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Supervisors: Dr. Hannes Kollist
Institute of Technology, University of Tartu, Estonia

Dr. Ebe Merilo
Institute of Technology, University of Tartu, Estonia

Opponent: Dr. Nathalie Leonhardt
Institute of Environmental Biology and Biotechnology.
CEA Cadarache. UMR 7265 CNRS-CEA-Université
Aix-Marseille II. France

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Author's contribution

I. I did part of the experiments, analyzed part of the data and participated in writing the manuscript.

II. I participated in the experimental design, did most of gas-exchange experiments, analyzed the data, wrote the first draft of the manuscript and participated in the editing process.

III. I participated in carrying out part of the experiments, writing the manuscript and analyzing the data.

ABBREVIATIONS

ABA	Abscisic acid; plant hormone associated with drought response
ABI1/ABI2	ABA insensitive 1/2; protein serine/threonine phosphatase 2C (PP2C); negative regulators of ABA signaling
AHA1	Arabidopsis H^+ATPase 1
AKT2	Arabidopsis potassium transport 2; also known as AKT2/3; AKT3; K^+ channel
AtABCB14	*Arabidopsis thaliana* ABC transporter B14, malate transporter
AtALMT12	*Arabidopsis thaliana* Aluminum-activated malate transporter 12; R-type anion channel; also known as QUAC1
$[Ca^{2+}]_{cyt}$	cytosolic Ca^{2+} concentration
CBL	Calcineurin B-Like protein; interacts with CIPKs; acts as Ca^{2+} sensor
CDPK	Calcium-Dependent Protein Kinase
CIPK	CBL-Interacting Protein Kinase
CPK	Calcium-dependent Protein Kinase
GHR1	Guard cell Hydrogen peroxide-Resistant 1; receptor kinase
HAB1	Hypersensitive to ABA 1; PP2C
GORK	Guard cell Outward Rectifying K^+ channel
K^+_{in}	K^+ uptake channel
KAT1/2	Potassium channel in *Arabidopsis thaliana* 1/2
OST1	Open Stomata 1 kinase
PP2C	Type 2C protein phosphatase
PYR	Pyrabactin Resistance; ABA receptor
PYL	PYR1-like; ABA receptor
QUAC1	Quickly-activating Anion Channel 1; R-type anion efflux channel; also known as AtALMT12 channel
RCAR	Regulatory Components of ABA Receptors
ROS	Reactive Oxygen Species
SLAC1	Slow Anion Channel 1; S-type anion efflux channel

1. INTRODUCTION

Photosynthesis is the basis of life on Earth. Plants use energy from the sun and water from soil to fix carbon and release oxygen. Water taken up by roots moves aboveground in xylem conduits and is transpired to atmosphere from leaves. Uptake of CO_2 from atmosphere and the release of water molecules take place through small pores on the surfaces of green parts of plants. However, when plants' access to water in the soil is limited, transpiration can lead to wilting and death; to prevent this, the pores are surrounded by two specialized cells – guard cells –, which can lose turgor and thereby close the pore. The structures consisting of two guard cells and a microscopic pore in between are called stomata.

The mechanisms that lead to changes in guard cell turgor and size have been extensively studied for decades, but novel methods and possibilities in molecular plant biology have made stomatal regulation research a fast-developing and competitive field. Earlier stomatal research focused on tobacco and broad bean, which have relatively large stomata; now *Arabidopsis thaliana* is used as a model organism to study various aspects of plant genetics, development and functioning, including stomatal regulation. Signaling pathways, which are mostly located in guard cells, can respond to environmental, biological or chemical triggers.

This thesis studied stomatal opening and closure mechanisms focusing on S-type anion channel (SLAC1) and its upstream regulation. SLAC1 is located in the plasma membrane of guard cells and mediates anion efflux during stomatal closure. SLAC1 anion efflux channel is activated by a signalosome, which is described as the major signaling pathway for abscisic acid (ABA)-induced stomatal closure; however we show here that this signalosome is also crucial for stomatal closure in response to various environmental factors. Furthermore, compensatory changes in *slac1* loss-of-function mutants caused stomatal opening to be slower via down-regulation of K^+_{in} channels.

1.1. Guard cells regulate stomatal pore size

Stomata, microscopic pores in the epidermal layer of leaves and stems in land plants, evolved more than 400 million years ago (Edwards et al., 1998; Ruszala et al., 2011). The size of stomatal pores balances uptake of CO_2 and transpirational water loss. Stomatal regulation is often studied using the model organism thale cress (*Arabidopsis thaliana*, Fig. 1) due to its relatively small size, fully sequenced genome and the availability of mutants of ion channels, transporters, and their regulators (The Arabidopsis Genome Initiative, 2000).

Figure 1. *Arabidopsis thaliana* wild type Col–0 plant (on the left) and stomata (on the right) as seen in the epidermal peel of Col–0.

Size of stomatal pore is directly affected by the turgor pressure of guard cells (Fischer, 1973; Ache et al., 2010). Changes in the activity of ion channels and transporters in guard cell plasma membrane and tonoplast affect the concentration of ions and guard cell osmotic pressure, leading to changes in guard cell water content and stomatal pore size (Pandey et al., 2007). Stomata respond to plant water status and environmental and endogenous factors: light intensity, CO_2 concentration, air humidity and plant hormone ABA (Fig.2), with many secondary messengers like Ca^{2+}, H_2O_2 and NO involved in this response (Agurla et al., 2014). In addition to chemical signaling, stomata are also affected by hydraulic signaling: they close in response to reduced leaf water potential (Comstock, 2002). Stomatal conductance, measured in mmol m^{-2} s^{-1}, represents the amount of water vapor exiting through the stomata per leaf area. Signaling pathways for stomatal closure and opening are highly regulated, and a stimulus can activate one while inhibiting the other, as has been shown for Ca^{2+} (De Silva et al., 1985; Schroeder and Hagiwara, 1990). In addition to stomatal responses to short- and long-term changes in the environment, stomatal conductance has a circadian cycle, which continues to operate even when plants are placed into continuous light (Dodd et al., 2004). The regulation of stomatal conductance helps to avoid unnecessary water loss while ensuring the uptake of photosynthetic CO_2 and reducing leaf temperature.

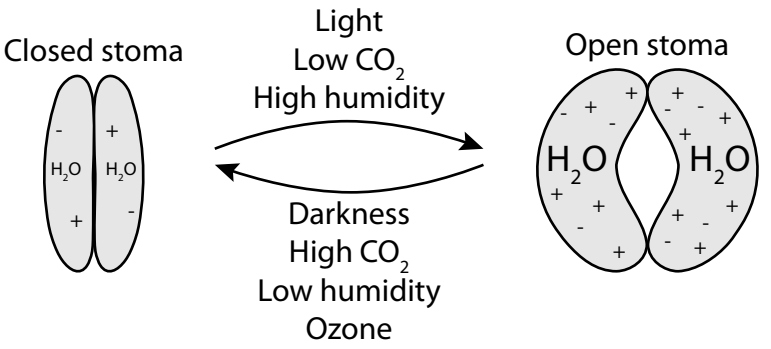

Figure 2. Guard cells respond to environmental factors causing stoma to close or open. Increase of cation (+) and anion (–) concentration in guard cells leads to water uptake and stomatal opening.

An increase in world population raises global food demand. Crop yields have increased in some areas, but are stagnating in many others (Edgerton, 2009; Ray et al., 2012). Agriculture already uses more than twice the amount of water compared to industry and municipalities combined (French and Schultz, 1984; Postel et al., 1996). Furthermore, there is a need to improve crop yield with much lower environmental impacts (Tilman et al., 2011). Water use efficiency (WUE), the ratio of the rate of photosynthesis to transpiration, is considered as an indicator of crop yield under drought stress and is dependent on stomatal conductance (Van Den Boogaard et al., 1997; Mei et al., 2013). However, WUE does not always predict plant yield, as plants with low yield and low water usage can have very high WUE (Blum, 2005). The challenge for the future is to improve plant WUE and its ability to cope with reduced water availability; solving this challenge requires fundamental research to understand stomatal regulation (Lawson and Blatt, 2014).

1.2. Regulation of stomatal opening

Rapid stomatal opening is induced by blue and red light, low CO_2 concentration and increase in air humidity (Kinoshita et al., 2001; Wang et al., 2010; Merilo et al., 2014). Stomatal opening requires ion movement into guard cells against concentration gradient: the majority of ion uptake is driven by the hyper-polarization of guard cells by H^+ATPase (Fig. 3). In case of light-induced stomatal opening, blue light causes autophosphorylation of guard cell phototropins PHOT1 and PHOT2 (Kinoshita et al., 2001; Ueno et al., 2005; Inoue et al., 2008), which in turn phosphorylate protein kinase BLUS1 (Takemiya et al., 2013). As a result of the activation of phototropins and BLUS1, H^+ATPase becomes activated by phosphorylation, followed by binding of 14-3-3 proteins (Svennelid et al., 1999; Takemiya et al., 2013) and, finally, H^+ extrusion from guard cells (Kinoshita and Shimazaki, 1999). ATP required

for the activity of H^+ATPase is mainly produced by photosynthesis in guard cell chloroplasts (Tominaga et al., 2001). Activation of H^+ATPases causes guard cell plasma membrane hyperpolarization (Roelfsema et al., 2001).

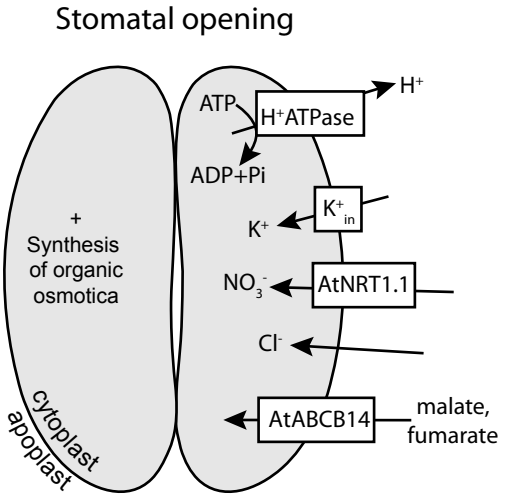

Figure 3. Stomatal opening begins with the activation of H^+ATPases, which actively transport protons out of guard cell, hyperpolarizing plasma membrane. This activates K^+_{in} channels. Malate and NO_3^- uptake is mediated by AtABCB14 transporter (Lee et al., 2008) and AtNRT1.1 (Guo et al., 2003), respectively. The Cl^- uptake transporter is not known (Kollist et al., 2014).

Hyperpolarization activates voltage-dependent K^+_{in} channels (Schroeder et al., 1987), resulting in the accumulation of K^+ in guard cells and stomatal opening (Humble and Raschke, 1971). K^+ ions are balanced with anions in the guard cells. The amount of Cl^- taken up by guard cells is insufficient to balance out the positive charge of K^+ (Humble and Raschke, 1971). Uptake of NO_3^- is mediated by AtNRT1.1 (CHL1) channel (Guo et al., 2003) which has two distinct nitrate affinity modes determined by phosphorylation-controlled dimerization (Sun et al., 2014). Organic anions like malate and fumarate participate in regulating guard cell turgor (Lee et al., 2008). In the morning, K^+ uptake is very important to increase guard cell turgor pressure, but later in the day, uptake of sugars such as sucrose also plays a role (Tallman, 2004).

Light-induced stomatal opening depends partly on photosynthesis (Talbott and Zeiger, 1998; Wang et al., 2011): in the blue and green light-induced opening responses, both photosynthesis-dependent and -independent components are involved, whereas red light response relies only on photosynthesis (Wang et al., 2011). Thus, although ATP and osmotica are produced in photosynthesis, inhibition of photosynthesis does not necessarily prevent light-induced stomatal responses. As regards CO_2- and humidity-induced stomatal opening, many details are still missing, however it is known that β-carbonic anhydrases βCA1

and βCA4, MATE-type transporter RHC1 and HT1 kinase are needed for CO_2-induced stomatal responses (Hashimoto et al., 2006; Hu et al., 2010; Tian et al., 2015).

1.3. SLAC1 is crucial during stomatal closure

Differently from ion pumps involved in stomatal opening, the ion channels involved in stomatal closure let selected ions diffuse across the electrical and concentration gradients without the need of added energy (Gadsby, 2009). Stomatal closure involves the release of ions through cation channels and slow- (S-) and rapid- (R-) type anion channels (Hosy et al., 2003; Negi et al., 2008; Vahisalu et al., 2008; Meyer et al., 2010). The two types of anion channels differ in kinetics: R-type channels are activated within 50 ms by depolarization and inactivated over time and by hyperpolarized potentials, whereas S-type channels have weak voltage-dependent activation and lack time-dependent inactivation (Schroeder and Keller, 1992; Raschke et al., 2003). In recent years, the genes encoding both slow and rapid type anion channels have been identified and this allows for more precise studies of the role of these proteins in stomatal regulation. Ion efflux channels found in Arabidopsis guard cells and functioning in stomatal closure are: S-type anion channels SLAC1 (Negi et al., 2008; Vahisalu et al., 2008) and its homologue SLAH3 (Geiger et al., 2011), R-type anion channel QUAC1 (Meyer et al., 2010) and cation efflux channel GORK (Hosy et al., 2003) (Fig. 4, Table 1).

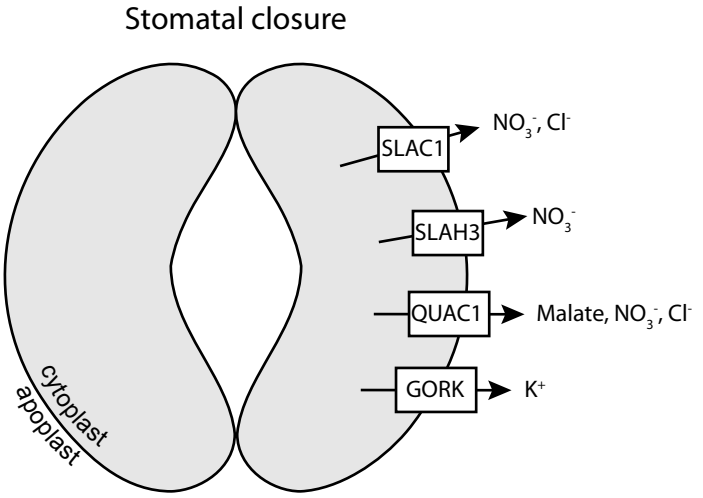

Figure 4. Stomatal closure depends on the activation of anion channels SLAC1, SLAH3, QUAC1 and K^+ channel GORK by phosphorylation or by depolarization of plasma membrane (see Table 1 for details).

Table 1. Guard cell ion efflux channels.

Abbreviation	Ions	Channel description	Regulation	Stomatal responses due to loss-of-function mutation	Source
SLAC1	NO_3^-, Cl^-	S-type anion channel	Phosphorylated and activated by OST1, GHR1 and CPKs (CPK21, CPK23 CPK3, CPK6, and CIPK23 which interacts with CBL1/CBL9). SLAC1 is slightly voltage-dependent and sensitive to (activated by) external Cl^- concentration	Clearly impaired darkness-, CO_2-, ABA-, ozone-induced stomatal closures.	(Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009; Lee et al., 2009; Hua et al., 2012; Maierhofer et al., 2014)
SLAH3	NO_3^-	S-type anion channel	Phosphorylated and activated by CPKs (CPK21,CPK23, and CIPK23 which interacts with CBL1/CBL9); interacts with CPK6. SLAH3 is slightly voltage-dependent and sensitive to (activated by) external NO_3 concentration	No significant phenotype	(Geiger et al., 2011; Scherzer et al., 2012; Maierhofer et al., 2014)
QUAC1/ AtALMT12	Malate, NO_3^-, Cl^-	R-type anion channel	Activated by PM depolarization and phosphorylation by OST1; extracellular malate shifts the activation threshold towards more hyperpolarized potentials	Impaired darkness- and CO_2-induced stomatal closure	(Meyer et al., 2010; Sasaki et al., 2010; Imes et al., 2013)
GORK	K^+	Voltage-gated outwardly rectifying K^+ channel	Activated by PM depolarization	Slightly slower closure	(Hosy et al., 2003)

SLAC1 channel is necessary for effective closure in response to elevated CO_2, darkness, low air humidity and ozone (Vahisalu et al., 2008). Plants lacking functional R-type anion channel QUAC1 (also called AtALMT12) have less impaired stomatal closure (Meyer et al., 2010), whereas *slah3* mutant behaves similarly to wild type (Geiger et al., 2011). The critical role of SLAC1 for anion efflux is also indicated by a strong reduction of S-type anion currents in *slac1* mutant guard cells (Vahisalu et al., 2008).

SLAC1 was first characterized as a Cl^- and malate efflux channel, but later it was shown that SLAC1 is eight times more permeable to NO_3^- compared to Cl^- (Geiger et al., 2009). SLAH3, another plasma membrane slow type anion channel is approximately 20 times more permeable to NO_3^- than Cl^- and it was shown that extracellular NO_3^- activated SLAH3 (Geiger et al., 2011). Thus it is considered that SLAC1 is involved in the efflux of Cl^- and NO_3^- whereas SLAH3 is more important for the release of NO_3^- (Roelfsema et al., 2012; Kollist et al., 2014). Basic properties and regulation of guard cell ion channels is summarized in Table I. SLAC1 anion channel is not involved in active anion uptake during stomatal opening (Negi et al., 2008; Vahisalu et al., 2008; Chen et al., 2010).

1.4. ABA signalosome

ABA is a plant hormone involved in a diverse array of processes in the plant, from seed germination to leaf senescence (Cutler et al., 2010; Finkelstein, 2013). ABA affects stomata by inducing closure and inhibiting opening. The concentration of ABA is affected by its synthesis, conjugation, transport and catabolism. Higher plants synthesize ABA indirectly from carotenoids (Finkelstein, 2013). *ABA1* encodes enzyme zeaxanthin epoxidase that converts zeaxanthin to violaxanthin (Rock and Zeevaart, 1991) and *ABA3* encodes sulfurase that produces molybdenum cofactor for abscisic aldehyde oxidase converting abscisic aldehyde to abscisic acid (Léon-Kloosterziel et al., 1996; Finkelstein, 2013). ABA concentration is reduced by catabolism: CYP707A1- CYP707A4 catalyze ABA 8'- hydroxylation (Kushiro et al., 2004; Saito et al., 2004) and as a side-reaction, also ABA 9'- hydroxylation (Okamoto et al., 2011).

ABA receptors and the signaling pathway for ABA-induced stomatal closure with the activation of SLAC1 were described in 2009 (Ma et al., 2009; Park et al., 2009). PYR/RCAR family of cytosolic ABA receptors consists of 14 members in Arabidopsis. PYRs are structurally distinct: PYR1, PYL1 and PYL2 form dimers, whereas PYL4, PYL5, PYL6, PYL8, PYL9 and PYL10 are monomeric, and PYL3 can be mono- and dimer (Dupeux et al., 2011; Hao et al., 2011). Both monomeric and dimeric PYRs can form high-affinity complexes with Protein Phosphatases 2C (PP2Cs) (Hao et al., 2011). PYL5-PYL10 belong to a subclass, which shows ABA-independent inhibition of PP2Cs (Hao et al., 2011) (Fig. 5B). Perception of ABA by PYR/RCAR receptors leads to the inhibition of

PP2Cs) and activation of protein kinase Open Stomata 1 (OST1) (Ma et al., 2009; Park et al., 2009), possibly by autophosphorylation of OST1 (Belin et al., 2006). OST1 then activates SLAC1 by phosphorylation, followed by anion efflux and stomatal closure (Geiger et al., 2009; Lee et al., 2009; Vahisalu et al., 2010; Brandt et al., 2012). In the absence of ABA, PP2Cs keep OST1 inactive by direct binding and dephosphorylation (Belin et al., 2006; Yoshida et al., 2006) (Fig. 5A).

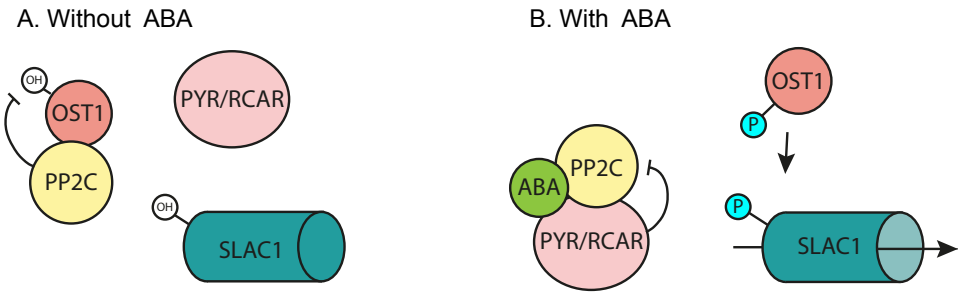

Figure 5. ABA-signaling leads to the activation of SLAC1. In the absence of ABA, PP2C phosphatases keep OST1 inactive and SLAC1 anion channel is closed (A). When ABA binds to PYR/RCAR, a complex is formed between ABA, PYR/RCAR receptors and PP2C phosphatases (B), which enables the activation of OST1 and SLAC1 (Ma et al., 2009; Park et al., 2009). SLAC1 can also be activated by other kinases (Fig. 6).

PP2Cs ABA insensitive 1 and 2 (ABI1 and ABI2), hypersensitive to ABA 1 (HAB1) and Protein phosphatase 2A (PP2AC-2) act as negative regulators in ABA signaling pathway (Merlot et al., 2001; Mustilli et al., 2002; Robert et al., 2006; Pernas et al., 2007; Hua et al., 2012). Well-studied *abi1-1* and *abi2-1* mutants lack ABA induced activation of guard cell S-type anion channels and stomatal closure (Pei et al., 1997) because these mutants have a single amino acid exchange point mutations, $ABI1^{G180D}$ and $ABI2^{G168D}$, that abolish their interaction with ABA receptors (Ma et al., 2009). Thus, in these mutants ABI1 and ABI2 phosphatases are not inactivated by ABA and OST1 remains inactive. Compared to these constitutively active *abi1-1* and *abi2-1* mutants, loss of function mutants of ABI1 and ABI2 have wild type-like response to ABA and loss of function double mutant is hypersensitive to ABA (Rubio et al., 2009). Mutation $HAB1^{G246D}$ prevents the mutated phosphatase from binding to ABA receptors similarly to the mutations in *abi1-1* and *abi2-1*, resulting in reduced sensitivity to ABA (Robert et al., 2006).

Plant lines carrying mutations in genes involved in the core ABA- signaling complex are available for studying stomatal responses to different factors. OST1 kinase is a major regulator of CO_2-induced stomatal closure and necessary for the activation of S-type anion channels in guard cells in response to bicarbonate and low humidity (Yoshida et al., 2006; Xue et al., 2011). As regards stomatal closure in response to darkness, *ost1* mutants were initially shown to have wild

type-like phenotypes (Mustilli et al., 2002), but Ache et al. (2010), showed that *ost1-2* has reduced stomatal closure during night. Dominant active mutants *abi1-1* and *abi2-1* had impaired CO_2-induced stomatal closure (Webb and Hetherington, 1997; Leymarie et al., 1998) and wild type-response to change in air humidity (Assmann et al., 2000), although ABA concentration increases in low air humidity (Monda et al., 2011), indicating that ABA is involved in humidity response. Apparently, links between ABA signaling and stomatal responses to environmental factors such as elevated CO_2, darkness, air humidity and air pollutant ozone deserve further clarification.

1.5. Role of Ca^{2+} in stomatal regulation

ABA-induced stomatal closure is Ca^{2+}-independent in *Vicia faba*, but *Nicotiana tabacum* and *Arabidopsis thaliana* exhibit both Ca^{2+}-dependent and Ca^{2+}-independent stomatal closure in response to ABA (Levchenko et al., 2005; Marten et al., 2007; Siegel et al., 2009). There are around 250 proteins in Arabidopsis, which contain Ca^{2+}-binding EF-hand motif (Day et al., 2002), among them 34 Ca^{2+}-dependent Protein Kinases (CPKs) (Cheng et al., 2002) and 10 Calcineurin B-Like proteins (CBLs) (Kolukisaoglu et al., 2004). CBLs form complexes with CBL-Interacting Protein Kinases (CIPKs) (Luan, 2009). There is a high structural similarity among CBLs and CIPKs, but these complexes have very limited functional redundancy (Cheng et al., 2002).

Calcium has long been known to be involved in the regulation of stomatal closure (Schwartz, 1985). Measuring Ca^{2+} changes in guard cells using Yellow Chameleon construct revealed that stomata respond to specific patterns of calcium oscillations, also termed as calcium signatures (Allen et al., 2001). Ca^{2+} oscillations during stomatal closure originate mostly from extracellular Ca^{2+} and can be prevented or decreased by extracellular Ca^{2+}-chelators (Schwartz, 1985; Knight et al., 1996; Klüsener et al., 2002; Siegel et al., 2009). Besides, Ca^{2+} can be released into the cytosol from vacuole (Knight et al., 1996). Even when Ca^{2+} oscillations are prevented by Ca^{2+}-chelators, guard cells are still able to respond to ABA, but the response is decreased (Siegel et al., 2009; Hubbard et al., 2012). Conditions that prevent Ca^{2+} oscillations strongly inhibit CO_2-induced stomatal closing as well (Hubbard et al., 2012)

Enhanced Ca^{2+} sensitivity, or Ca^{2+} priming, has been shown for S-type anion channel activation. Intracellular cytosolic free calcium ($[Ca^{2+}]_{cyt}$) alone did not activate S-type anion channels in Arabidopsis, but if guard cell protoplasts were pre-exposed to high external Ca^{2+} during isolation, then high Ca^{2+} treatment rapidly activated S-type anion currents during recordings (Allen et al., 2002). Increased Ca^{2+} sensitivity of S-type anion channel activation was triggered by elevated CO_2 and pre-incubation with ABA (Siegel et al., 2009; Xue et al., 2011).

1.6. Activation of SLAC1 anion channel

Guard cell anion efflux channel SLAC1 is closed unless it is phosphorylated (Brandt et al., 2012). Several protein kinases are able to phosphorylate SLAC1 in guard cells: OST1 (Geiger et al., 2009; Lee et al., 2009; Vahisalu et al., 2010), CPKs (Geiger et al., 2010; Brandt et al., 2012), Guard cell Hydrogen peroxide Resistant 1 (GHR1) (Hua et al., 2012) and CIPK23 interacting with CBL1/CBL9 (Maierhofer et al., 2014) (Fig. 6). OST1 and GHR1 are both crucial for the initiation of stomatal closure (Mustilli et al., 2002; Hua et al., 2012) and both phosphorylate SLAC1 in *Xenopus* oocytes (Lee et al., 2009; Geiger et al., 2010; Hua et al., 2012), yet they are not compensatory as stomatal closure is nearly abolished in single mutants of either OST1 or GHR1. Loss of function mutations in OST1 and GHR1 have very slow stomatal closure and high stomatal conductance (Xue et al., 2011; Hua et al., 2012). Although many CPKs are able to activate SLAC1 in *Xenopus* oocytes, plants with all CPKs functional show no SLAC1 activation if either OST1 or GHR1 is missing (Geiger et al., 2009; Hua et al., 2012). To explain this, Scherzer et al. (2012) discussed the possibility that OST1 is required for the activation of CPKs or OST1 controls $[Ca^{2+}]_{cyt}$, which in turn determines CPK activation.

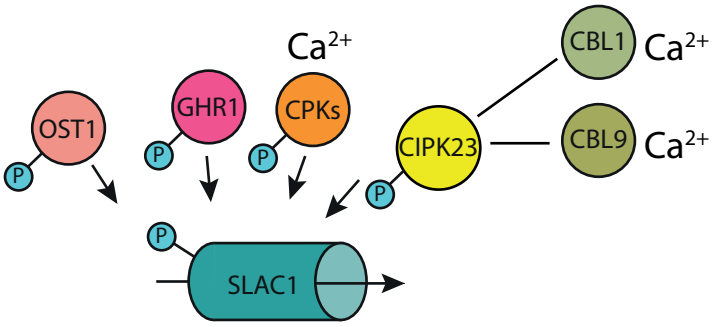

Figure 6. SLAC1 anion efflux channel, which is opened by phosphorylation, has been shown to be phosphorylated by many protein kinases: OST1 (Geiger et al., 2009), GHR1 (Hua et al., 2012), CPKs (Geiger et al., 2010; Brandt et al., 2012) and CIPK23-CBL1/CBL9 (Maierhofer et al., 2014).

SLAC1 has many activation sites phosphorylated by different kinases: CPK6 phosphorylates SLAC1 site S59, whereas OST1 phosphorylates sites S59, S86, S113 and S120 in the N-terminal region of SLAC1 (Vahisalu et al., 2010; Brandt et al., 2012). SLAC1 site S59 can also be phosphorylated by CPK23 and CIPK23-CBL1/CBL9 (Maierhofer et al., 2014). The activation of SLAC1 by OST1 is very unlikely to require Ca^{2+} because neither SLAC1 nor OST1 have Ca^{2+}-binding EF hands. Furthermore, OST1 is able to phosphorylate SLAC1 in *Xenopus* oocyte system without plant Ca^{2+}-binding proteins (Day et al., 2002; Geiger et al., 2009; Hua et al., 2012). When SLAC1 was first described, both

slac1-1 and *slac1-3* were shown to have reduced response to Ca^{2+} oscillations, which indicates that Ca^{2+}-dependent stomatal closure also requires SLAC1 anion channel activation (Vahisalu et al., 2008).

SLAC1 can be activated by Ca^{2+}-dependent protein kinases CPK6 (Brandt et al., 2012), CPK21 and CPK23 (Geiger et al., 2010) and CPK3 (Scherzer et al., 2012). Ca^{2+}-dependence of CPKs can vary remarkably: the kinase activities of CPK3 and CPK21 are much more Ca^{2+}-dependent compared to CPK6 and CPK23. At low Ca^{2+} concentration CPK3 and CPK21 are mostly inactive, while CPK6 and CPK23 still have relatively high kinase activity (Scherzer et al., 2012). CPK6, CPK21 and CPK23 all have 4 EF-hands, as is typical for Arabidopsis CPK/CDPKs, but CPK3 has only one EF-hand (Day et al., 2002). SLAC1 and SLAH3 anion channels are also activated by CIPK23, which interacts with CBL1 and CBL9 (Maierhofer et al., 2014). Many other CPKs have been investigated for their potential involvement in stomatal closure; *cpk10*, *cpk4cpk11* and *cpk32cpk7cpk8* mutants had impaired Ca^{2+}-induced stomatal closure, but ABA-induced closure was similar to wild type (Hubbard et al., 2012). Previously, *cpk10* was shown to have strongly impaired ABA- and Ca^{2+}-induced stomatal closure (Zou et al., 2010).

In addition to mediating the Ca^{2+}-independent pathway of stomatal closure by directly phosphorylating SLAC1 (Geiger et al., 2009), OST1 also activates Ca^{2+} -dependent pathway of stomatal closure through ROS production. OST1 phosphorylates and activates guard cell NADPH oxidase Respiratory Burst Oxidase Homolog F (RBOHF) (Kwak et al., 2003; Geiger et al., 2009). This plasma membrane bound protein produces superoxide, which is rapidly transformed to hydrogen peroxide (H_2O_2) in the apoplast (Zhang et al., 2004). H_2O_2 in turn can activate plasma membrane Ca^{2+} channels, causing Ca^{2+} oscillations in guard cells (Pei et al., 2000). This leads to the activation of SLAC1, possibly by Ca^{2+}-dependent kinases and CIPK23 (Geiger et al., 2010; Brandt et al., 2012; Maierhofer et al., 2014). Ca^{2+} also promotes further production of H_2O_2 by activating RBOHFs through binding to their EF-hand domains (Ogasawara et al., 2008) and activating CBL1/9-CIPK26 complex (Drerup et al., 2013). This could act as an enhancement loop to increase $[Ca^{2+}]_{cyt}$.

1.7. Co-regulation of stomatal opening and closure

In natural conditions, guard cells are affected by several stimuli at once. It would be inefficient to activate opening and closure pathways simultaneously, thus it is reasonable to expect that plants have developed co-regulation and cross-talk of these pathways.

There are at least four ways to separate stomatal opening from stomatal closure. 1) OST1 kinase, which is active during stomatal closure, simultaneously activates SLAC1 anion efflux channel and inactivates potassium

uptake channel KAT1 in *Arabidopsis thaliana* (Geiger et al., 2009; Sato et al., 2009), resulting in reduced potassium uptake during stomatal closure. 2) Ion channels are regulated by plasma membrane potential: hyperpolarization leads to the activation of potassium uptake channels KAT1/KAT2, whereas depolarization leads to the activation of rapid type anion efflux channel AtALMT12 and potassium efflux channel GORK (Schroeder et al., 1987; Roelfsema et al., 2001; Hosy et al., 2003; Meyer et al., 2010). 3) Ca^{2+} oscillations: Ca^{2+} activates many CPKs involved in stomatal closure and inactivates K^{+}_{in} channels (Grabov and Blatt, 1999; Mori et al., 2006; Geiger et al., 2010). 4) ABA signaling induces stomatal closure and inhibits stomatal opening (De Silva et al., 1985), e.g. ABA inhibits blue light-induced phosphorylation of H^{+}ATPase (Hayashi et al., 2011).

Due to tight cross-talk between stomatal opening and closure pathways, stomatal opening response might be affected in mutants with impaired stomatal closure.

2. AIMS

The ability to regulate stomatal openness ensures plant survival and enables to optimize photosynthesis in continuously changing environment. SLAC1 anion channel mediates anion efflux from guard cells and is crucial for stomatal closure in response to several stimuli. During stomatal opening, SLAC1 must be inactivated to avoid anion efflux and depolarization. This implies that stomatal opening might be faster in *slac1* plants.

Stomatal closure in response to ABA is mediated by ABA signalosome: PYR/RCAR receptors, PP2C phosphatases and OST1 kinase. Some mutants of ABA signalosome have impaired closure in response to some environmental factors, but their role in CO_2-, darkness-, air humidity- and ozone-induced closures remained unclear.

The aims of my thesis were to:

- Study the role of ABA signalosome components in stomatal closure induced by environmental factors: reduced air humidity, elevated CO_2 concentration, darkness and air pollutant ozone.
- Study the role of SLAC1 in stomatal opening in response to light, increased air humidity and decreased CO_2 concentration and the mechanism behind potential changes in opening responses of *slac1* mutants.
- Study stomatal opening responses of mutants with similar phenotype to *slac1* to reveal general patterns in stomatal regulation.

3. MATERIALS AND METHODS

3.1. Plant growth

Seeds were stratified in distilled water at 4°C and then planted in the soil. Plants were grown in controlled conditions in peat:vermiculite mixture. Light cycle of 12 hours day (light intensity 100–150 μmol m^{-2} s^{-1}) and 12 hours night was used, with temperatures 23/18 °C.

Plants used for gas exchange measurements were grown through a hole in a glass plate. This was achieved by securing a glass plate on top of a pot filled with soil. Pre-stratified seeds were placed in the soil inside 3 mm hole in the middle of the glass plate and placed in growth chamber. Excessive seedlings were removed after several days to get one plant growing in each pot. Approximately 23–28 days old plants were used for gas-exchange experiments; this corresponds to total leaf area between 5 and 15 cm^2. However, some mutants (*aba1-1*, *aba3-1*, *abi1-1C*, and $hab1^{G246D}$) had slower growth rates; thus, older plants (26–32 d) were analyzed.

3.2. Gas exchange measurements

Plant stomatal conductance was measured using an 8-cuvette flow-through gas exchange measurement device (Kollist et al., 2007). This device measures air humidiy, CO_2 concentration, ozone and temperature inside the cuvettes. Intact plants are inserted into the device and can be treated with changes in CO_2, light, air humidity and ozone. From differences in CO_2 and H_2O concentrations of the air going into the cuvette and coming out, net assimilation rate and transpiration were calculated, then stomatal conductance was derived from transpiration using a custom-written programme.

3.3. qPCR

Guard cells from plants grown in pots without glass cover were isolated using the protocol of Leonhardt et al. (2004). Plant RNA was extracted using the RNeasy Plant Mini Kit and converted to cDNA using the QuantiTect Reverse Transcription Kit (both from Qiagen GmbH, Germany). The 7900HT Fast Real-Time PCR System was used with the MicroAmpTM Optical 384-well Reaction Plate (both from Applied Biosystems, Singapore) and MicroAmpTM Optical Adhesive Film (Applied Biosystems, Foster City, CA, USA). For qPCR reactions, the MaximaTM SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, Thermo Scientific Molecular Biology) was used. The TIP41-like family protein (At4g34270; (Czechowski et al., 2005)) was used as the normalization standard.

4. RESULTS AND DISCUSSION

4.1. ABA signalosome maintains steady-state stomatal conductance

The signaling pathway for ABA-induced activation of SLAC1 anion channel and concurrent stomatal closure were described a year after the characterization of SLAC1 (Ma et al., 2009; Park et al., 2009). ABA binds to PYR/RCAR receptors in the cytosol of guard cells. As a result, PP2Cs are inactivated and OST1 kinase is free to activate SLAC1 anion channel (Ma et al., 2009; Park et al., 2009). We studied the mutants involved in ABA signaling pathway (listed in I, Table I) to address the role of ABA signalosome for steady-state stomatal conductance and for stomatal closure induced by darkness, elevated CO_2, low air humidity and ozone (I).

These experiments showed that ABA signaling is important for determining basal steady-state stomatal conductance as the stomatal conductance values of ABA signalosome mutants varied 7-fold (I, Fig. 2). Stomatal conductances of *slac1-3* and *ost1-3* were higher than wild type (I, Fig. 2, Fig. 4). In *slac1-3* mutant, impaired anion efflux channel leads to ion accumulation and more open stomata (Negi et al., 2008; Vahisalu et al., 2008). The mechanism can be explained similarly in mutants with impaired OST1 (Mustilli et al., 2002). OST1 kinase is a central regulator for anion efflux, activating SLAC1 and QUAC1 and inactivating KAT1 (Geiger et al., 2009; Sato et al., 2009; Imes et al., 2013). Therefore, without OST1, anion channels are not activated to initiate stomatal closure and stomatal conductance is constantly high.

Plants carrying dominant *abi1-1* and *abi2-1* mutations that prevent inhibition of ABI1 and ABI2 protein phosphatases by PYR/RCAR receptors keep OST1 constantly inactive, explaining why ABA does not induce the activation of S-type anion channels in these mutants (Pei et al., 1997). However, *abi1-1* and *abi2-1* had even higher stomatal conductance than *slac1-3* and *ost1-3* (I, Fig. 2). If the high stomatal conductance of *abi1-1* and *abi2-1* was only caused by the inhibition of OST1 and SLAC1, then it would be at the same level as that of *ost1-3* and *slac1-3*. Since the stomatal conductance of *abi1-1* and *abi2-1* was higher, it is likely that ABI1 and ABI2 affected stomatal openness partly independently of OST1 and SLAC1. The activation of H^+ATPases, which hyperpolarize plasma membrane and induce stomatal opening is inhibited by ABA; moreover, *abi1-1* and *abi2-1* mutants had higher basal and blue light-induced activation of H^+ATPases (Hayashi et al., 2011). While dominant active mutations in PP2Cs increase stomatal conductance, opposite effect can be observed in triple mutant *abi1-2hab1-1pp2ca-1*, an ABA hypersensitive loss-of-function mutant of three PP2Cs, which had nearly two times lower stomatal conductance than wild type plants (Rubio et al., 2009) (I, Fig. 2).

Steady-state stomatal conductance of PYR/RCAR mutants clearly depended on the number of PYR/RCARs affected (I, Fig. 2, Fig. 7). Stomatal conductance

of triple PYR/RCAR mutants was higher than in wild type and it increased further when more ABA receptors were knocked out. The sextuple mutant *112458* had the highest stomatal conductance – approximately 4 times of wild type value. Similarly with *abi1-1* and *abi2-1* mutants, *112458* may show higher basal guard cell activation of H^+ATPases, further increasing stomatal conductance. In roots, *112458* exhibited about 30% higher basal plasma membrane H^+ATPase activity compared to wild type (Planes et al., 2014). Disruptions in ABA synthesis also led to higher steady-state stomatal conductance in *aba3-1* and *aba1-1* (Ler), whereas ABA catabolism mutants *cyp707a1* and *cyp707a3* had slightly lower stomatal conductance than wild type.

The large variation in steady-state stomatal conductance of ABA signalosome mutants indicates that basal ABA signaling through PYR/RCAR receptors plays a fundamental role in controlling plant steady-state stomatal conductance.

4.2. ABA signalosome in the regulation of stomatal closure

Our aim was to study the role of ABA signalosome components for O_3-, darkness-, low humidity- and elevated CO_2-induced stomatal closure in intact plants (I). It has been shown that guard cell anion channel SLAC1 mediates stomatal closure regardless of stimulus (Vahisalu et al., 2008). OST1, the primary regulator of SLAC1 is involved in stomatal response to ABA (Mustilli et al., 2002), CO_2 (Xue et al., 2011), ozone (Vahisalu et al., 2010), low air humidity (Yoshida et al., 2006) and darkness (Ache et al., 2010) (I). OST1 and SLAC1 are thus necessary for stomatal closure in response to all studied factors, whereas the level of impairment was stronger in *ost1* than *slac1* mutants (I). This makes sense: besides SLAC1, OST1 has other substrates that play a role in the regulation of stomatal movements, such as QUAC1 and potassium uptake channel KAT1. Dominant *abi1-1* and *abi2-1* mutations also caused impaired darkness-, reduced humidity- and O_3-induced stomatal closure and showed reduced bicarbonate-induced activation of S-type anion channels and partial impairment of CO_2-induced stomatal responses (I).

Plant line carrying mutations in six PYR/RCAR ABA receptors – *112458* – had impaired stomatal closure in response to darkness, reduced air humidity, elevated CO_2 and O_3 (I). PYR/RCAR receptors specifically bind ABA, meaning that ABA itself is important for studied stomatal responses. The sextuple mutant *112458* that displayed the strongest impairment of stomatal responses among the tested PYR/RCAR mutants, lacks functional dimeric PYR1, PYL1 and PYL2 (Gonzalez-Guzman et al., 2012). Another mutant, *1124C*, also lacking these three receptors in addition to PYL4, had impaired response to reduced air humidity and ozone (I, Fig. 7). Thus, dimer-forming PYR1, PYL1 and PYL2 and monomeric PYL4 might be important for stomatal responses to abiotic factors. Furthermore, our results indicate that there might be a functional

specificity among PYR/RCARs necessitating further studies. Dimeric PYR1, PYL1 and PYL2 have lower ABA-binding affinity, with K_D values approximately 50–100 times higher than for monomeric PYL5, PYL6 and PYL8 (Dupeux et al., 2011). It is therefore possible that monomeric PYRs participate in determining the steady-state stomatal conductance, whereas dimeric PYRs are more important in determining closure responses. Accordingly, Dupeux et al. (2011) discussed that when cytosolic ABA levels are low, monomeric receptors control PP2Cs, whereas dimeric receptors become activated when stress-induced ABA is synthesized.

ABA biosynthesis mutants *aba1-1* and *aba3-1* still contain ABA and show some stress-induced ABA biosynthesis (Rock and Zeevaart, 1991; Léon-Kloosterziel et al., 1996); explaining why *aba1-1* and *aba3-1* mutants had wild type-like or even greater stomatal closure responses (I). Thus, the residual level of ABA in *aba3-1* and *aba1-1* (Rock and Zeevaart, 1991; Szostkiewicz et al., 2010) appears to be sufficient to trigger stomatal closure in response to different stimuli.

4.3. Link between high conductance and reduced stomatal responses

Generally, mutants and accessions with higher steady-state stomatal conductance often show impaired responses to environmental factors (I) (Webb and Hetherington, 1997; Vahisalu et al., 2008; Monda et al., 2011; Xue et al., 2011). This is to be expected: ion extrusion is impaired in these lines, affecting both steady-state conductance and stimuli-induced closure. The degree of impairment depends on the defect, e.g. *abi1-1* and *abi2-1*, although showing larger steady-state conductance compared to *slac1* and *ost1*, revealed stronger closure responses. This can be explained by stimulus-induced inactivation of other, intact PP2Cs in *abi1-1* and *abi2-1*. However, several PYR/PYL mutants (triple mutants listed in I, Table II*, 1458, 12458)* had high stomatal conductance combined with wild type like or even stronger stomatal closure (I, Fig. 7, Fig. S7). This result further indicates that PYR/PYL receptors have distinct functions in stomatal regulation with some affecting basal stomatal conductance, whereas others affecting stomatal responsiveness. It is also possible that in PYR/PYL mutants, compensatory changes might have taken place to reduce the effect of missing receptors (see discussion below).

It is important to consider whether we compare stomatal responses in absolute or relative units. For example, after 32 minutes in darkness, PYR pentuple mutant *12458* closed 74 units compared to 70 in Col-0. In relative terms, however, this was 14% versus 40% of closure in *12458* and Col-0, respectively (I, Fig. 7). On the other hand, *abi1-2hab1-1pp2ca-1* mutant with very low stomatal conductance closed 41 units with relative closure of 67% in response to darkness (I, Fig. 5). Thus comparing only absolute or relative

responses can easily result in misleading conclusions, when initial stomatal conductance is different.

To describe stomatal closure kinetics and to find additional characteristics that can be used to compare closure responses of plant lines with different initial conductances, we fitted an exponential function to stomatal closure curves and calculated maximum stomatal closure rate. In many cases, stomatal closure kinetics was affected by the mutation and fitting stomatal response to exponential function was not possible, indicating that the closure was impaired (I, Table II). When deciding whether a particular defect has affected stomatal regulation, we considered all calculated closure characteristics: absolute response, subjection to an exponential fitting and maximum closure rate.

After keeping plants in elevated CO_2 or darkness for 60 minutes, we switched to normal CO_2 or light and measured the re-opening of stomata. Generally, upon re-opening, plants tended to restore initial stomatal conductance values within 1 hour (I), indicating that there is an innate plant- and line-specific stomatal conductance value that is regained after stress alleviates.

To study stomatal opening of *slac1* mutants in comparison with wild type (Col-0), we tried different sets of conditions and exposure times to achieve similar stomatal conductance values, as this would enable straightforward comparison of their opening responses. We found that keeping plants at low air humidity (relative air humidity 17.9 ± 0.7%) for 2–3 hours allows stomatal conductance of *slac1* and wild type to achieve similar values. This is not unexpected: as shown in Fig. 4 of I, stomata of *slac1* started to close in dry air after an initial delay.

4.4. Stomatal opening is slower in *slac1* mutant

We studied stomatal opening responses of *slac1* mutants after stabilizing steady-state stomatal conductance to similar level to that as in wild type (II). After 2–3 hours at low air humidity, the differences between stomatal conductance values of wild type, *slac1-1* and *slac1-3* were non-significant in all experiments (Fig. 7A). To quantify stomatal opening, we calculated the rate of stomatal opening, which shows how much stomatal conductance increased per minute during the linear, fastest phase of stomatal opening. The linear opening usually occurred about 5–15 minutes after the onset of stimulus, but the time interval was adjusted separately for each experiment to ensure that the whole linear phase of stomatal opening was included. These experiments revealed that the rate of stomatal opening was reduced in *slac1-1* and *slac1-3* during light-, reduced CO_2- and high humidity-induced stomatal opening (Fig. 7B).

We also calculated the half-times of stomatal opening response, which indicate the time it takes to reach half of the maximum stimulus-induced response. The stomatal opening responses were measured for 52, 60–66 or 102 minutes after the onset of light, reduced CO_2 or high air humidity, respectively. Within this time, wild type reached a new steady-state stomatal conductance,

but the stomatal conductance of *slac1* mutants was still increasing, therefore the real half-times of stomatal opening of *slac1* mutants could be even higher. Half-times of stomatal opening were higher for *slac1* mutants compared to wild type in light-, reduced CO_2-, and humidity-induced stomatal opening responses (Fig. 7C). Together these results show that stomatal opening is slower in *slac1-1* and *slac1-3* regardless of the stimulus inducing stomatal opening. Since the upstream regulation pathways are different for these stimuli, it is likely that mutations in SLAC1 affect a universal step in stomatal opening.

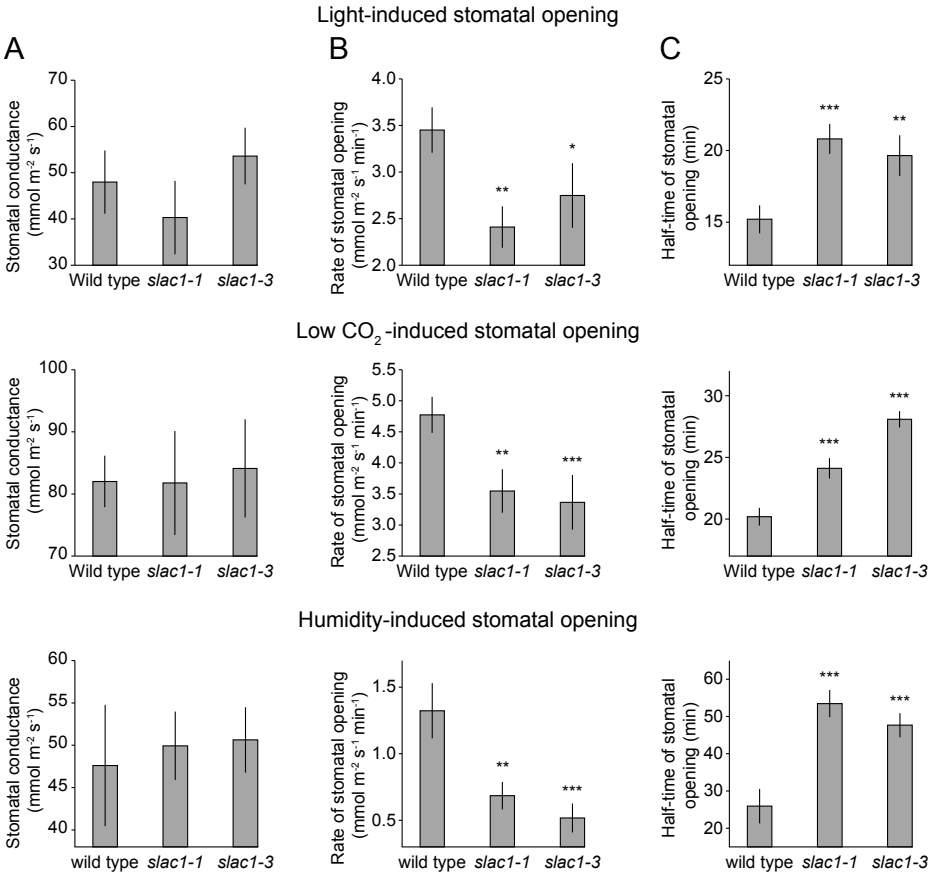

Figure 7. Stomatal opening is slower in *slac1-1* and *slac1-3* in response to light, reduced CO_2 and increased air humidity. Column A. Pre-treatment stomatal conductance. Column B. Rate of increase in stomatal conductance during the linear phase of stomatal opening. Column C. Half-time of stomatal opening (time when stomatal conductance had increased by 50%) with initial stomatal conductance as 0% and maximum measured stomatal conductance as 100%. n=5–8, n=9–14, and n=3 for light-, reduced CO_2- and humidity-induced stomatal opening, respectively. Error bars indicate SEM. Statistics: one-way ANOVA, post-hoc Fisher LSD was used for comparison with wild type. * $p<0.1$; ** $p<0.05$; *** $p<0.01$.

4.5. Mutations in SLAC1 lead to increased Ca^{2+} sensitivity of K^+_{in} channels

Regardless of stimulus, stomatal opening requires ion uptake, including potassium through K^+_{in} channels. Patch clamp method allows the measurement of ion currents in the protoplasts of plant cells. We showed that K^+ uptake activity in the guard cell protoplasts of *slac1-1* and *slac1-3* was impaired by 84% and 79% respectively, at membrane potential of –180 mV, when experiments were performed at 250 nM free $[Ca^{2+}]_{cyt}$ (I).

Stomatal opening was similarly slow when K^+_{in} channels were blocked or genetically impaired (Kelly et al., 1995; Kwak et al., 2001; Lebaudy et al., 2008). A 75% reduction of K^+_{in} activity at –180 mV leads to 38% to 45% reduction in light-induced stomatal opening, as was shown with plant lines expressing dominant negative point mutations in K^+_{in} channel subunit KAT1 (Kwak et al., 2001). Another mutant, *kincless*, which has no K^+_{in} current, had less stomatal opening in 4 hours than wild type in 1 hour (Lebaudy et al., 2008). Therefore, it is likely that the slow stomatal opening of *slac1* mutants was caused by a reduction in K^+_{in} activity.

K^+_{in} channels are regulated in several ways. K^+_{in} activity is decreased by increasing $[Ca^{2+}]_{cyt}$ (Schroeder and Hagiwara, 1989) and by site-specific phosphorylation of KAT1 by OST1 (Sato et al., 2009). Even more importantly, K^+_{in} activity depends directly on plasma membrane potential and decreases when plasma membrane is depolarized (Schroeder et al., 1987). K^+_{in} channels can also be inhibited by ABA without elevation in $[Ca^{2+}]_{cyt}$, meaning that ABA inhibits K^+_{in} channels independently of Ca^{2+} (Romano et al., 2000). The latter is probably mediated by OST1, which is activated by ABA (Ma et al., 2009; Park et al., 2009) and reduces K^+_{in} activity (Sato et al., 2009).

To test the hypothesis that reduced K^+_{in} activity of *slac1* mutants could be related with Ca^{2+}, we measured K^+_{in} activity at subphysiological $[Ca^{2+}]_{cyt}$ concentration of less than 10 nM. Interestingly, at such conditions K^+_{in} activity of *slac1* mutants was rapidly increased, indicating that decreased K^+_{in} activity of *slac1* mutants was related to increased Ca^{2+} sensitivity of K^+_{in} channels (II). We could also show that *slac1* guard cells had slightly higher $[Ca^{2+}]_{cyt}$ levels, further supporting the view that Ca^{2+}-related mechanisms are involved in reduced K^+_{in} activity of *slac1* mutants. However, even at low $[Ca^{2+}]_{cyt}$, *slac1* still had roughly 20% lower K^+_{in} activity compared to wild type suggesting that in addition to Ca^{2+} there are probably other mechanisms that contribute to major downregulation of guard cell K^+_{in} activity and slower stomatal opening of plants that carry mutations in SLAC1 anion channel.

4.6. Other possible causes for reduced stomatal opening and K^+_{in} activity in *slac1* mutants

Slower stomatal opening of *slac1* has been explained by elevated pH of *slac1* guard cells in combination with increased $[Ca^{2+}]_{cyt}$ (II). In guard cells of both wild type and *slac1*, cytosolic pH is lower when stomata are closed and higher when stomata are open, however pH is higher in *slac1* even when comparing wild type with open stomata and *slac1* with closed stomata (Wang et al., 2012). Nevertheless, K^+_{in} channel activity data shown in II and III were measured at controlled pH in the bath solution surrounding protoplasts and controlled pH inside the pipette. Ca^{2+} concentration was also controlled, but still K^+_{in} channel activity at the same plasma membrane potential was much lower in *slac1*.

While it is known that K^+_{in} currents are inhibited by elevated $[Ca^{2+}]_{cyt}$ (Schroeder and Hagiwara, 1989), it is not known whether Ca^{2+} reduces K^+_{in} activity directly or through a Ca^{2+} -binding intermediary. However, it is likely that a Ca^{2+}-binding mediator is involved, as KAT1 does not have Ca^{2+}-binding EF hand motifs (Day et al., 2002). It is tempting to hypothesize that similarly to SLAC1 there might exists a co-regulation of K^+_{in} channels by OST1 and CPKs and K^+_{in} channels are inactivated by those kinases Ca^{2+}-independently and dependently, respectively. Recently, CPK13 was found to inhibit K^+_{in} channels, but remarkably, CPK13 is Ca^{2+}-independent, despite having 2 EF-hands (Cheng et al., 2002; Hrabak et al., 2003; Ronzier et al., 2014). Thus it remains to be addressed whether CPK13 is involved in the Ca^{2+}-dependent inactivation of K^+_{in} channels in *slac1* mutants.

ABA also inhibits stomatal opening and the effects of ABA and increased Ca^{2+} are additive (De Silva et al., 1985). We measured the concentration of ABA in the leaves of wild type, *slac1-1* and *slac1-3*, but could not detect any differences in the baseline leaf ABA concentrations (II). This does not exclude, however, a potential difference in the guard cell ABA concentration.

Disruption of SLAC1 might cause a change in the expression of genes for other channels or transporters in order to slow down further stomatal opening. We compiled a list of identified channels and transporters that have been proven or suggested to be involved in stomatal regulation and used qPCR method to measure the expression level of these genes in wild type, *slac1-1* and *slac1-3* mutants. Stomata comprise only roughly 1% of leaf mass, so we extracted RNA from isolated guard cells. This experiment showed that H^+ATPases AHA1, AHA2, ABC transporter B14 (AtABCB14) and K^+ channels KAT2 and AKT2 had slightly lower expression levels in *slac1* mutant guard cells (II). Although these channels/transporters are involved in stomatal opening it is unlikely that observed downregulation of these genes is enough to explain the slow stomatal opening of *slac1* mutants since even greater changes in gene expression have shown to have little effect on stomatal opening. For example, KAT1 potassium uptake channel is important for potassium uptake during stomatal opening, yet a mutation which makes KAT1 nonfunctional had very little effect on stomatal

opening (Szyroki et al., 2001). AtABCB14 is important for malate uptake, yet plants with AtABCB14 knocked out showed only minor reduction in stomatal opening efficiency (Lee et al., 2008).

4.7. Stomatal opening of *ost1-3* and *abi2-1*

Our next aim was to find out whether stomatal opening is also slower in other mutants with similar phenotype to *slac1*. We studied the stomatal opening in two other mutants with high stomatal conductance and reduced closure due to impaired S-type anion channel activation: *ost1-3* and *abi2-1*. Stomatal conductance of *ost1-3* and *abi2-1* did not decrease much in low air humidity or darkness and remained significantly higher than in Col-0 and Ler, respectively, even after 12 hours in darkness (III). These experiments confirm that in the absence of OST1, stomatal closure is greatly impaired but in the absence of SLAC1, other anion channels partly compensate for the deficiency and stomata slowly close.

Stomatal opening responses of *ost1-3* and *abi2-1* in response to low CO_2 and light were slow (III, Table 1). K^+_{in} activity was also lower in these mutants, but not as low as in *slac1-1* and *slac1-3* (III, Fig. 1). When $[Ca^{2+}]_{cyt}$ was chelated to less than 10 nM, then K^+_{in} activity did not increase in *ost1-3* and increased only slightly in *abi2-1,* but the increase in K^+_{in} activity at low $[Ca^{2+}]_{cyt}$ of *abi2-1* was much less than in wild type or *slac1* mutant (III, Fig. 1). K^+_{in} activity phenotype of *abi2-1* might be caused by constitutive inhibition of OST1 (III, Fig. 1). Slowed stomatal opening and reduced K^+_{in} activity phenotypes of *ost1-3* and *abi2-1* suggest that these phenotypes might be a general characteristic of plants with more open stomata or alternatively characteristic of plants that are defective in S-type anion channel activation. To clarify this point further, additional research with mutants that carry more open stomata phenotype, but have intact S-type anion channel activity would be needed.

4.8. Compensatory changes in stomatal regulation.

Plants grown in controlled conditions have spontaneous mutation rate of 7×10^{-9} base substitutions per site per generation (Ossowski et al., 2010). There are many examples of single nucleotide substitutions causing a very strong phenotype, e.g. *slac1-1* , *abi1-1* and *abi2-1* (Pei et al., 1997; Negi et al., 2008; Vahisalu et al., 2008). Thus, being able to compensate for changes that affect important processes like stomatal regulation is vital for plants.

Often, there are several genes that encode proteins with the same function. Examples from stomatal regulation are CPKs: mutants where only one CPK was knocked out (*cpk21*, *cpk23*), had no inhibition or very little inhibition of stomatal closure, despite the fact that CPKs activate anion channels SLAC1 and SLAH3 (I). Furthermore, the critical role of PYR/RCARs for stomatal closure

became evident only when six PYR/RCAR receptors were knocked out (I). Steady-state stomatal conductance, however, increased in accordance with the number of receptors knocked out, showing clear phenotype already in plants having three impaired PYR/RCARs. Comparing stomatal responses of *1124C*, *12458*, *112458*, it seems that PYL1 is very important. Thus, there is a functional distinction among PYR/RCARs: some of them cannot be fully compensated by others. Compensation is partly the case with SLAC1 and SLAH3 as well, as both mediate anion efflux from guard cells and are activated partly by the same regulators (see Table I). Strongly impaired initial stomatal responses to environmental factors of *slac1* mutants (I, Vahisalu et al., 2008) suggest that functional SLAH3 and other channels mediating anion efflux could not compensate for missing SLAC1. However, when kept in darkness and low humidity for longer time, *slac1* mutants started to close after initial delay (I, Fig. 4), indicating that in long-term responses other anion efflux channels were able to compensate for missing SLAC1 (Vahisalu et al., 2008; Geiger et al., 2011).

A mutation can be compensated on genomic level by downregulating or upregulating the expression of genes coding proteins with similar function or by modifying the activities of respective proteins by posttranslational modifications and by protein-protein interactions. It is likely that the posttranslational mechanisms serve to change the sensitivity of the pathway. The regulation of PP2C activity can become less or more sensitive to ABA, depending on the PP2C:PYR/RCAR ratio (Szostkiewicz et al., 2010), representing compensation within the ABA signalosome. When PP2C:PYR/RCAR ratio increases, the regulation of PP2Cs becomes less sensitive to ABA and vice versa (Szostkiewicz et al., 2010). The change in PP2C:PYR/RCAR ratio may serve as a way to sensitize guard cells to ABA in ABA-deficient mutants that were shown to be hypersensitive to exogenous ABA (Szostkiewicz et al., 2010). Reduced ABA concentration of *aba1-1* and *aba3-1* (Rock and Zeevaart, 1991; Léon-Kloosterziel et al., 1996) led to high stomatal conductance but did not cause impairment in stomatal closure responses. The latter could be explained by compensatory changes in PP2C:PYR/RCAR ratio, resulting in high sensitivity of ABA signalosome to ABA, so that even moderate increase in ABA concentration, as in ABA deficient mutants, may activate the stomatal closure pathway (I, Fig. 8). ABA receptor-PP2C complex has an apparent affinity for ABA in the low nanomolar range (Dupeux et al., 2011), further explaining intact responses of ABA-deficient mutants that have residual amounts of ABA.

This study illustrated that compensatory changes to reduce the effect of a mutation can sometimes be even more indirect. Plants with impaired S-type anion channels have downregulated stomatal opening to reduce excessive water loss (II, III). The downregulation of K^+_{in} channel activity in *slac1* mutants is a compensatory change that could be explained as a strategy to prevent further stomatal opening. This allows the plant to maintain some control over stomatal openness.

5. CONCLUSIONS

Stomatal pores are surrounded by two guard cells that control plant gas exchange. Guard cell plasma membrane anion efflux channel SLAC1 is involved in stomatal closure induced by many stimuli, including plant hormone ABA. We tested whether the signaling proteins leading to ABA-induced stomatal closure have a role in stomatal responses induced by different environmental factors. We found that signaling through ABA signalosome consisting of PYR/RCARs, PP2Cs, OST1, and SLAC1 is important for stomatal closure in response to elevated CO_2, darkness, decreased air humidity, and ozone (I). Therefore, ABA signalosome is not exclusive to ABA-induced closure, but is also involved in stomatal closure in response to environmental factors.

We found that defects in ABA signalosome increased steady-state stomatal conductance (I). PYR/RCAR ABA receptors were particularly important for maintaining stomatal conductance, as the sextuple PYR/RCAR mutant *112458* had more than three times higher stomatal conductance compared to wild type (I). ABA biosynthesis mutants *aba1-1* and *aba3-1* also had very open stomata. Mutants with very high stomatal conductance show higher photosynthetic rate, but are more susceptible to excessive water loss. A SLAC-deficient mutant of rice was smaller compared to wild type in field conditions, indicating that stress induced by more open stomata outbalances the positive effect on photosynthesis in natural conditions (Kusumi et al., 2012).

Stomatal opening requires uptake of ions and involves a different set of channels and transporters compared to stomatal closure, yet stomatal opening of *slac1* mutants was slower in response to light, reduced atmospheric CO_2, and increased air humidity (II). K^+_{in} channel activity of *slac1* guard cell protoplasts was ~80 % lower than in wild type when measured at 250 nM Ca^{2+} (II) and also reduced at 100 nM Ca^{2+} (III). $[Ca^{2+}]_{cyt,}$ is known to inhibit K^+ uptake channels. Lowering Ca^{2+} concentration to <10 nM increased K^+_{in} channel activity in *slac1* mutants, therefore the reduction in K^+_{in} channel activity is caused by $[Ca^{2+}]_{cyt}$. K^+_{in} channels in *slac1* guard cells thus exhibit increased sensitivity to Ca^{2+} (II, III). In addition to increased Ca^{2+}-sensitivity of K^+_{in} channels, guard cells of *slac1* mutant had higher $[Ca^{2+}]_{cyt}$ (II). These results show that the compensatory changes that can outbalance the negative effects of defects in crucial proteins, such as SLAC1 anion channel, can be indirect and unpredictable.

Similarly to *slac1-1* and *slac1-3*, mutants of OST1 and ABI2 lack S-type anion channel activation and have slow stomatal opening in response to light and low CO_2. Guard cells of *ost1-3* and *abi2-1* also showed impaired K^+_{in} activity at 100 nM Ca^{2+}. Mutations in *ost1-3* and *abi2-1* slowed stomatal opening response to light and low CO_2 associated with reduced K^+_{in} activity, indicating that a reduction in K^+_{in} activity is a compensatory change, which is not unique to *slac1* mutant (III). However, K^+_{in} activity was not clearly reversible at low $[Ca^{2+}]_{cyt}$ in *ost1-3* and was only partly reversible in *abi2-1* (III).

Taken together, short- and long-term adaptation processes in guard cells involve compensatory changes and adjustment of ABA- and Ca^{2+}-sensitivity. The existence of compensatory changes has implications for both the research that is done with mutants to find out the role of certain proteins and also for designing breeding strategies that involve development of plants with new traits. Changes in some proteins are countered with other changes – direct and indirect – in a bid to minimize effect of the mutation. Therefore we should admit that the role of proteins is challenging to research and could even stay un-detected when using phenotypic studies of mutants, considering that compensatory changes might have taken place.

The intertwined nature of signaling pathways creates a challenge for both plant genetic research and genetic engineering as single mutations can 1) either be compensated for by adaptive changes and result in the lack of phenotype, 2) have a strong effect on other pathways that can lead to stronger phenotype and 3) even have an effect on different pathways leading to unexpected phenotype.

REFERENCES

Ache P, Bauer H, Kollist H, Al-Rasheid KAS, Lautner S, Hartung W, Hedrich R (2010) Stomatal action directly feeds back on leaf turgor: new insights into the regulation of the plant water status from non-invasive pressure probe measurements. *The Plant Journal* **62**: 1072–1082

Agurla S, Gayatri G, Raghavendra AS (2014) Nitric oxide as a secondary messenger during stomatal closure as a part of plant immunity response against pathogens. *Nitric Oxide* **43**: 89–96

Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffmann T, Tang YY, Grill E, Schroeder JI (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements. *Nature* **411**: 1053–1057

Allen GJ, Murata Y, Chu SP, Nafisi M, Schroeder JI (2002) Hypersensitivity of Abscisic Acid–Induced Cytosolic Calcium Increases in the Arabidopsis Farnesyltransferase Mutant *era1-2*. *The Plant Cell* **14**: 1649–1662

Assmann SM, Snyder JA, Lee Y-RJ (2000) ABA-deficient (*aba1*) and ABA-insensitive (*abi1-1*, *abi2-1*) mutants of *Arabidopsis* have a wild-type stomatal response to humidity. *Plant, Cell & Environment* **23**: 387–395

Belin C, Franco P-O de, Bourbousse C, Chaignepain S, Schmitter J-M, Vavasseur A, Giraudat J, Barbier-Brygoo H, Thomine S (2006) Identification of Features Regulating OST1 Kinase Activity and OST1 Function in Guard Cells. *Plant Physiology* **141**: 1316–1327

Blum A (2005) Drought resistance, water-use efficiency, and yield potential – are they compatible, dissonant, or mutually exclusive? *Australian Journal of Agricultural Research* **56**: 1159–1168

Brandt B, Brodsky DE, Xue S, Negi J, Iba K, Kangasjarvi J, Ghassemian M, Stephan AB, Hu H, Schroeder JI (2012) Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 10593–10598

Cheng S-H, Willmann MR, Chen H-C, Sheen J (2002) Calcium Signaling through Protein Kinases. The Arabidopsis Calcium-Dependent Protein Kinase Gene Family. *Plant Physiology* **129**: 469–485

Chen Y, Hu L, Punta M, Bruni R, Hillerich B, Kloss B, Rost B, Love J, Siegelbaum SA, Hendrickson WA (2010) Homologue structure of the SLAC1 anion channel for closing stomata in leaves. *Nature* **467**: 1074–1080

Comstock JP (2002) Hydraulic and chemical signalling in the control of stomatal conductance and transpiration. *Journal of Experimental Botany* **53**: 195–200

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic Acid: Emergence of a Core Signaling Network. *Annual Review of Plant Biology* **61**: 651–679

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R (2005) Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis. *Plant Physiology* **139**: 5–17

Day IS, Reddy VS, Shad Ali G, Reddy ASN (2002) Analysis of EF-hand-containing proteins in *Arabidopsis*. *Genome Biology* **3**: RESEARCH0056

Van Den Boogaard R, Alewijnse D, Veneklaas EJ, Lambers H (1997) Growth and water-use efficiency of 10 *Triticum aestivum* cultivars at different water availability in relation to allocation of biomass. *Plant, Cell & Environment* **20**: 200–210

Dodd AN, Parkinson K, Webb AAR (2004) Independent circadian regulation of assimilation and stomatal conductance in the *ztl-1* mutant of *Arabidopsis*. *New Phytologist* **162**: 63–70

Drerup MM, Schlücking K, Hashimoto K, Manishankar P, Steinhorst L, Kuchitsu K, Kudla J (2013) The Calcineurin B-like calcium sensors CBL1 and CBL9 together with their interacting protein kinase CIPK26 regulate the Arabidopsis NADPH oxidase RBOHF. *Molecular Plant* **6**: 559–569

Dupeux F, Santiago J, Betz K, Twycross J, Park S-Y, Rodriguez L, Gonzalez-Guzman M, Jensen MR, Krasnogor N, Blackledge M, et al (2011) A thermodynamic switch modulates abscisic acid receptor sensitivity. *The EMBO Journal* **30**: 4171–4184

Edgerton MD (2009) Increasing Crop Productivity to Meet Global Needs for Feed, Food, and Fuel. *Plant Physiology* **149**: 7–13

Edwards D, Kerp H, Hass H (1998) Stomata in early land plants: an anatomical and ecophysiological approach. *Journal of Experimental Botany* **49**: 255–278

Finkelstein R (2013) Abscisic Acid Synthesis and Response. *The Arabidopsis Book / American Society of Plant Biologists*. doi: 10.1199/tab.0166

Fischer RA (1973) The Relationship of Stomatal Aperture and Guard-cell Turgor Pressure in *Vicia faba*. *Journal of Experimental Botany* **24**: 387–399

French R, Schultz J (1984) Water use efficiency of wheat in a Mediterranean-type environment. I. The relation between yield, water use and climate. *Australian Journal of Agricultural Research* **35**: 743–764

Gadsby DC (2009) Ion channels versus ion pumps: the principal difference, in principle. *Nature reviews Molecular cell biology* **10**: 344–352

Geiger D, Maierhofer T, AL-Rasheid KAS, Scherzer S, Mumm P, Liese A, Ache P, Wellmann C, Marten I, Grill E, et al (2011) Stomatal Closure by Fast Abscisic Acid Signaling Is Mediated by the Guard Cell Anion Channel SLAH3 and the Receptor RCAR1. *Science Signaling* **4**: ra32–ra32

Geiger D, Scherzer S, Mumm P, Marten I, Ache P, Matschi S, Liese A, Wellmann C, Al-Rasheid KAS, Grill E, et al (2010) Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca^{2+} affinities. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 8023–8028

Geiger D, Scherzer S, Mumm P, Stange A, Marten I, Bauer H, Ache P, Matschi S, Liese A, Al-Rasheid KAS, et al (2009) Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 21425–21430

Gonzalez-Guzman M, Pizzio GA, Antoni R, Vera-Sirera F, Merilo E, Bassel GW, Fernández MA, Holdsworth MJ, Perez-Amador MA, Kollist H, et al (2012) Arabidopsis PYR/PYL/RCAR Receptors Play a Major Role in Quantitative Regulation of Stomatal Aperture and Transcriptional Response to Abscisic Acid. *The Plant Cell* **24**: 2483–2496

Grabov A, Blatt MR (1999) A Steep Dependence of Inward-Rectifying Potassium Channels on Cytosolic Free Calcium Concentration Increase Evoked by Hyperpolarization in Guard Cells. *Plant Physiology* **119**: 277–288

Guo F-Q, Young J, Crawford NM (2003) The Nitrate Transporter AtNRT1.1 (CHL1) Functions in Stomatal Opening and Contributes to Drought Susceptibility in Arabidopsis. *The Plant Cell* **15**: 107–117

Hao Q, Yin P, Li W, Wang L, Yan C, Lin Z, Wu JZ, Wang J, Yan SF, Yan N (2011) The Molecular Basis of ABA-Independent Inhibition of PP2Cs by a Subclass of PYL Proteins. *Molecular Cell* **42**: 662–672

Hashimoto M, Negi J, Young J, Israelsson M, Schroeder JI, Iba K (2006) Arabidopsis HT1 kinase controls stomatal movements in response to CO_2. *Nature Cell Biology* **8**: 391–397

Hayashi M, Inoue S, Takahashi K, Kinoshita T (2011) Immunohistochemical Detection of Blue Light-Induced Phosphorylation of the Plasma Membrane H^+-ATPase in Stomatal Guard Cells. *Plant and Cell Physiology* **52**: 1238–1248

Hosy E, Vavasseur A, Mouline K, Dreyer I, Gaymard F, Poree F, Boucherez J, Lebaudy A, Bouchez D, Very A-A, et al (2003) The *Arabidopsis* outward K^+ channel *GORK* is involved in regulation of stomatal movements and plant transpiration. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 5549–5554

Hrabak EM, Chan CWM, Gribskov M, Harper JF, Choi JH, Halford N, Kudla J, Luan S, Nimmo HG, Sussman MR, et al (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiology* **132**: 666–680

Hua D, Wang C, He J, Liao H, Duan Y, Zhu Z, Guo Y, Chen Z, Gong Z (2012) A Plasma Membrane Receptor Kinase, GHR1, Mediates Abscisic Acid- and Hydrogen Peroxide-Regulated Stomatal Movement in *Arabidopsis*. *The Plant Cell* **24**: 2546–2561

Hubbard KE, Siegel RS, Valerio G, Brandt B, Schroeder JI (2012) Abscisic acid and CO_2 signalling via calcium sensitivity priming in guard cells, new CDPK mutant phenotypes and a method for improved resolution of stomatal stimulus-response analyses. *Annals of Botany* **109**: 5–17

Hu H, Boisson-Dernier A, Israelsson-Nordström M, Böhmer M, Xue S, Ries A, Godoski J, Kuhn JM, Schroeder JI (2010) Carbonic Anhydrases are Upstream Regulators in Guard Cells of CO_2-Controlled Stomatal Movements. *Nature cell biology* **12**: 87–18

Humble GD, Raschke K (1971) Stomatal Opening Quantitatively Related to Potassium Transport Evidence from Electron Probe Analysis. *Plant Physiology* **48**: 447–453

Imes D, Mumm P, Böhm J, Al-Rasheid KAS, Marten I, Geiger D, Hedrich R (2013) Open stomata 1 (OST1) kinase controls R-type anion channel QUAC1 in Arabidopsis guard cells. *The Plant Journal: For Cell and Molecular Biology* **74**: 372–382

Inoue S, Kinoshita T, Matsumoto M, Nakayama KI, Doi M, Shimazaki K (2008) Blue light-induced autophosphorylation of phototropin is a primary step for signaling. *Proceedings of the National Academy of Sciences* **105**: 5626–5631

Kelly WB, Esser JE, Schroeder JI (1995) Effects of cytosolic calcium and limited, possible dual, effects of G protein modulators on guard cell inward potassium channels. *The Plant Journal* **8**: 479–489

Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, Shimazaki K (2001) phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* **414**: 656–660

Kinoshita T, Shimazaki K i (1999) Blue light activates the plasma membrane H^+-ATPase by phosphorylation of the C-terminus in stomatal guard cells. *The EMBO Journal* **18**: 5548–5558

Klüsener B, Young JJ, Murata Y, Allen GJ, Mori IC, Hugouvieux V, Schroeder JI (2002) Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. *Plant Physiology* **130**: 2152–2163

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. *The Plant Cell* **8**: 489–503

Kollist H, Nuhkat M, Roelfsema MRG (2014) Closing gaps: linking elements that control stomatal movement. *New Phytologist* **203**: 44–62

Kollist T, Moldau H, Rasulov B, Oja V, Rämma H, Hüve K, Jaspers P, Kangas-järvi J, Kollist H (2007) A novel device detects a rapid ozone-induced transient stomatal closure in intact Arabidopsis and its absence in abi2 mutant. *Physiologia Plantarum* **129**: 796–803

Kolukisaoglu U, Weinl S, Blazevic D, Batistic O, Kudla J (2004) Calcium Sensors and Their Interacting Protein Kinases: Genomics of the Arabidopsis and Rice CBL-CIPK Signaling Networks. *Plant Physiology* **134**: 43–58

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The Arabidopsis cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. *The EMBO Journal* **23**: 1647–1656

Kusumi K, Hirotsuka S, Kumamaru T, Iba K (2012) Increased leaf photosynthesis caused by elevated stomatal conductance in a rice mutant deficient in SLAC1, a guard cell anion channel protein. *Journal of Experimental Botany* **63**: 5635–5644

Kwak JM, Mori IC, Pei Z-M, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI (2003) NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *The EMBO Journal* **22**: 2623–2633

Kwak JM, Murata Y, Baizabal-Aguirre VM, Merrill J, Wang M, Kemper A, Hawke SD, Tallman G, Schroeder JI (2001) Dominant Negative Guard Cell K^+ Channel Mutants Reduce Inward-Rectifying K^+ Currents and Light-Induced Stomatal Opening in Arabidopsis. *Plant Physiology* **127**: 473–485

Lawson T, Blatt MR (2014) Stomatal Size, Speed, and Responsiveness Impact on Photosynthesis and Water Use Efficiency. *Plant Physiology* **164**: 1556–1570

Lebaudy A, Vavasseur A, Hosy E, Dreyer I, Leonhardt N, Thibaud J-B, Véry A-A, Simonneau T, Sentenac H (2008) Plant adaptation to fluctuating environment and biomass production are strongly dependent on guard cell potassium channels. *Proceedings of the National Academy of Sciences* **105**: 5271–5276

Lee M, Choi Y, Burla B, Kim Y-Y, Jeon B, Maeshima M, Yoo J-Y, Martinoia E, Lee Y (2008) The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to CO_2. *Nature Cell Biology* **10**: 1217–1223

Lee SC, Lan W, Buchanan BB, Luan S (2009) A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. *Proceedings of the National Academy of Sciences* **106**: 21419–21424

Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI (2004) Microarray Expression Analyses of Arabidopsis Guard Cells and Isolation of a Recessive Abscisic Acid Hypersensitive Protein Phosphatase 2C Mutant. *The Plant Cell* **16**: 596–615

Léon-Kloosterziel KM, Gil MA, Ruijs GJ, Jacobsen SE, Olszewski NE, Schwartz SH, Zeevaart JAD, Koornneef M (1996) Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. *The Plant Journal* **10**: 655–661

Levchenko V, Konrad KR, Dietrich P, Roelfsema MRG, Hedrich R (2005) Cytosolic abscisic acid activates guard cell anion channels without preceding Ca^{2+} signals. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 4203–4208

Leymarie J, Vavasseur A, Lascève G (1998) CO_2 sensing in stomata of *abi1-1* and *abi2-1* mutants of *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* **36**: 539–543

Luan S (2009) The CBL–CIPK network in plant calcium signaling. *Trends in Plant Science* **14**: 37–42

Maierhofer T, Diekmann M, Offenborn JN, Lind C, Bauer H, Hashimoto K, S. Al-Rasheid KA, Luan S, Kudla J, Geiger D, et al (2014) Site- and kinase-specific phosphorylation-mediated activation of SLAC1, a guard cell anion channel stimulated by abscisic acid. *Science Signaling* **7**: ra86–ra86

Marten H, Konrad KR, Dietrich P, Roelfsema MRG, Hedrich R (2007) Ca^{2+}-Dependent and -Independent Abscisic Acid Activation of Plasma Membrane Anion Channels in Guard Cells of *Nicotiana tabacum*. *Plant Physiology* **143**: 28–37

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors. *Science* **324**: 1064–1068

Mei X, Zhong X, Vincent V, Liu X (2013) Improving Water Use Efficiency of Wheat Crop Varieties in the North China Plain: Review and Analysis. *Journal of Integrative Agriculture* **12**: 1243–1250

Merilo E, Jõesaar I, Brosché M, Kollist H (2014) To open or to close: species-specific stomatal responses to simultaneously applied opposing environmental factors. *New Phytologist* **202**: 499–508

Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *The Plant Journal* **25**: 295–303

Meyer S, Mumm P, Imes D, Endler A, Weder B, Al-Rasheid KAS, Geiger D, Marten I, Martinoia E, Hedrich R (2010) AtALMT12 represents an R-type anion channel required for stomatal movement in Arabidopsis guard cells. *The Plant Journal* **63**: 1054–1062

Monda K, Negi J, Iio A, Kusumi K, Kojima M, Hashimoto M, Sakakibara H, Iba K (2011) Environmental regulation of stomatal response in the Arabidopsis Cvi-0 ecotype. *Planta* **234**: 555–563

Mori IC, Murata Y, Yang Y, Munemasa S, Wang Y-F, Andreoli S, Tiriac H, Alonso JM, Harper JF, Ecker JR, et al (2006) CDPKs CPK6 and CPK3 Function in ABA Regulation of Guard Cell S-Type Anion- and Ca^{2+}- Permeable Channels and Stomatal Closure. *PLoS Biology*. doi: 10.1371/journal.pbio.0040327

Mustilli A-C, Merlot S, Vavasseur A, Fenzi F, Giraudat J (2002) Arabidopsis OST1 Protein Kinase Mediates the Regulation of Stomatal Aperture by Abscisic Acid and Acts Upstream of Reactive Oxygen Species Production. *The Plant Cell* **14**: 3089–3099

Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K (2008) CO_2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature* **452**: 483–486

Ogasawara Y, Kaya H, Hiraoka G, Yumoto F, Kimura S, Kadota Y, Hishinuma H, Senzaki E, Yamagoe S, Nagata K, et al (2008) Synergistic activation of the

Arabidopsis NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. *The Journal of Biological Chemistry* **283**: 8885–8892

Okamoto M, Kushiro T, Jikumaru Y, Abrams SR, Kamiya Y, Seki M, Nambara E (2011) ABA 9'-hydroxylation is catalyzed by CYP707A in Arabidopsis. *Phytochemistry* **72**: 717–722

Ossowski S, Schneeberger K, Lucas-Lledó JI, Warthmann N, Clark RM, Shaw RG, Weigel D, Lynch M (2010) The Rate and Molecular Spectrum of Spontaneous Mutations in *Arabidopsis thaliana*. *Science* **327**: 92–94

Pandey S, Zhang W, Assmann SM (2007) Roles of ion channels and transporters in guard cell signal transduction. *FEBS Letters* **581**: 2325–2336

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, et al (2009) Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins. *Science* **324**: 1068–1071

Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and *abi1* and *abi2* mutants. *The Plant Cell* **9**: 409–423

Pei Z-M, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406**: 731–734

Pernas M, García-Casado G, Rojo E, Solano R, Sánchez-Serrano JJ (2007) A protein phosphatase 2A catalytic subunit is a negative regulator of abscisic acid signalling. *The Plant Journal* **51**: 763–778

Planes MD, Niñoles R, Rubio L, Bissoli G, Bueso E, García-Sánchez MJ, Alejandro S, Gonzalez-Guzmán M, Hedrich R, Rodriguez PL, et al (2014) A mechanism of growth inhibition by abscisic acid in germinating seeds of *Arabidopsis thaliana* based on inhibition of plasma membrane H^+-ATPase and decreased cytosolic pH, K^+, and anions. *Journal of Experimental Botany* **66**: 813–825

Postel SL, Daily GC, Ehrlich PR (1996) Human Appropriation of Renewable Fresh Water. *Science* **271**: 785–788

Raschke K, Shabahang M, Wolf R (2003) The slow and the quick anion conductance in whole guard cells: their voltage-dependent alternation, and the modulation of their activities by abscisic acid and CO_2. *Planta* **217**: 639–650

Ray DK, Ramankutty N, Mueller ND, West PC, Foley JA (2012) Recent patterns of crop yield growth and stagnation. *Nature Communications* **3**: 1293

Robert N, Merlot S, N'Guyen V, Boisson-Dernier A, Schroeder JI (2006) A hypermorphic mutation in the protein phosphatase 2C HAB1 strongly affects ABA signaling in Arabidopsis. *FEBS Letters* **580**: 4691–4696

Rock CD, Zeevaart JA (1991) The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 7496–7499

Roelfsema MRG, Hedrich R, Geiger D (2012) Anion channels: master switches of stress responses. *Trends in Plant Science* **17**: 221–229

Roelfsema MRG, Steinmeyer R, Staal M, Hedrich R (2001) Single guard cell recordings in intact plants: light-induced hyperpolarization of the plasma membrane. *The Plant Journal* **26**: 1–13

Romano LA, Jacob T, Gilroy S, Assmann SM (2000) Increases in cytosolic Ca^{2+} are not required for abscisic acid-inhibition of inward K^+ currents in guard cells of *Vicia faba* L. *Planta* **211**: 209–217

Ronzier E, Corratgé-Faillie C, Sanchez F, Prado K, Brière C, Leonhardt N, Thibaud J-B, Xiong TC (2014) CPK13, a Noncanonical Ca^{2+}-Dependent Protein Kinase, Specifically Inhibits KAT2 and KAT1 Shaker K^+ Channels and Reduces Stomatal Opening. *Plant Physiology* **166**: 314–326

Rubio S, Rodrigues A, Saez A, Dizon MB, Galle A, Kim T-H, Santiago J, Flexas J, Schroeder JI, Rodriguez PL (2009) Triple Loss of Function of Protein Phosphatases Type 2C Leads to Partial Constitutive Response to Endogenous Abscisic Acid. *Plant Physiology* **150**: 1345–1355

Ruszala EM, Beerling DJ, Franks PJ, Chater C, Casson SA, Gray JE, Hetherington AM (2011) Land Plants Acquired Active Stomatal Control Early in Their Evolutionary History. *Current Biology* **21**: 1030–1035

Saito S, Hirai N, Matsumoto C, Ohigashi H, Ohta D, Sakata K, Mizutani M (2004) Arabidopsis CYP707As Encode (+)-Abscisic Acid 8′-Hydroxylase, a Key Enzyme in the Oxidative Catabolism of Abscisic Acid. *Plant Physiology* **134**: 1439–1449

Sasaki T, Mori IC, Furuichi T, Munemasa S, Toyooka K, Matsuoka K, Murata Y, Yamamoto Y (2010) Closing Plant Stomata Requires a Homolog of an Aluminum-Activated Malate Transporter. *Plant and Cell Physiology* **51**: 354–365

Sato A, Sato Y, Fukao Y, Fujiwara M, Umezawa T, Shinozaki K, Hibi T, Taniguchi M, Miyake H, Goto DB, et al (2009) Threonine at position 306 of the KAT1 potassium channel is essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2.6 protein kinase. *Biochemical Journal* **424**: 439–448

Scherzer S, Maierhofer T, Al-Rasheid KAS, Geiger D, Hedrich R (2012) Multiple Calcium-Dependent Kinases Modulate ABA-Activated Guard Cell Anion Channels. *Molecular Plant* **5**: 1409–1412

Schroeder JI, Hagiwara S (1990) Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of nonselective Ca^{2+} permeable channels. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 9305–9309

Schroeder JI, Hagiwara S (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* **338**: 427–430

Schroeder JI, Keller BU (1992) Two types of anion channel currents in guard cells with distinct voltage regulation. *Proceedings of the National Academy of Sciences* **89**: 5025–5029

Schroeder JI, Raschke K, Neher E (1987) Voltage dependence of K^+ channels in guard-cell protoplasts. *Proceedings of the National Academy of Sciences of the United States of America* **84**: 4108–4112

Schwartz A (1985) Role of Ca^{2+} and EGTA on Stomatal Movements in *Commelina communis* L. *Plant Physiology* **79**: 1003–1005

Siegel RS, Xue S, Murata Y, Yang Y, Nishimura N, Wang A, Schroeder JI (2009) Calcium Elevation- and Attenuated Resting Calcium-dependent Abscisic Acid Induction of Stomatal Closure and Abscisic Acid-Induced Enhancement of Calcium Sensitivities of S-type Anion and K^+ Channels in Arabidopsis guard cells. *The Plant journal : for cell and molecular biology* **59**: 207–220

De Silva DLR, Hetherington AM, Mansfield TA (1985) Synergism Between Calcium Ions and Abscisic Acid in Preventing Stomatal Opening. *New Phytologist* **100**: 473–482

Sun J, Bankston JR, Payandeh J, Hinds TR, Zagotta WN, Zheng N (2014) Crystal structure of the plant dual-affinity nitrate transporter NRT1.1. *Nature* **507**: 73–77

Svennelid F, Olsson A, Piotrowski M, Rosenquist M, Ottman C, Larsson C, Oecking C, Sommarin M (1999) Phosphorylation of Thr-948 at the C Terminus of the Plasma Membrane H^+-ATPase Creates a Binding Site for the Regulatory 14-3-3 Protein. *The Plant Cell Online* **11**: 2379–2391

Szostkiewicz I, Richter K, Kepka M, Demmel S, Ma Y, Korte A, Assaad FF, Christmann A, Grill E (2010) Closely related receptor complexes differ in their ABA selectivity and sensitivity. *The Plant Journal* **61**: 25–35

Szyroki A, Ivashikina N, Dietrich P, Roelfsema MRG, Ache P, Reintanz B, Deeken R, Godde M, Felle H, Steinmeyer R, et al (2001) KAT1 is not essential for stomatal opening. *Proceedings of the National Academy of Sciences* **98**: 2917–2921

Takemiya A, Sugiyama N, Fujimoto H, Tsutsumi T, Yamauchi S, Hiyama A, Tada Y, Christie JM, Shimazaki K (2013) Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening. *Nature Communications*. doi: 10.1038/ncomms3094

Talbott LD, Zeiger E (1998) The role of sucrose in guard cell osmoregulation. *Journal of Experimental Botany* **49**: 329–337

Tallman G (2004) Are diurnal patterns of stomatal movement the result of alternating metabolism of endogenous guard cell ABA and accumulation of ABA delivered to the apoplast around guard cells by transpiration? *Journal of Experimental Botany* **55**: 1963–1976

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**: 796–815

Tian W, Hou C, Ren Z, Pan Y, Jia J, Zhang H, Bai F, Zhang P, Zhu H, He Y, et al (2015) A molecular pathway for CO_2 response in *Arabidopsis* guard cells. *Nature Communications*. doi: 10.1038/ncomms7057

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences* **108**: 20260–20264

Tominaga M, Kinoshita T, Shimazaki K (2001) Guard-Cell Chloroplasts Provide ATP Required for H^+ Pumping in the Plasma Membrane and Stomatal Opening. *Plant and Cell Physiology* **42**: 795–802

Ueno K, Kinoshita T, Inoue S, Emi T, Shimazaki K (2005) Biochemical Characterization of Plasma Membrane H^+-ATPase Activation in Guard Cell Protoplasts of *Arabidopsis thaliana* in Response to Blue Light. *Plant and Cell Physiology* **46**: 955–963

Vahisalu T, Kollist H, Wang Y-F, Nishimura N, Chan W-Y, Valerio G, Lamminmäki A, Brosché M, Moldau H, Desikan R, et al (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature* **452**: 487–491

Vahisalu T, Puzõrjova I, Brosché M, Valk E, Lepiku M, Moldau H, Pechter P, Wang Y-S, Lindgren O, Salojärvi J, et al (2010) Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. *The Plant Journal* **62**: 442–453

Wang F-F, Lian H-L, Kang C-Y, Yang H-Q (2010) Phytochrome B Is Involved in Mediating Red Light-Induced Stomatal Opening in *Arabidopsis thaliana*. *Molecular Plant* **3**: 246–259

Wang Y, Noguchi K, Terashima I (2011) Photosynthesis-Dependent and -Independent Responses of Stomata to Blue, Red and Green Monochromatic Light: Differences Between the Normally Oriented and Inverted Leaves of Sunflower. *Plant and Cell Physiology* **52**: 479–489

Wang Y, Papanatsiou M, Eisenach C, Karnik R, Williams M, Hills A, Lew VL, Blatt MR (2012) Systems Dynamic Modeling of a Guard Cell Cl^- Channel Mutant Uncovers an Emergent Homeostatic Network Regulating Stomatal Transpiration. *Plant Physiology* **160**: 1956–1967

Webb A, Hetherington AM (1997) Convergence of the Abscisic Acid, CO_2, and Extracellular Calcium Signal Transduction Pathways in Stomatal Guard Cells. *Plant Physiology* **114**: 1557–1560

Xue S, Hu H, Ries A, Merilo E, Kollist H, Schroeder JI (2011) Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO_2 signal transduction in guard cell. *The EMBO Journal* **30**: 1645–1658

Yoshida R, Umezawa T, Mizoguchi T, Takahashi S, Takahashi F, Shinozaki K (2006) The Regulatory Domain of SRK2E/OST1/SnRK2.6 Interacts with ABI1 and Integrates Abscisic Acid (ABA) and Osmotic Stress Signals Controlling Stomatal Closure in *Arabidopsis*. *Journal of Biological Chemistry* **281**: 5310–5318

Zhang X, Wang H, Takemiya A, Song C, Kinoshita T, Shimazaki K (2004) Inhibition of Blue Light-Dependent H^+ Pumping by Abscisic Acid through Hydrogen Peroxide-Induced Dephosphorylation of the Plasma Membrane H^+-ATPase in Guard Cell Protoplasts. *Plant Physiology* **136**: 4150–4158

Zou J-J, Wei F-J, Wang C, Wu J-J, Ratnasekera D, Liu W-X, Wu W-H (2010) Arabidopsis Calcium-Dependent Protein Kinase CPK10 Functions in Abscisic Acid- and Ca^{2+}-Mediated Stomatal Regulation in Response to Drought Stress. *Plant Physiology* **154**: 1232–1243

SUMMARY IN ESTONIAN

SLAC1 anioonkanali ja tema regulaatorite roll õhulõhede avanemisel ja sulgumisel müürloogas (*Arabidopsis thaliana*)

Taimede roheliste osade pinnal on õhulõhed – kahe sulgraku poolt ümbritsetud poorid, mis reguleerivad taimede gaasivahetust. Õhulõhede kaudu pääseb taime fotosünteesiks vajalik süsihappegaas ja paratamatult kaasneb sellega vee aurumine taimest atmosfääri ehk transpiratsioon. Õhulõhed avanevad valguse, madala süsihappegaasi ja kõrge õhuniiskuse toimel ning sulguvad pimeduse, kõrge süsihappegaasi, madala õhuniiskuse, osooni ja taimehormoon abstsiishappe (ABA) toimel. Õhulõhede avanemisel kasvab sulgrakkudes osmooselt aktiivsete ioonide kontsentratsioon ja sellega kaasneb turgorrõhu kasv ning vee sissevool. Sulgrakkude anatoomiline ehitus tagab selle, et paisudes nad kõverduvad ja moodustavad enda vahele poori ehk õhupilu. Õhulõhede sulgumisel käivituvad mehhanismid, mille käigus osmooselt aktiivseid ioone väljalaskvad kanalid aktiveeruvad, sulgrakkude turgor väheneb ja õhulõhe sulgub.

Mõni aasta tagasi kirjeldati signaalrada, mille kaudu ABA aktiveerib olulise anioonide väljavoolukanali SLAC1. ABA seondub PYR/RCAR retseptoritega, inhibeerides sel moel 2C tüüpi fosfataase (PP2C), mis on omakorda proteiinkinaas OST1 inhibiitorid. ABA seondumisel retseptoritega vabaneb OST1 inhibitsioonist ja aktiveerib anioonkanali SLAC1; järgneb anioonide väljavool ja õhulõhe sulgumine. Selleks, et uurida ABA signaalraja rolli õhulõhede basaalse avatuse määramisel ja sulgumisreaktsioonide käivitamisel erinevate keskkonnafaktorite toimel, kasutati käesolevas töös taimeliine, mis kandsid mutatsioone ABA signaalraja erinevates komponentides (I). Selgus, et 1) ABA signaalrada omab võtmerolli õhulõhede basaalse juhtivuse määramisel: PYR/RCAR mitmikmutantide juhtivus kasvas puuduolevate PYR/RCAR retseptorite arvu tõustes; basaalne juhtivus oli metsiktüübi omast oluliselt kõrgem ka taimeliinides, milles SLAC1 ja OST1 olid välja lülitatud, PP2C fosfataas konstitutiivselt aktiveeritud või ABA süntees defektne. 2) ABA-st ja PYR/RCAR retseptoritest lähtuv PP2C kinaaside inhibitsioon oli vajalik õhulõhede kiireks vastuseks pimedusele, madalale õhuniiskusele ja osoonile ning mõjutas ka õhulõhede CO_2 vastust.

Varasemast on teada, et anioonkanal SLAC1 on oluline õhulõhede sulgumiseks praktiliselt kõigi stiimulite toimel; käesolevas töös uuriti toimiva SLAC1 puudumise mõju õhulõhede avanemisele (II). SLAC1 kanal vahendab ainult anioonide väljavoolu, mistõttu võiks eeldada, et *slac1* mutantides on avanemine isegi kiirem. Selgus, et 1) *slac1* mutantide õhulõhed avanevad aeglasemalt valguse, õhuniiskuse tõusu ja madala süsihappegaasi toimel; selle põhjuseks on kaaliumi sissevoolukanalite (K^+_{in}) vähenenud aktiivsus. 2) K^+_{in} kanalite aktiivsust vähendab sulgrakkude Ca^{2+} kontsentratsiooni ($[Ca^{2+}]_{cyt}$) tõus, $[Ca^{2+}]_{cyt}$ oli *slac1* sulgrakkudes tõesti kõrgem kui metsiktüübis. 3) *slac1* K^+_{in} kanalite Ca^{2+}-tundlikkus oli oluliselt tõusnud: kui Ca^{2+} kontsentratsioon

langetati <10 nM, mis on oluliselt madalam füsioloogilisest Ca^{2+} kontsentratsioonist, siis tõusis *slac1* mutantides K^{+}_{in} kanalite aktiivsus sisuliselt metsiktüübi tasemele. Seega, *slac1* mutantide õhulõhede aeglasem avanemine tuleneb kõrgemast $[Ca^{2+}]_{cyt}$ tasemest *slac1* sulgrakkudes, ning K^{+}_{in} kanalite suuremast Ca^{2+} -tundlikkusest võrreldes metsiktüübiga (II).

Järgmisena uurisime õhulõhede avanemist teistes kõrge õhulõhede juhtivusega mutantides: *abi2-1*, kus PP2C ABI2 on püsivalt aktiveeritud ja *ost1-3*, kus OST1 kinaas on välja lülitatud. Selgus, et sarnaselt *slac1* mutantidele oli *abi2-1* ja *ost1-3* õhulõhede avanemine metsiktüübist aeglasem ja K^{+}_{in} kanalite aktiivsus vähenenud. $[Ca^{2+}]_{cyt}$ *in vitro* langetamisel tõusis *abi2-1* mutandi sulgrakkude K^{+}_{in} kanalite aktiivsus, ehkki mitte nii palju, kui *slac1* mutantides, kuid ei tõusnud *ost1-3* sulgrakkude K^{+}_{in} kanalite aktiivsus (III). Kõigis kolmes avatud õhulõhedega ja aeglase õhulõhede sulgumisega mutandis on seega toimunud kompensatoorne muudatus, mille tulemusel on K^{+}_{in} kanalite aktiivsus langenud ja õhulõhede edasine avanemine aeglustunud. *slac1* mutantides oli selle põhjuseks K^{+}_{in} kanalite kõrgem Ca^{2+} -tundlikkus ja sulgrakkude kõrgem $[Ca^{2+}]_{cyt}$. Mutantides *ost1-3* ja *abi2-1* oli K^{+}_{in} kanalite aktiivus samuti langenud ning see ei taastunud ka madala Ca^{2+} kontsentratsiooni juures täielikult. Seega, K^{+}_{in} kanalite aktiivsuse langus iseloomustas kõiki uuritud avatud õhulõhedega mutante, kuid pöörduv kompensatoorne K^{+}_{in} kanalite aktiivuse Ca^{2+}-sõltuv regulatsioon võib olla unikaalne *slac1* mutantide tunnus.

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PUBLICATIONS

CURRICULUM VITAE

Name:	Kristiina Laanemets (*née* Hallaste)
Date of birth:	January 12th, 1984
Citizenship:	Estonian
Marital status:	Married, 2 children (born in 2009 and 2011)
Contact:	Nooruse 1–302A, Tartu 50411, Estonia
	kristiina.laanemets@ut.ee, +372 52 20038

Education

2008– ...	PhD studies in engineering and technology, University of Tartu
2006–2008	Master studies in molecular and cell biology, University of Tartu, MSc
2003–2006	Bachelor studies in biology, University of Tartu, BSc
1994–2002	Viimsi High school
1990–1994	Kadrina High school

Professional employment

2014– ...	Junior research fellow of plant biology, University of Tartu
2013–2014	Technician, University of Tartu
2008–2010	Laboratory technician, University of Tartu
2005 summer	Field experiments, University of Tartu

List of publications

Ebe Merilo, Pirko Jalakas, **Kristiina Laanemets**, Omid Mohammadi, Hanna Hõrak, Hannes Kollist and Mikael Brosché. Abscisic acid transport and homeostasis in the context of stomatal regulation. *Molecular Plant*, provisionally accepted

Ebe Merilo, **Kristiina Laanemets**, Honghong Hu, Shaowu Xue, Liina Jakobson, Ingmar Tulva, Miguel Gonzalez-Guzman, Pedro L. Rodriguez, Julian I. Schroeder, Mikael Broschè, Hannes Kollist (2013) PYR/RCAR Receptors Contribute to Ozone-, Reduced Air Humidity-, Darkness- and CO_2-Induced Stomatal Regulation. *Plant Physiology*. **162** (3): 1652–1668

Kristiina Laanemets, Benjamin Brandt, Junlin Li, Ebe Merilo, Yong-Fei Wang, Malik M. Keshwani, Susan S. Taylor, Hannes Kollist, Julian I. Schroeder (2013) Calcium-Dependent and -Independent Stomatal Signaling Network and Compensatory Feedback Control of Stomatal Opening via Ca^{2+} Sensitivity Priming. *Plant Physiology*. **163 (2)**: 504–513

Kristiina Laanemets, Yong-Fei Wang, Ove Lindgren, Juyou Wu, Noriyuki Nishimura, Stephen Lee, Daniel Caddell, Ebe Merilo, Mikael Brosche, Kalle Kilk, Ursel Soomets, Jaakko Kangasjärvi, Julian I. Schroeder, Hannes Kollist (2013) Mutations in the SLAC1 anion channel slow stomatal opening and severely reduce K^+ uptake channel activity via enhanced cytosolic [Ca^{2+}] and increased Ca^{2+} sensitivity of K^+ uptake channels. *New Phytologist.* **197 (1)**: 88–98

Hannes Kollist, Mathieu Jossier, **Kristiina Laanemets**, Sébastien Thomine (2011) Anion channels in plant cells. *FEBS Journal.* **278** (22): 4277–4292

ELULOOKIRJELDUS

Nime: Kristiina Laanemets (*née* Hallaste)
Sünniaeg: 12. jaanuar, 1984
Kodakondsus: Eesti
Perekonnaseis: Abielus, 2 last (sündinud aastatel 2009 ja 2011)
Kontakt: Nooruse 1–302A, Tartu 50411, Eesti
kristiina.laanemets@ut.ee, +372 522 0038

Haridus

2008– ... Doktoriõpe, tehnika ja tehnoloogia, Tartu Ülikool
2006–2008 Magistriõpe, molekulaar- ja rakubioloogia, Tartu Ülikool, MSc
2003–2006 Bakalaureuseõpe, bioloogia, Tartu Ülikool, BSc
1994–2002 Viimsi Keskkool
1990–1994 Kadrina Keskkool

Erialane teenistuskäik

2014– ... Taimebioloogia nooremteadur, Tartu Ülikool
2013–2014 Tehnik, Tartu Ülikool
2008–2010 Laborant, Tartu Ülikool
2005 suvi Tööettevõtja, välikatsed, Tartu Ülikool

Publikatsioonid

Ebe Merilo, Pirko Jalakas, **Kristiina Laanemets**, Omid Mohammadi, Hanna Hõrak, Hannes Kollist and Mikael Brosché. Abscisic acid transport and homeostasis in the context of stomatal regulation. *Molecular Plant*, ajutiselt vastu võetud

Ebe Merilo, **Kristiina Laanemets**, Honghong Hu, Shaowu Xue, Liina Jakobson, Ingmar Tulva, Miguel Gonzalez-Guzman, Pedro L. Rodriguez, Julian I. Schroeder, Mikael Broschè, Hannes Kollist (2013) PYR/RCAR Receptors Contribute to Ozone-, Reduced Air Humidity-, Darkness- and CO_2-Induced Stomatal Regulation. *Plant Physiology*. **162** (3): 1652–1668

Kristiina Laanemets, Benjamin Brandt, Junlin Li, Ebe Merilo, Yong-Fei Wang, Malik M. Keshwani, Susan S. Taylor, Hannes Kollist, Julian I. Schroeder (2013) Calcium-Dependent and -Independent Stomatal Signaling Network and Compensatory Feedback Control of Stomatal Opening via Ca^{2+} Sensitivity Priming. *Plant Physiology*. **163 (2)**: 504–513

Kristiina Laanemets, Yong-Fei Wang, Ove Lindgren, Juyou Wu, Noriyuki Nishimura, Stephen Lee, Daniel Caddell, Ebe Merilo, Mikael Brosche, Kalle Kilk, Ursel Soomets, Jaakko Kangasjärvi, Julian I. Schroeder, Hannes Kollist (2013) Mutations in the SLAC1 anion channel slow stomatal opening and severely reduce K^+ uptake channel activity via enhanced cytosolic [Ca^{2+}] and increased Ca^{2+} sensitivity of K^+ uptake channels. *New Phytologist.* **197 (1)**: 88–98

Hannes Kollist, Mathieu Jossier, **Kristiina Laanemets**, Sébastien Thomine (2011) Anion channels in plant cells. *FEBS Journal.* **278** (22): 4277–4292

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