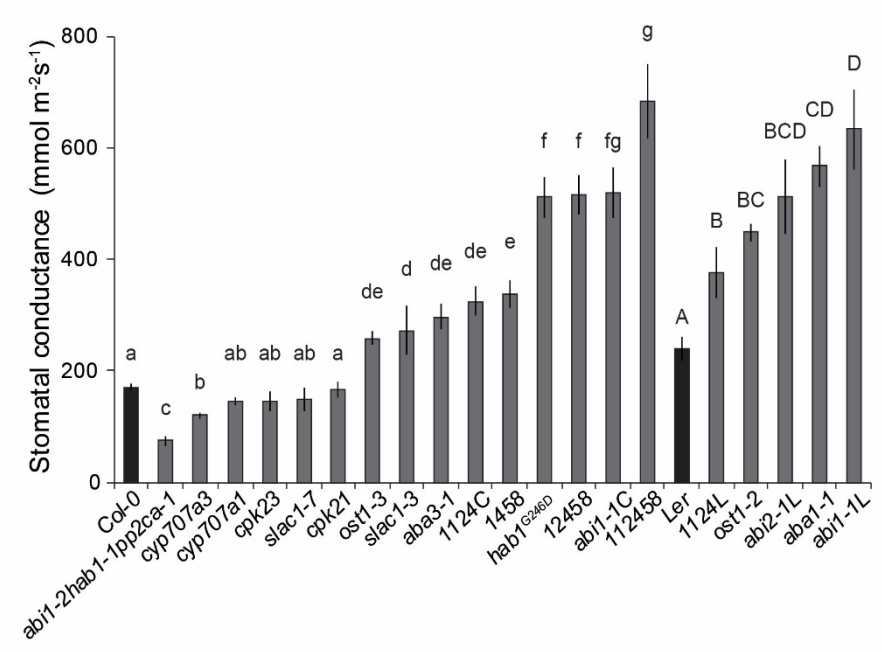


Figure 6. Whole-plant steady-state stomatal conductance of ABA signalosome mutants (copied from ref I). The bars represent the average stomatal conductance values together with SEM (n = 4–64). Significant differences ($p < 0.05$) according to ANOVA with Fisher's LSD test are marked with different small letters for plants in Col-0 background and with capital letters for plants in L*er* background.

The values of g_{st} differed more than nine times among the studied mutants, ranging from 74 to 683 mmol m^{-2} s^{-1} (figure 6). Plants with the highest g_{st} belonged to either PYR/RCAR loss-of-function mutants or to dominant active PP2C mutants: sextuple *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* (*112458;* 638 mmol m^{-2} s^{-1}), pentuple *pyr1 pyl2 pyl4 pyl5 pyl8 (12458*; 515 mmol m^{-2} s^{-1}), *abi1-1C* (519 mmol m^{-2} s^{-1}), and $hab1^{G246D}$ (512 mmol m^{-2} s^{-1}) (figure 6). Plants with the lowest g_{st} were triple loss-of-function mutants of PP2Cs *abi1-2 hab1-1 pp2ca-1* (figure 6). The values of g_{st} were altered in accordance with the functioning of the ABA signalling pathway. Reduced ABA content, gradually reduced levels of functional PYR/RCAR proteins, the presence of constitutively active PP2Cs, and the lack of functional OST1 and SLAC1 resulted in higher g_{st} (I). In contrast, a lack of three PP2Cs in *abi1-2 hab1-1 pp2ca-1* and higher ABA content in *cyp707a3* resulted in lower g_{st} (I). These results indicate that ABA signalling through PYR/RCAR receptors and PP2C phosphatases plays a fundamental role in controlling water loss through stomata at the whole plant level.

4.1.2. Stomatal closure to abiotic stimuli in the ABA signalosome mutants

Stomata close in response to environmental stimuli such as high CO_2, darkness, reduction in air humidity, and short-term O_3 increase. It is hypothesized that the pathways of stomatal closure induced by abiotic stimuli and by ABA should converge at some point, but the convergence point is yet to be elucidated (Engineer et al., 2016). Thus, the involvement of ABA signalosome components in the regulation of stomatal closure to abiotic stimuli such as high CO_2, darkness, ozone and reduced air humidity was studied (figure 7). A considerable amount of data on stomatal signalling has been collected using isolated leaf epidermises or guard cell protoplasts (e.g. Mustilli et al., 2002; Geiger et al., 2010), but here gas-exchange analysis of whole plants was employed to study stomatal responses and the impact of the mutations on stomatal conductance.

The mutants of *SLAC1* and *OST1* showed impaired stomatal response to all studied stimuli (figures 7, 8). Initial stomatal closure of *cpk23* was also significantly reduced, although to a lesser degree than that of *slac1-3* or *ost1-3* (figure 8, I). However, *cpk21* reacted like WT to the studied stimuli (figure 8, I). Thus, these results indicate that SLAC1 and OST1 are the central components during stomatal closure in response to all the studied stimuli and add new players to the current model of CO_2-induced stomatal regulation.

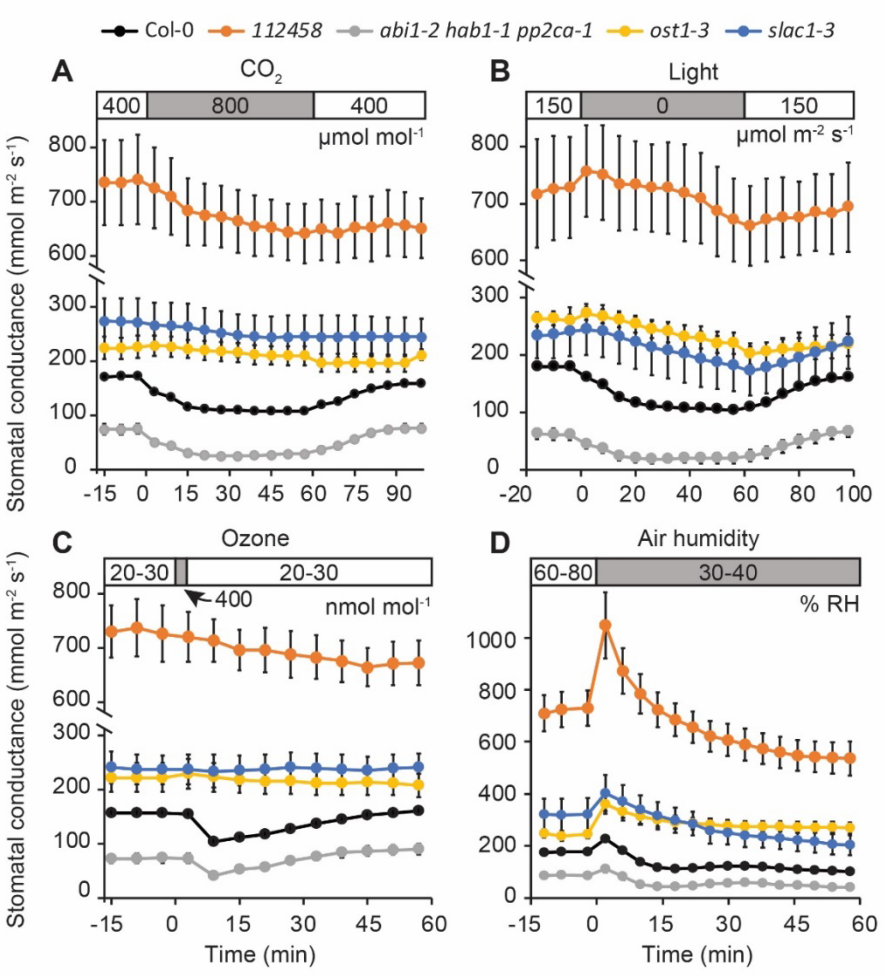


Figure 7. Stomatal responses of selected mutants of the ABA signalosome to different abiotic stimuli (based on ref I). Time courses of stomatal conductance in response to changes in aerial [CO_2] (A), in light intensity (B), in O_3 concentration (C), and in air humidity (D) are shown (n = 5–50).

PP2C phosphatases such as ABI1, ABI2, HAB1, and PP2CA are negative regulators of the ABA signalling pathway (Gosti et al., 1999; Merlot et al., 2001; Saez et al., 2004; Kuhn et al., 2006). PP2C activity is suppressed when PYR/RCAR receptors together with ABA molecules bind to the phosphatase (Ma et al., 2009; Park et al., 2009). Humidity- and O_3-induced stomatal responses were reduced in mutants with constitutively active ABI1 and ABI2 (*abi1-1C*, *abi1-1L* and *abi2-1L*), whereas plants with constitutively active HAB1 ($hab1^{G246D}$) showed reduced response only to O_3 (figure 8). The function of PP2Cs in darkness- and CO_2-induced stomatal closure was less clear. Although the initial closure rates for all the constitutively active mutants for

both stimuli were WT-like, on several occasions their stomatal closure could not be described with an exponential function, as in the case of WT response (I). Moreover, guard cell protoplasts from *abi1-1L* and *abi2-1L* showed reduced but functional HCO_3^--induced activation of anion currents and during the stepwise elevation of CO_2 concentration stomatal closure was reduced in *abi2-1L* (I). The partial CO_2- and darkness-induced stomatal closure could be explained by the functioning of the remaining PP2Cs. The triple knockout mutant of PP2Cs exhibited WT-like stomatal closure rates except in response to O_3, which might indicate the activity of other functional PP2Cs, e.g. ABI2 (figures 7, 8). Thus, for O_3- and humidity-induced stomatal closure, the inhibition of PP2C activity is important, but it is less important for darkness- and CO_2-induced closure.

From the PYR/RCAR mutants the most insistent differences from WT responses were displayed by the sextuple mutant, indicating a functional redundancy among these ABA receptor proteins (figure 8). The *112458* sextuple mutant displayed impaired stomatal responses and reduced initial stomatal closure to all studied stimuli but high CO_2 (figure 8). However, during a stepwise increase in CO_2 concentration (from 50 ppm to 800 ppm) the CO_2-induced stomatal closure was impaired in *112458* (I). Hence, PYR/RCARs, especially PYL1, are required for rapid stomatal closure in response to darkness, reduced air humidity, and O_3 (I), but their contribution to high CO_2-induced stomatal closing might be yet unrevealed due to redundancy among 14 PYR/RCAR proteins or absent due to other parallel molecular mechanisms.

Chater and colleagues recently showed CO_2 insensitivity in the quadruple ABA receptor mutant (Chater et al., 2015), but another report had showed earlier that the same mutant has slowed but functional CO_2 responses (Xue et al., 2011). The latest model for the convergence of CO_2 and ABA pathways suggests that the CO_2 response is weakened in the complete absence of an ABA response (Engineer et al., 2016). Two different scenarios could be considered here: (a) ABA and ABA receptors function upstream of the convergence point of CO_2 and ABA signalling, while synergistically amplifying common downstream signalling mechanisms; or (b) CO_2 rapidly causes an elevation of guard cell [ABA] within 1–3 min and thus mediates stomatal closure (Engineer et al., 2016).

Surprisingly, the stomatal closure rates in response to reduced air humidity in *aba1-1* and *aba3-1* were significantly larger than WT responses (figure 8, I). As residual amounts of ABA were still detected in the mutants of *ABA1* and *ABA3* (Rock and Zeevaart, 1991; Léon-Kloosterziel et al., 1996), this could be enough for activating the ABA signalling pathway. Although the mutants defective in ABA catabolism (*cyp707a1* and *cyp707a3*) contain higher amounts of ABA (Okamoto et al., 2009), their initial stomatal closure rates for all studied stimuli were WT-like (I). Thus, ABA metabolism mutants showed weaker impairment of stomatal closure than ABA signalling mutants.

All-in-all, the results of the current thesis contribute to the understanding that the ABA signalling pathway plays an important role in stomatal closure to reduced air humidity, darkness, and O_3 (figure 8). High CO_2-induced stomatal

closure involves partially the proteins belonging to the ABA signalosome, but also the presence of a parallel, yet unidentified signalling pathway that enables the activation of OST1, SLAC or both is possible. OST1 and SLAC1 were important in response to all studied stimuli (figures 7, 8).

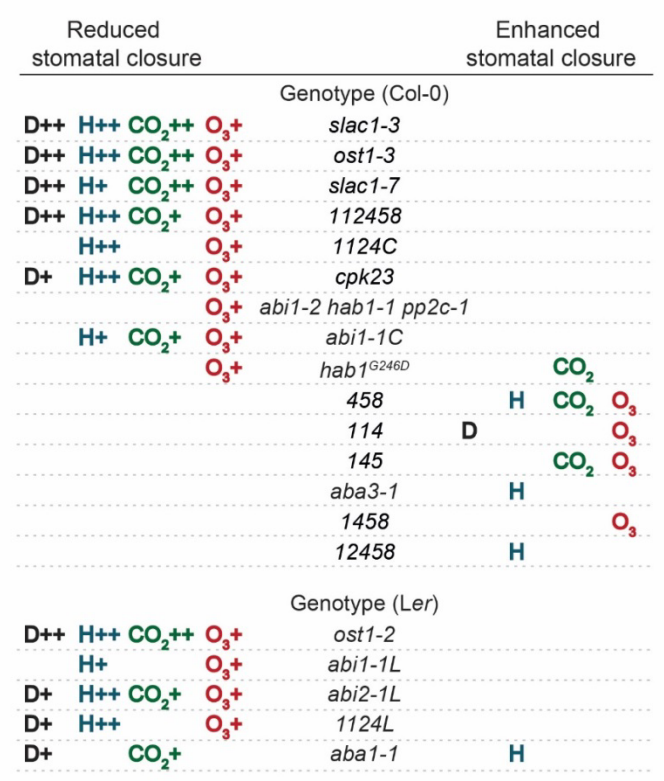


Figure 8. Overview of stomatal closure responses to all the studied stimuli (copied from ref I figure 9). Mutants showing WT-like responses to all studied stimuli (*148*, *cpk21*, *cyp707a1*, and *cyp707a3*) were discarded from the list. The order of the mutants is presented according to their phenotypic severity. H and D stand for air humidity and darkness, respectively. + shows that either initial change in g_{st} or curve fitting was significantly different from the WT response. ++ shows that both initial change in g_{st} and curve fitting were significantly different from the WT response. The absence of a symbol shows WT-like stomatal closure to the corresponding stimulus.

4.2. MPK12 is involved in guard cell CO_2 signalling

While studying the role of the Ca^{2+}-sensing receptor (CAS) in stomatal signalling, it was revealed that the mutation responsible for the CO_2-insenstivity phenotype in *cas-2* was located in *MPK12* (II). In parallel, mapping the ozone sensitivity of Arabidopsis Cvi-0 accession at the University of Helsinki identified a

QTL in *MPK12* (II, Brosché et al., 2010). Thus, the subsequent research was targeted to elucidate the role of MPK12 in stomatal signalling, especially in CO_2-induced stomatal responses.

During the characterization of stomatal responses of *cas* mutants, phenotypic discrepancy between different alleles of *cas* was observed (II). The CO_2-insensitivity phenotype of *cas-2* was not linked to the T-DNA insert in the *CAS* gene (II). After removing the *CAS* T-DNA insert by crossing *cas-2* with Col-0, a novel mutant called *cis* (*CO_2 insensitive*) was created (II). The mapping of the mutation was conducted by crossing *cis* with C24 accession, which has more closed stomata than Col-0, and by whole-genome sequencing of pooled DNA from selected individuals of the F3 population (figure 9A). As *cis* exhibited a deletion in *MPK12*, it was renamed to *mpk12-4* (II).

The earlier QTL mapping of ozone sensitivity in Cvi-0 had pointed towards the lower ends of chromosomes 2 and 3 (Brosché et al., 2010). To pinpoint the causative genomic changes of Cvi-0, the NIL (near-isogenic line) called Col-S (for Col-0 ozone sensitive) was created through eight generations of backcrossing of Cvi-0 with Col-0 (figure 9B). With the help of Col-S and other created lines, the mapping results pointed towards a region of 90 kb on chromosome 2 and 17.70–18.18 Mbp on chromosome 3 (II). Thereafter it was possible to separate the QTLs of chromosome 2 and 3 by backcrossing Col-S with Col-0 and the NILs Col-S2 and Col-S3 were obtained (figure 9B). In Col-S2 a single point mutation G157C was verified in *MPK12*, leading to a glycine to arginine substitution at position 53 of the protein (II). The mapping resolution on chromosome 3 was insufficient to identify the causative gene there (II).

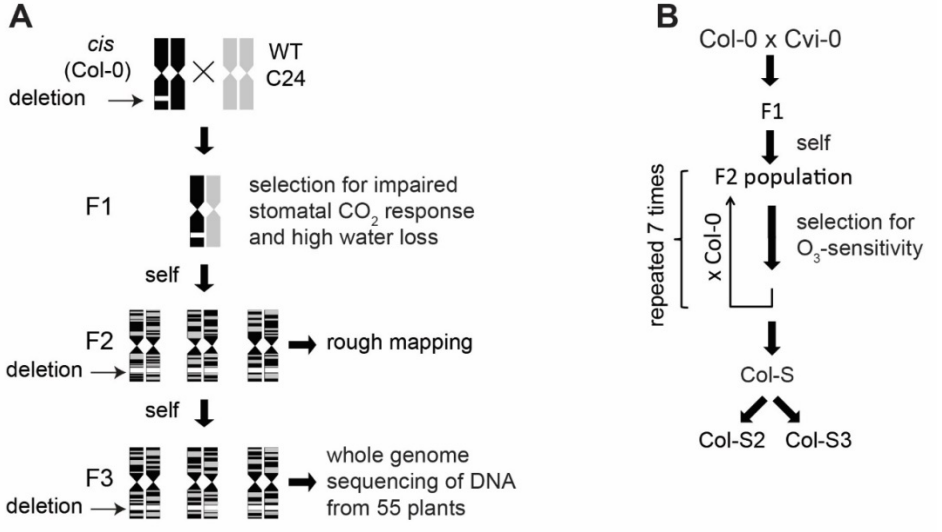


Figure 9. Identification of *cis* and Col-S2 mutation (based on ref II). (A) Mapping scheme of *cis* mutation obtained from *cas-2* T-DNA line. (B) Scheme of mapping the ozone sensitive trait of Cvi-0.

To study the function of MPK12 in guard cells in further detail, the following plant lines were used. First, the *mpk12-4* deletion mutant, identified from the GABI-Kat T-DNA line with a deletion of 4772 bp, had the coding region and regulatory sequences of *MPK12* deleted (II). Second, the Col-S2 line with the *MPK12* G157C point mutation was used (II). Third, the *mpk12-3* insertion mutant was identified that possesses a SAIL T-DNA insertion in the second exon of *MPK12* (II). Previous studies had reported lethal effects of SALK T-DNA insertion in *MPK12* (Jammes et al., 2009; Lee et al., 2009a). Other lines used previously in the reverse genetics characterization were either point mutation alleles in the Col-*er* background (Jammes et al., 2009; Salam et al., 2012; Salam et al., 2013), NIL lines (Des Marais et al., 2014), or mutant lines with inducible RNA interference (RNAi) suppression (Lee et al., 2009a). As mutations in *ERECTA* modify transpiration efficiency and stomatal density (Masle et al., 2005), this might have influenced some of the previously described phenotypes with lines in the Col-*er* and L*er* background. Thus, the new *mpk12* lines fully or partly in Col-0 background allowed a more detailed analysis of the role of MPK12 in stomatal regulation due to the variety of mutation alleles (II).

In addition, it was recently shown that a natural accession of Arabidopsis carries an amino acid substitution in MPK12, which causes reduction in water use efficiency (Des Marais et al., 2014). Des Marais and co-workers had isolated the NIL containing a small introgression from the Cvi-0 genome in a L*er* accession by selecting for decreased WUE (Des Marais et al., 2014).

Lack of functional MPK12 in Arabidopsis induced a decrease in WUE, increase in stomatal conductance, reduction in stomatal responses to aerial [CO_2] changes, and impairment of bicarbonate-induced activation of S-type anion channels (figure 10, II). Interestingly, stomatal closure responses to ABA, darkness, ozone, and reduced air humidity were WT-like in *mpk12* mutants (II). These results implicate that MPK12 is a specific regulator of CO_2 signalling. Recently it was shown that the high CO_2 signal may involve the transmembrane region of SLAC1, but the ABA activation of SLAC1 requires intact N- and C-terminus and not the transmembrane region (Yamamoto et al., 2016). As ABA and CO_2 regulation of SLAC1 could use at least partly different signalling pathways, this might explain the lack of strong ABA phenotypes in *mpk12*.

To find potential interaction partners for MPK12, Y2H pairwise comparisons were carried out against several kinases and phosphatases involved in either stomatal signalling or related to MPK12 function (II). It was found that MPK12 interacted with the protein kinase HT1 and with the MPK phosphatase IBR5 (INDOLE-3-BUTYRIC ACID RESPONSE 5) (II), which regulates auxin signalling in roots and inactivates MPK12 by dephosphorylation (Lee et al., 2009a). Despite the exceptionally strong CO_2-insensitivity phenotype of *ht1* mutants (Hashimoto et al., 2006), the regulators of HT1 had remained unrevealed until recent developments (Tian et al., 2015). RHC1 was shown to interact with HT1 and suggested to inhibit HT1 activity (Tian et al., 2015). Thus, these data provide the first evidence for the interaction between HT1 and MPK12 (II).

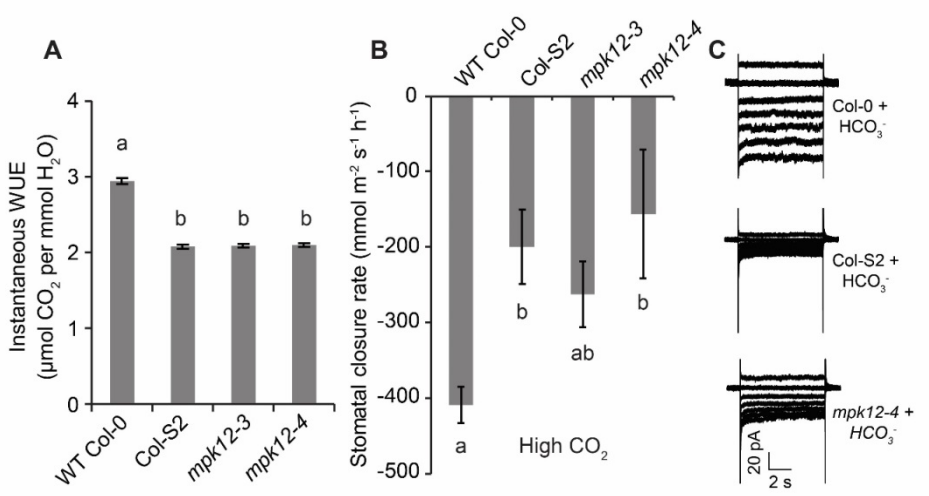


Figure 10. MPK12 affects guard cell functioning (based on ref II). (A) Instantaneous WUE is reduced in *mpk12* compared to WT (n = 13–16). Means ± SEM are represented. (B) Stomatal response to 800 ppm of CO_2 was impaired in *mpk12* compared to WT. Stomatal closure rate was measured as the decrease in stomatal conductance within the first 10 minutes after onset of the stimulus (n = 12–13). Means ± SEM are represented. (C) Representative patch clamp recordings of the bicarbonate (HCO_3^-)-induced slow type anion currents in guard cell protoplasts. Small letters (in A, B) denote statistically significant differences according to one-way ANOVA with Tukey's HSD *post hoc* test for unequal sample size.

To test whether MPK12 could regulate HT1 activity directly, *in vitro* kinase assays with casein as the substrate for HT1 were performed. Interestingly, the interaction between MPK12 and HT1 had inhibitory effects on HT1 kinase activity, which was suppressed in the presence of Col-0 MPK12, but not in the presence of the Cvi-0 version of MPK12 (MPK12 G53R) (II). Lack of HT1 inhibition by MPK12 G53R was probably due to weaker interaction with HT1. Moreover, the inhibitory effect was independent of MPK12 kinase activity, since both HT1 autophosphorylation and casein phosphorylation were reduced by the kinase inactive version MPK12 K70R, as well as by the kinase hyperactive version MPK12 Y122C (II). Thus, these results suggest that the inhibition of HT1 is independent of MPK12 kinase activity, but depends on the amino acid in position 53.

To find out whether this phenomenon is only MPK12-specific or has a wider spectrum, the inhibitory effect of MPK12 with other similar MPKs was compared. MPK12 belongs to the same group of MPKs as MPK4 and MPK11 (Andreasson and Ellis, 2010), and the two latter are both one of the more highly expressed MPKs in the guard cells after MPK12 (Yang et al., 2008). MPK4 is a crucial regulator of pathogen and stress responses and it is also expressed in guard cells (Petersen et al., 2000). MPK11 is paralogous to MPK4 (Andreasson and Ellis, 2010), but little is known about its function and physiological role.

Interestingly, MPK4, but not MPK11 inhibited HT1 kinase activity similarly to MPK12 (figure 11A-B, II). MPK4-induced inhibition of HT1 activity *in vitro* was blocked by the introduction of a G55R mutation in MPK4, which corresponded to the G53R mutation in MPK12 (II). Thus, the HT1 inhibition mechanism is not specific to MPK12, and the conserved residue corresponding to G53 in MPK12 is of high importance in the inhibition of HT1.

Based on the high-resolution Arabidopsis MPK6 crystal structure published just recently (Wang et al., 2016a), modelling the structures of MPK4 and MPK12 was undertaken in order to address the role of the G55R and G53R mutations, respectively. The substitution of Gly to Arg in those MPKs induced the protrusion of the arginine sidechain on the surface of the protein, which alters the structure of the loop region and could affect the binding affinity for other proteins (figure 11C, II).

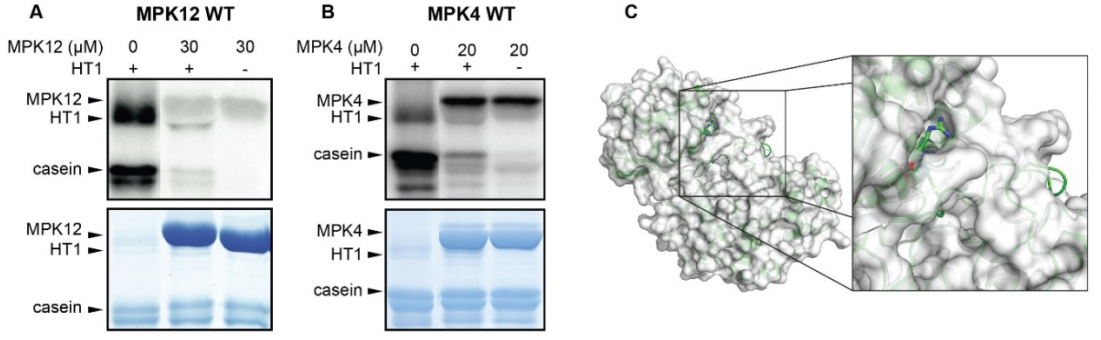


Figure 11. Regulation of HT1 by MPK12 and MPK4 (based on ref II). (A) Inhibition of HT1 kinase activity in vitro by MPK12. Upper panel: autoradiography of the SDS PAGE gel; lower panel: Coomassie-stained SDS PAGE. Reaction mixture was incubated for 30 min. (B) Inhibition of HT1 kinase activity in vitro by MPK4. Upper panel: autoradiography of the SDS PAGE gel; lower panel: Coomassie-stained SDS PAGE. Reaction mixture was incubated for 30 min. (C) Whole protein (left) and close-up (right) view of the superposition of models for MPK12 WT (secondary structure and surface in white) and MPK12 G53R (secondary structure in green) conducted with SWISS-MODEL. There is a close structural similarity between the structures except where the arginine at position 53 protrudes from the mutant protein surface and changes the loop region of the mutant protein.

All in all, these results suggest that the CO_2 signal leading to stomatal closure is conducted via MPK12 and MPK4 that results in inhibition of HT1, which in turn enables SLAC1 activation. However, neither MPK12 G53R from Cvi-0 nor MPK4 G55R are able to fully inhibit HT1 activity.

The protein kinase HT1 has a central role in guard cell CO_2 responses as a negative regulator (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). The *ht1-2* mutant with a kinase-dead HT1 exhibits

constitutive high CO_2 response, it has more closed stomata in ambient conditions and lacks the ability to open or close in response to changes in CO_2 concentrations (Hashimoto et al., 2006). Recently, Hõrak and co-authors described the regulatory mechanisms and substrates of HT1 by showing that HT1 kinase activity is inhibited by MPK4 and MPK12 (Hõrak et al., 2016). In addition to the data that HT1 inhibits OST1-mediated SLAC1 activation (Tian et al., 2015), it was also shown that HT1 can inhibit SLAC1 activation by GHR1 in the oocytes (Hõrak et al., 2016). Moreover, ABA-induced stomatal closure and ABA-induced inhibition of stomatal opening were functional in *ht1* mutants, suggesting that HT1 is involved in the ABA-independent stomatal regulation (Hõrak et al., 2016).

The current model of high CO_2 perception and signalling in the guard cells is depicted in figure 12. CO_2 could enter the guard cells through the PIP2;1 aquaporin (Wang et al., 2016b), and its conversion to bicarbonate in the cytosol is catalysed by carbonic anhydrases βCA4 and βCA1 (Hu et al., 2010). Although it is not known how the bicarbonate signal is further sensed in guard cells, it is plausible that in high CO_2 conditions inhibition of HT1 kinase activity is induced by MPK12 and MPK4 (figure 12). Bicarbonate-induced inhibition of HT1 by RHC1 was also shown to take place (Tian et al., 2015). As a result, HT1 inhibition in turn enables activation of the slow-type anion channel SLAC1 e.g. by OST1 or by GHR1 (figure 12). It was recently shown that GHR1 is involved in CO_2-induced stomatal closure, it is phosphorylated by HT1 and facilitates SLAC1 activation in the oocytes (Hõrak et al., 2016). In the case of the MPK12 G53R mutation in Cvi-0 or MPK4 G55R mutation (right guard cell in figure 12), the glycine to arginine substitution decreases the ability of these MPKs to inhibit HT1 kinase activity under high CO_2 conditions. As a consequence, SLAC1 activity is continually inhibited directly or indirectly by HT1, which results in decreased sensitivity to CO_2-induced stomatal closure and more open stomata.

Recently it was shown by two different groups and by two different experimental setups that in a heterologous system with the oocytes of *X. laevis*, the CO_2 signalling pathway leading to the activation of the SLAC1 anion channel could be reconstituted. Wang and co-authors expressed CO_2-permeable aquaporin PIP2;1 together with the carbonic anhydrase βCA4, OST1, and SLAC1 in the oocyte and observed SLAC1 anion channel activity in the presence of bicarbonate HCO_3^- (Wang et al., 2016b). However, a stomatal CO_2 signalling pathway without involving HT1 is rather unlikely, since based on *ht1* mutant phenotypes, the HT1 kinase seems to be the central and crucial component in stomatal CO_2 responses. Another study suggested that CO_2-induced SLAC1 activation could be reconstituted by expressing a MATE transporter RHC1, βCA4, HT1, OST1 and SLAC1 in the oocytes (Tian et al., 2015). However, Wang and others (2016b) presented that by expressing RHC1 alone in the *X. laevis* oocytes, ionic currents could also be observed, which does not exclude a role for RHC1 in CO_2 signalling, but points to the need to use other experimental methods for investigating the role of RHC1. Thus, the full stomatal CO_2 signalling pathway is yet to be clarified and several different approaches are needed to be engaged into the investigation.

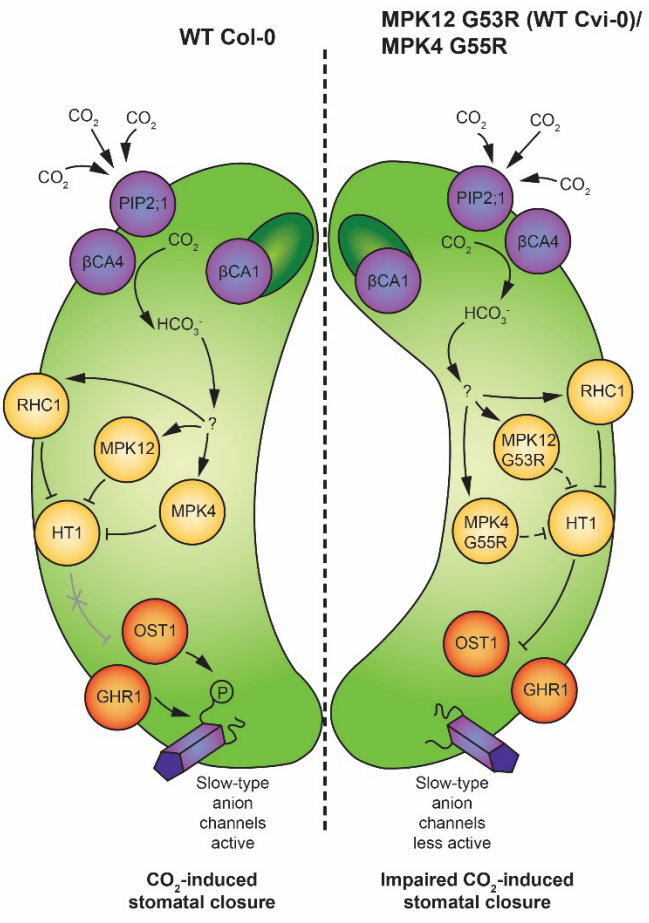


Figure 12. The molecular signalling pathway during high CO_2-induced stomatal closure (copied from ref II). The left guard cell depicts high CO_2-induced signalling in WT Col-0, where functional MPK12 and MPK4 can inhibit HT1 activity and thus enable activation of the slow-type anion channel SLAC1 by OST1 and GHR1. The guard cell on the right depicts high CO_2-induced signalling in WT Cvi-0 and in the case of MPK4 G55R mutation, where HT1 kinase activity is not fully inhibited and thus SLAC1 anion channel activity is also partly inhibited. This results in a smaller rate of stomatal closure in response to high CO_2.

4.3. Screening for mutants impaired in transpiration

Increased transpiration as a phenotype has been used in thermal imaging screens to monitor lower leaf temperatures of intact plants, and has been successfully applied to find mutants impaired in stomatal regulation (Merlot et al., 2002; Mustilli et al., 2002; Hashimoto et al., 2006). Isolation of cuticle mutants based on mutant screens has focused on various phenotypes, including increased leaf

permeability to toluidine blue or Calcofluor dye (Tanaka et al., 2004; Bessire et al., 2007), altered stem or leaf reflectance (Jenks et al., 1996; Chen et al., 2005), occurrence of organ fusions (Kurdyukov et al., 2006), altered resistance to *Botrytis cinerea* or *Pseudomonas syringae* (Bessire et al., 2007; Tang et al., 2007), and tomato fruit surface appearance (Isaacson et al., 2009; Petit et al., 2014).

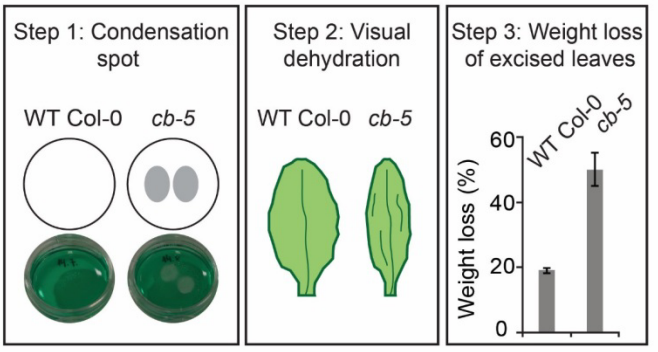


Figure 13. The outline of the 3-step screening method together with illustrative images and figures (copied from ref III). The method comprised three different steps for assessing transpiration in leaves. In the first step, excised leaves were placed abaxial side down on the lid of a Petri dish filled with water and the occurrence of a condensation spot underneath the leaf and the lid was monitored. In the second step, visual dehydration of the cut leaves was assessed. In the third step, weight loss of excised leaves was measured.

Here a method for screening mutants for impaired transpiration is introduced, which is based on temperature-dependent water spot condensation, visual wilting, and weight loss of excised leaves (figure 13, III). An activation-tagged T-DNA mutant library was used as a pool of mutants with altered gene expression (Weigel et al., 2000). Over 12,000 plants from this library were grown and screened for impaired transpiration (III). As a result of this screening, a set of *cool breath* (*cb*) mutants with enhanced transpiration was identified (III).

4.4. BODYGUARD is required for the biosynthesis of cutin

A mutant *cool breath5 (cb5)* was isolated from the abovementioned screen and was subjected to further investigation. The insertion in *cb5* was verified to locate in the first exon of *BDG*, thus, *cb5* was allelic to the previously reported mutants (Kurdyukov et al., 2006; Wang et al., 2011), and was therefore renamed as *bdg-6*. BODYGUARD (BDG) is a cuticle-related α/β-hydrolase domain protein with a yet unknown function. The aim of this thesis was to study the role of BODYGUARD protein regarding cuticle formation and plant transpiration. For this, a SAIL T-DNA line (SAIL_425_C10) with the insertion

159 bp upstream from *BDG* start codon, named as *bdg-7*, was also included. Additionally, *BDG* cDNA was ectopically expressed in WT Col-0 under the 35S promoter and two lines (*35S:BDG* #13, *35S:BDG* #21) with the highest expression level were also included.

First, I aimed to determine the expression sites of *BDG* and to verify if it was also transcribed elsewhere than published by Kurdyukov et al. (2006). For this, *BDG* promoter activity pattern was studied by expressing *GUS* and *GFP* marker genes under the control of the 2.6 kb *BDG* promoter (III). *BDG* expression was observed throughout the plant life cycle in the developing leaf buds, including nodes and buds of cauline leaves (III), which was an indicator for its importance in the developing leaf tissues. Interestingly, the expression of *BDG* was also present in regions characteristic to suberin deposition, such as the abscission zone of the silique, the chalaza/micropyle region of the seed, and the lateral roots (III). This prompted me to analyse the suberin monomer composition of roots. An overall 5–15% decrease in total aliphatic monomer content of roots was observed in *bdg* mutants, with the strongest reduction in C18 unsaturated monomers (III). Thus, these data suggest a more general role of BDG in the synthesis of plant lipid polyesters.

To address the molecular function of BDG in the cuticle biosynthesis over time, I analysed the cutin monomer content at various developmental stages of leaves and in flowers. It was previously shown that *bdg* loss-of-function mutants have increased wax and cutin loads (Kurdyukov et al., 2006). Indeed, the rosette leaves of 36-day-old *bdg-6* and *bdg-7* had up to 20% higher total cutin load than WT (figure 14A), consistent with previous observations (Kurdyukov et al., 2006). However, analysis of younger leaves revealed that the cutin load was up to 60% lower in the mutants than in WT (figure 14A). A decrease in total cutin load was also observed in mature flowers of the mutants (figure 14B). Up to a four-fold increase in total cutin load in leaves and up to a 40% increase in the flowers was detected in the overexpressors (figure 14A-B). Interestingly, these changes could not be observed for all monomers, and they were statistically significant for only some of them, such as 18:2 dicarboxylic acid (DCA) and C18 polyunsaturated ω-OH fatty acids (III). The notable decrease of some cutin monomer loads in *bdg* mutants and increase in the *BDG* overexpressors support the idea that BDG is a limiting factor in the biosynthesis pathway of those monomers.

Regarding its subcellular localization, BDG was found only in the extracellular space (Kurdyukov et al., 2006). The aim of this work was to provide additional proofs for the subcellular localization with another approach. For this transient expression of BDG:sGFP fusion protein in *N. benthamiana* was used. I detected BDG:sGFP as a network of strings within the cytoplasm resembling the pattern of ER (figure 15A-B). As a control for ER localization, AtFAAH, the fatty acid amide hydrolase from Arabidopsis involved in the N-acylethanolamine signalling, was used (Kang et al., 2008). AtFAAH showed a similar fluorescence pattern to BDG:sGFP (figure 15C). Moreover, sGFP:BDG could not be detected in the plasma membrane or cell wall in the plasmolysis

experiment (figure 15D-F). GFP fusion to the slow-type anion channel SLAC1 was used as a control for plasma membrane localization (Vahisalu et al., 2008), which showed clear plasmolysis-induced Hechtian strands as the structures of the plasma membrane (figure 15G-I). Interestingly, GFP fusion to the C-terminus of BDG induced weaker fluorescence compared to the fusion to the N-terminus, so the intracellular fluorescence pattern was verified only with the sGFP:BDG fusion protein. Thus, these data suggest that BDG could be located in the ER.

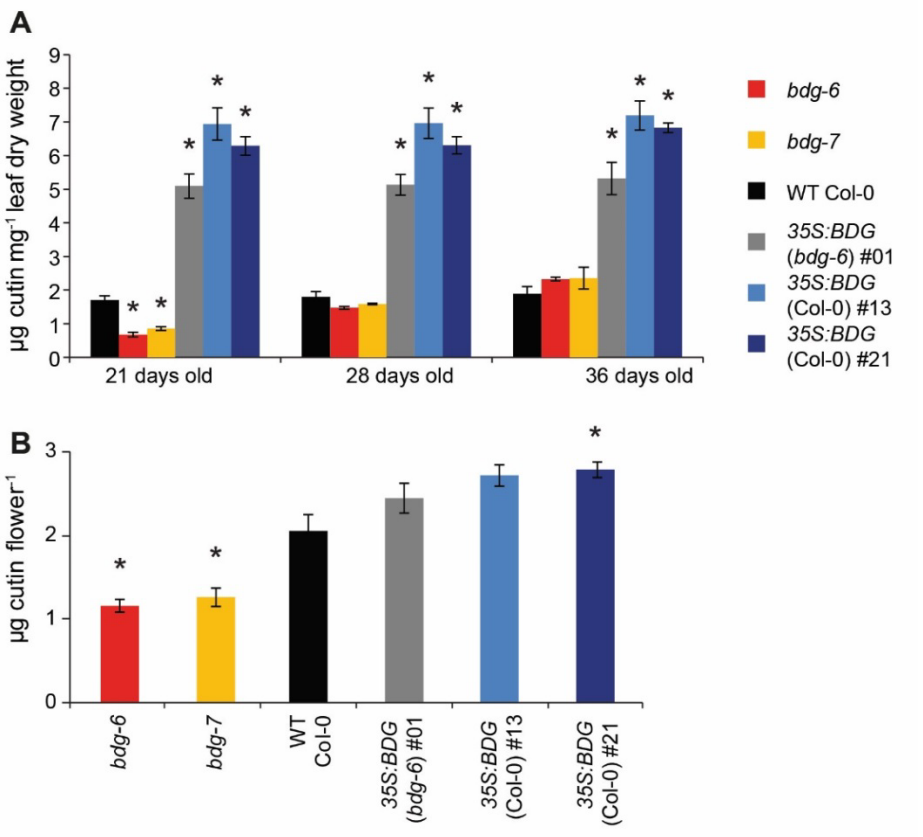


Figure 14. Analysis of cutin monomers in leaves and mature flowers of Arabidopsis (copied from ref III). (A) Total cutin load in rosette leaves from 21-, 28- and 36-day-old plants (mean ± SEM). Averages represent the mean of three to five biological repeats from altogether 11–18 plants. (B) Total cutin load of mature flowers on the first day of flowering (mean ± SEM). Averages represent the mean of five biological repeats, each containing 10 flowers. Statistically significant differences from WT are depicted with asterisks (2-sided T-test with Bonferroni correction; $p<0.001$).

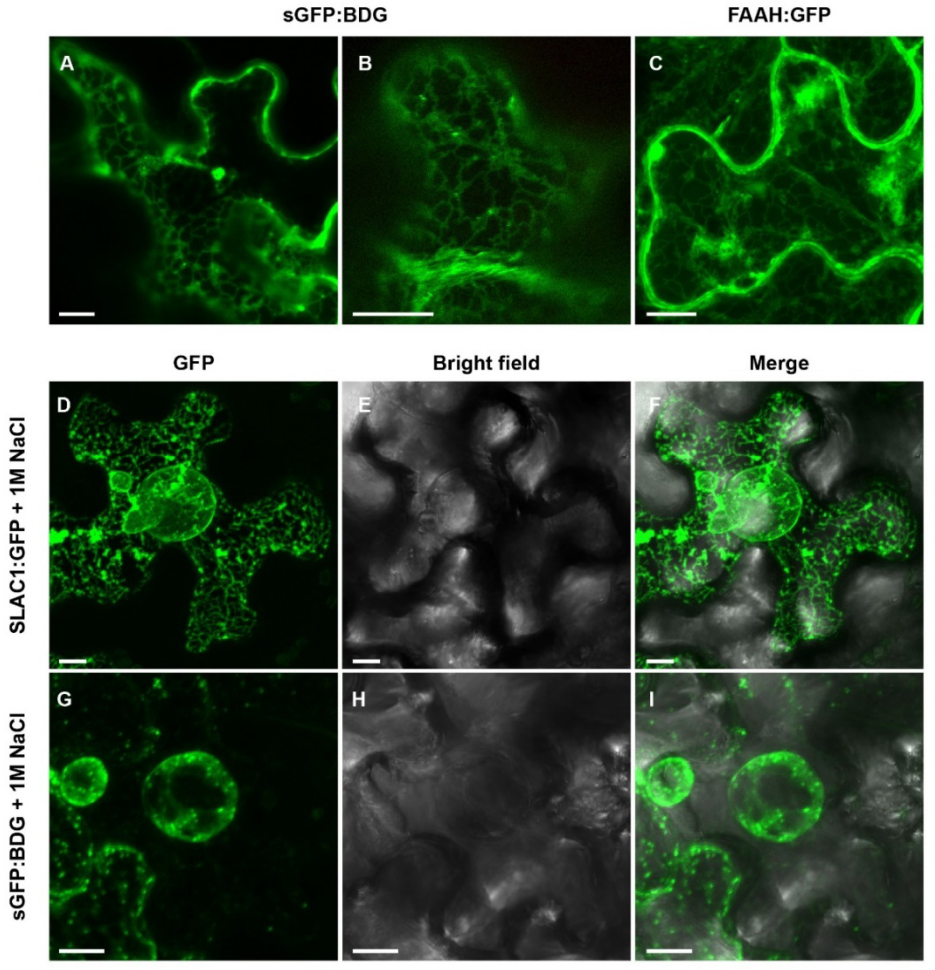


Figure 15. Subcellular localization of BDG. Confocal images of transiently expressed AtBDG:sGFP in *N. benthamiana* leaves show intracellular and a typical ER-specific pattern. (A-B) BDG N-terminally fused with GFP(S65T) shows the typical pattern of ER. (C) ER-localized AtFAAH C-terminally fused with sGFP(S65T) as a control. (D-F) Membrane-localized SLAC1 C-terminally fused with GFP after 2h of 1M NaCl incubation to induce plasmolysis. (G-I) BDG N-terminally fused with sGFP(S65T) after 2h of 1M NaCl incubation to induce plasmolysis. All scale bars denote 10 µm.

This data strongly suggest that BDG plays a crucial role for the content of C18 unsaturated cutin monomers, especially in young developing leaf tissues. BDG could affect the monomer load either via synthesis or incorporation of these monomers into the cutin polyester. BDG belongs to the α/β-hydrolase fold protein superfamily comprising many hydrolases and lipases. Thus, BDG might catalyse intracellular deacylation steps that could occur between various oxidation reactions. For example, deacylation might be required to move acyl

chains from the membrane lipids to the acyl-CoA pool. However, this would require that BDG localizes inside the cells, preferably in the ER, which is supported by this study (figure 15). Which of the localization patterns are correct and which factors influence the subcellular positioning of BDG, need further clarification. Moreover, biochemical studies to determine the exact reaction catalysed by BDG in the plant lipid biosynthesis pathway are also required.

The water barrier properties of the plant cuticle are known to depend mostly on the cuticular waxes (Schreiber, 2010). However, it has been shown that not only a decrease but also an increase in cutin amounts can lead to enhanced leaf water loss (Li et al., 2007). Cuticular permeability and cuticular conductance were strongly affected by the decrease in *BDG* expression level, but not by its several-fold increase (III). These results support the hypotheses that not only wax molecules are important in determining the water homeostasis on a whole plant level, but also that cutin monomer composition and cuticular integrity are crucial. Modulating the level of *BDG* expression could be a new way to modify the cutin biopolymer without affecting the water barrier properties of the plant. All-in-all, these results indicate that BDG might be used as a novel tool in breeding for the improvement of plant performance via cuticular properties.

5. CONCLUSIONS

- The ABA signalosome plays a major role in the regulation of plant stomatal conductance. High stomatal conductance was observed in plants with reduced ABA content, with non-operating PYR/RCAR proteins, with constitutively active PP2Cs, and in plants lacking OST1 or SLAC1. Low stomatal conductance was observed in plants lacking some of the PP2Cs and in plants with higher ABA content.
- The components of the ABA signalling pathway play an important role in stomatal closure due to reduced air humidity, darkness, and O_3. High CO_2-induced stomatal closure involves some of the ABA signalosome proteins, but presence of a parallel signalling pathway activating OST1 or SLAC1 is possible. All the studied closure-inducing stimuli require functional SLAC1 and OST1.
- The CO_2-related phenotypes of Cvi-0 and *cis* were both caused by mutations in *MPK12*. Lack of MPK12 function resulted in reduced stomatal responses to [CO_2] changes, impaired bicarbonate-induced activation of S-type anion channels, but ABA-induced stomatal closure remained intact. A model was suggested, where the high CO_2 signal is passed through MPK12 and MPK4 leading to inhibition of HT1, which in turn enables SLAC1 activation.
- A new method for screening mutants with impaired transpiration was introduced, which was based on temperature-dependent water spot condensation, visual wilting and weight loss of excised leaves. The method was used to screen 12,000 plants from an activation-tagged T-DNA mutant library to identify *cool breath* mutants.
- BDG plays a crucial role for the content of C18 unsaturated cutin monomers especially in young developing leaf tissues and flowers, and could hypothetically facilitate deacylation of acyl chains in the ER for the biosynthesis of these monomers.
- All-in-all, ABA, MPKs, and the cuticle all have their distinct role in regulating plant transpiration and coordinating gas exchange. ABA, together with the components of the ABA signalling pathway, seem to regulate stomatal closure in response to several abiotic stimuli, but high CO_2 might also trigger other regulators in the guard cells. Some plant MPKs, especially MPK12 and MPK4, have a role in stomatal CO_2 signalling via inhibiting HT1, which in turn allows SLAC1 activation. Cuticular permeability was not affected by increasing the expression level of BDG, indicating that BDG could be used to engineer the cutin polyester framework without affecting its water barrier properties.

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SUMMARY IN ESTONIAN

Abstsiishappe, CO_2 ning kutiikula roll taime transpiratsiooni regulatsioonis

Veatu gaasivahetuse regulatsioon on äärmiselt oluline taime elus püsimiseks ning aluseks põllumajandustaimede kõrgele saagikusele. Gaasivahetuse all peetakse silmas süsihappegaasi sisenemist taime lehte ning vee väljumist lehest atmosfääri. Süsihappegaasi on taimel vaja fotosünteesi käimas hoidmiseks, kuid veekadu peab taim võimalusel piirama, et vältida kuivale jäämist ja põua tõttu suremist. Enamik taime veekaost toimub läbi taime pinnal olevate väikeste pooride, õhulõhede, kuna teisi epidermiserakke katab vett hülgav lipiidne kutiikula kiht. Selleks, et saaksime tõsta saagikust või parandada taimede ellujäämust ekstreemsetes oludes, on oluline mõista molekulaarseid mehhanisme, millega reguleeritakse taime gaasivahetust nii õhulõhede kui kutiikula kaudu.

Esiteks oli töö eesmärk hinnata abstsiishappe signaaliraja valkude mõju taime õhulõhede juhtivusele ning abiootiliste stiimulite (pimedus, madal niiskus, kõrge CO_2 kontsentratsioon, lühiajaline osooni kontsentratsiooni tõus) poolt esile kutsutud õhulõhede sulgumisele. Õhulõhede juhtivus erines uuritud mutantide vahel 9 korda. Kõrge õhulõhede juhtivus oli taimedel, mis sisaldasid vähem abstsiishapet, millel oli maksimaalne hulk PYR/RCAR retseptorvalke välja lülitatud, milles olid konstitutiivselt aktiveeritud PP2C fosfataasid ja mis olid defektsed OST1 või SLAC1 ekspressiooni poolest. Madal õhulõhede juhtivus oli taimedel, milles ei ekspresseerunud mõned PP2C fosfataasid või mis sisaldasid tavapärasest rohkem abstsiishapet. Kõigi uuritud stiimulite korral oli normaalse õhulõhede sulgumise jaoks vajalik valkude SLAC1 ja OST1 olemasolu taimes. PP2C fosfataaside aktiivsus oli vajalik osooni ning madala niiskuse mõjul õhulõhede sulgumise jaoks, kuid vähem oluline pimeduse ning kõrge süsihappegaasi kontsentratsiooni mõjul sulgumise jaoks. PYR/RCAR abstsiishappe retseptorite olemasolu oli vajalik pimeduse, madala niiskuse ja osooni mõjul sulgumise jaoks, kuid süsihappegaasi reaktsioonides oli olulisus vähem selge ning viitas võimalike alternatiivsete signaaliradade olemasolule. Uuritud retseptoritest oli õhulõhede sulgumise jaoks olulisim PYL1. Lisaks, abstsiishappe metabolismi mutantide õhulõhede reaktsioonid olid vähem häirunud kui signaaliraja mutantidel. Kokkuvõtteks, saadud tulemuste põhjal võib järeldada, et abstsiishappe signaaliraja komponendid omavad olulist rolli osooni, madala niiskuse ja pimeduse toimel õhulõhede sulgumise reaktsioonides. Kõrge süsihappegaasi mõjul toimuvas õhulõhede sulgumises osalevad osad abstsiishappe signaaliraja komponendid, kuid tõenäoliselt aktiveerib OST1 kinaasi või SLAC1 anioonkanalit ka mõni teine, seni tuvastamata regulaator.

Teiseks, töö eesmärgiks oli ka kaardistada süsihappegaasiga seotud fenotüüpe põhjustavad mutatsioonid hariliku müürlooga ökotüübis Cvi-0 ja mutandis *cis* ning lisaks uurida ka kinaasi MPK12 rolli õhulõhede süsihappegaasi signaalirajas. Häirunud õhulõhede vastused süsihappegaasi kontsentratsiooni muutustele *cis* ja Cvi-0 taimedes olid tingitud vastavalt *MPK12* ekspressiooni

puudumisest või punktmutatsioonist funktsionaalselt olulises valgu piirkonnas. Leidsime, et *mpk12* mutantides oli vähenenud veekasutuse efektiivsus, madal õhulõhede juhtivus, nõrgenenud õhulõhede reaktsioonid süsihappegaasi kontsentratsioonile ning puudulik bikarbonaadi toimel aeglast tüüpi anioonkanalite aktivatsioon sulgrakkudes. Samas selgus, et abstsiishappe mõjul toimus mutantides õhulõhede sulgumine normaalselt. Biokeemilistest katsetest selgus, et MPK12 interakteerub HT1 kinaasiga ning et MPK12 inhibeerib HT1 kinaasi aktiivsust. Lisaks oli ka MPK4, kuid mitte MPK11 võimeline inhibeerima HT1 kinaasi aktiivust. Inhibitsioon ei sõltunud MPK12 enda kinaassest aktiivsusest, küll aga oli selles oluline roll MPK12 aminohappel glütsiin 53, mis on kõikides taime MPK valkudes konserveerunud. Seega saadud tulemuste põhjal pakuti välja mudel, mille järgi signaal süsihappegaasi kontsentratsiooni tõusust edastatakse taimes valkudele MPK12 ja MPK4, mis omakorda inhibeerivad HT1 kinaasi aktiivsust interaktsiooni käigus ning seeläbi võimaldatakse SLAC1 kanali aktivatsioon OST1 ja GHR1 poolt.

Kolmandaks, töö eesmärgiks oli kasutusele võtta uus meetod hariliku müürlooga taimede skriininguks, et selekteerida muutunud päevase transpiratsiooni tasemega mutante. Selleks töötati välja skriiningmeetodi, mis võtab arvesse taimelehtede temperatuuri, lõikamisjärgset närtsimist ning kaalukaotust. Meetodit on lihtne kasutada ning see ei vaja kallist ega spetsiaalset aparatuuri.

Neljandaks, töö eesmärgiks oli uurida valgu BODYGUARD (BDG) rolli taime transpiratsioonis ning kutiikula arengus. Leiti, et BDG puudumisel oli taimede kutikulaarne läbilaskvus ja transpiratsioon suurenenud, kuid BDG üleekspressiooni korral mainitud omadustes muutusi ei esinenud. Promooteri aktiivsuse järgi on BDG ekspresseeritud muu hulgas ka lehevõrsetes ning õites. Tuvastati, et *bdg* mutantide õites ning noortes lehtedes on kutiini monomeeride üldhulk vähenenud kuni 60% ning üleekspressiooni liinides on monomeeride üldhulk kuni neljakordistunud. Kuid muutunud polnud mitte kõikide monomeeride hulgad, vaid suurimateks muutujateks olid C18 dikarboksüülhapped ja C18 küllastumata ω-OH rasvhapped. BDG:sGFP valgu ekspressioon tubaka lehtedes viitas, et BDG valk võiks taimerakus asuda endoplasmaatilises retiikulumis, mis toetab hüpoteesi, et BDG valk on oluline seal toimuvas lipiidisünteesis. Seega, saadud tulemused viitavad, et BDG on oluline faktor C18 küllastumata kutiini monomeeride normaalse hulga saavutamiseks eelkõige noortes lehtedes ja õites ning et BDG võiks kaasa aidata endoplasmaatilises retiikulumis toimuvale monomeeride biosünteesile.

Kokkuvõtteks, abstsiishape, MPK kinaasid ja kutiikula on kõik olulised faktorid reguleerimaks taime transpiratsiooni ja gaasivahetust. Abstsiishape koos selle signaaliraja komponentidega mõjutab õhulõhede sulgumist mitmete erinevate abiootiliste stiimulite toimel, kuid kõrgenenud süsihappegaasi kontsentratsiooni korral on olulised ilmselt ka mõned abstsiishappest sõltumatud regulaatorid. Mõned taime MPK kinaasid nagu MPK12 ja MPK4 aktiveeritakse kõrgenenud süsihappegaasi kontsentratsiooni tingimustes selleks, et seeläbi inhibeerida HT1 kinaasi ja seejärel aktiveerida SLAC1 mõne aktivaatori nagu OST1 ja GHR1 poolt. Kuna kutikulaarne läbilaskvus ei muutunud kõrge BDG

ekspressiooni korral, siis oleks BDG valku võimalik kasutada disainimaks sellise kutikulaarse struktuuriga taimi, mille veevahetuse omadused poleks muutunud.

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