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Unravelling signalling pathways contributing to stomatal conductance and responsiveness

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Unravelling signalling pathways contributing to stomatal conductance and responsiveness

UNIVERSITY OF TARTU
Press

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

This dissertation was accepted for the commencement of the degree of Doctor of Philosophy in Environmental Engineering on June 27, 2019 by the Council of the Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

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Commencement: Auditorium 121, Nooruse 1, Tartu, at 10:15
on August 16th, 2019

ISSN 2228-0855
ISBN 978-9949-03-128-3 (print)
ISBN 978-9949-03-129-0 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

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

I. Merilo, E.; Yarmolinsky, D.; **Jalakas, P.**; Parik, H.; Tulva, I.; Rasulov, B; Kilk, K; Kollist, H. (2018) Stomatal VPD Response: There Is More to the Story Than ABA. *Plant Physiology*, 176(1): 851–864

II. **Jalakas, P.**; Merilo, E.; Kollist, H.; Brosché, M. (2018) ABA-mediated regulation of stomatal density is OST1-independent. *Plant Direct*, 2(9)

III. Merilo, E.; **Jalakas, P.**; Kollist, H.; Brosché, M. (2015) The Role of ABA Recycling and Transporter Proteins in Rapid Stomatal Responses to Reduced Air Humidity, Elevated CO_2, and Exogenous ABA. *Molecular Plant*, 8(4): 657–659

IV. **Jalakas, P.**; Huang, Y-C.; Yeh, Y-H.; Zimmerli, L.; Merilo, E; Kollist, H.; Brosché, M. (2017) The Role of ENHANCED RESPONSES TO ABA1 (ERA1) in *Arabidopsis* Stomatal Responses Is Beyond ABA Signalling. *Plant Physiology*, 174(2): 665–671

Author's contribution

I. I performed some of the experiments and commented on the manuscript.

II. I performed all of the experiments and analyzed the results. I wrote the first draft of the manuscript and participated in the editing process.

III. I performed some of the experiments and commented on the manuscript.

IV. I performed most of the experiments, except pathogen assays and ABA-inhibition of stomatal opening experiment. I analyzed the results and participated in writing of the manuscript.

ABBREVIATIONS

AAO3	ABSCISIC ALDEHYDE OXIDASE 3; protein involved in ABA biosynthesis
ABA	abscisic acid; a plant hormone
ABA-GE	ABA glucose ester; inactive form of ABA
ABA1/2/3/4	ABA DEFICIENT 1/2/3/4; proteins involved in ABA biosynthesis
ABI1	ABA INSENSITIVE 1; protein phosphatase of type 2C, negative regulator of ABA signalling
ABCG	ATP-binding cassette transporter subfamily G; several members have been identified as ABA transporters
AIT1/NPF4.6	ABA-IMPORTING TRANSPORTER 1/ NRT1/PTR FAMILY 4.6; ABA importer
ALMT12/QUAC1	ALUMINUM-ACTIVATED MALATE TRANSPORTER 12/ QUICK-ACTIVATING ANION CHANNEL 1; rapid-type guard cell anion channel
BG1/2	BETA-1,3-GLUCANASE 1/2; hydrolyzes ABA-GE to ABA
CPK	calcium-dependent kinase
CYP707A	CYTOCHROME P450, FAMILY 707, SUBFAMILY A; involved in ABA catabolism
DTX50	DETOXIFICATION EFFLUX CARRIER 50; mediates ABA efflux
ERA1	ENHANCED RESPONSE TO ABA 1; involved in ABA-mediated signalling pathway
GFP	GREEN FLUORESCENT PROTEIN; reporter protein used to visualize the subcellular localization of other proteins attached to it
GHR1	GUARD CELL HYDROGEN PEROXIDERESISTANT 1; a leucine-rich repeat receptor-like kinase which mediates SLAC1 activation
NCED	NINE-CIS-EPOXYCAROTENOID DIOXYGENASE; key enzyme in ABA biosynthesis pathway
OST1/SnRK2.6	OPEN STOMATA 1/ SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2–6; ABA-activated protein kinase
PP2Cs	type 2C protein phosphatases, negative regulators of ABA signalling

PYR/PYL/RCAR	PYRABACTIN RESISTANCE 1/PYR1-like/ REGULATORY COMPONENT OF ABA RECEPTOR; ABA receptor family
SLAC1	SLOW ANION CHANNEL 1; slow-type guard cell anion channel
SLAH3	SLAC1 HOMOLOUGE 3; slow-type guard cell anion channel
VPD	Vapour-pressure deficit, difference between the vapour pressures of the leaf and the atmosphere

INTRODUCTION

Plants, the primary producers on Earth, use energy from sunlight to convert carbon dioxide (CO_2) and water from the soil to produce organic compounds and oxygen, both of which are needed by humans and other heterotrophic organisms. In the epidermis of the aerial parts of plants are stomata, microscopic pores surrounded by a pair of guard cells that control gas exchange between the leaf and the atmosphere, i.e., the uptake of CO_2 for photosynthesis and the loss of water via transpiration. Guard cells integrate internal and various environmental signals such as light, CO_2, temperature and humidity to regulate the opening and closing of stomatal pores. These stomatal movements are accomplished by changes in guard cell turgor, which are driven by the uptake or release of osmotically active ions.

In future climate change scenarios, atmospheric CO_2 concentrations and temperature will continue to rise, whereas precipitation will decrease in some areas and increase in others. These phenomena affect the frequency and severity of drought periods and flooding, which in turn will influence agriculture by reducing crop yields, thereby affecting food availability. It is important for plants to respond and adapt to these changing conditions. Accurate control of stomatal aperture influences biomass production and plant water management. Therefore, one important focus of plant breeding is to develop more water use efficient plants with reduced transpiration and stomatal conductance. On the other hand, low stomatal conductance is associated with reduced photosynthesis and could limit growth. Therefore, it is reasonable to assume that there exists a threshold value below which the further reduction in stomatal conductance results in a negative effect on yield. Furthermore, reduced transpiration results in reduced leaf cooling, which is important under warm temperatures. Thus, understanding the mechanism behind stomatal regulation gives valuable information for breeding crop plants for different climatic conditions.

This thesis focuses on stomatal regulation on different timescales: 1.) regulation of stomatal movements in response to the plant hormone abscisic acid (ABA) and environmental factors, which can take place within minutes to hours and 2.) regulation of stomatal conductance via stomatal density, which takes place over a longer timescale. Methods involving genetics, molecular biology and plant physiology were used to characterize the important role of protein kinase OST1, a positive regulator in ABA signalling pathway, in stomatal responsiveness to environmental factors. Furthermore, the role of protein farnesylation, a post-translational modification of proteins, in regulating stomatal movements was studied, as well as the phenotypes of different proposed ABA transporters.

1. REVIEW OF THE LITERATURE

1.1. Stomatal closure

Stomata close in response to abiotic environmental stimuli, air pollutants (O_3), pathogen attack and endogenous plant hormones (ABA). Stomatal closure is induced by anion and K^+ efflux from guard cells, which leads to a reduction in their turgor pressure and, consequently, stomatal closure. Patch-clamp studies have revealed two types of anion channels in the guard cell plasma membrane based on their activation kinetics: rapid-type (R-type) and slow-type (S-type) channels (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Schroeder and Keller, 1992). R-type channels activate within milliseconds by depolarization and are deactivated during prolonged stimulation (Schroeder and Keller, 1992). In contrast, S-type channels show weak voltage-dependent activation, with activation times up to one minute (Schroeder and Keller, 1992). Both S-type and R-type channels are permeable to several anions, including malate^{2-}, NO_3^- and Cl^-, and can be activated by an increase in cytosolic Ca^{2+} concentrations (Hedrich et al., 1990; Schmidt and Schroeder, 1994). Furthermore, both R-type and S-type anion currents have been shown to be activated by ABA (Raschke et al., 2003; Roelfsema et al., 2004).

The gene encoding the S-type anion channel *SLOW ANION CHANNEL 1* (*SLAC1*) was identified in screens for O_3-sensitive or CO_2-insensitive mutants (Negi et al., 2008; Vahisalu et al., 2008). The guard cells of *slac1* mutants showed clear impairment in Ca^{2+}- and ABA-induced S-type anion currents (Vahisalu et al., 2008). Additionally, plants lacking functional SLAC1 had impaired stomatal closure responses to environmental and endogenous signals (Negi et al., 2008; Vahisalu et al., 2008). In addition to SLAC1, there are four homologues of SLAC1 in *Arabidopsis*: SLAH1 to SLAH4 (Negi et al., 2008; Vahisalu et al., 2008). Among them, only SLAH3 has been shown to be expressed in guard cells (Geiger et al., 2011). SLAC1 was characterized to be selective towards NO_3^- and Cl^- ions (Lee et al., 2009), whereas SLAH3 was more permeable to NO_3^- than to Cl^- (Geiger et al., 2011). To date, no stomatal phenotype has been revealed in *slah3* knockout plants. However, patch-clamp studies have shown that in nitrate-based buffers, S-type anion currents are active in the guard cells of *slac1* mutants but absent in the guard cells of *slah3* mutants (Geiger et al., 2011).

ALUMINUM-ACTIVATED MALATE TRANSPORTER 12 (ALMT12) was reported to be expressed in the guard cell plasma membrane and the loss-of-function *almt12* mutant showed impaired responses to darkness and ABA (Sasaki et al., 2010; Meyer et al., 2010). In *almt12* mutants, R-type currents were reduced in the presence of extracellular malate, and the expression of ALMT12 in *Xenopus* oocytes indicated that ALMT12 forms the malate-sensitive R-type anion channel (Meyer et al., 2010). Moreover, unlike other genes in the ALMT family, ALMT12 is not activated by Al^{3+} (Meyer et al., 2010), and thus,

ALMT12 was named QUICK-ACTIVATING ANION CHANNEL 1 (QUAC1). In the guard cells of *almt12* mutants, the R-type anion currents were reduced by 40% (Meyer et al., 2010), which implies that there might be other R-type anion channels in addition to QUAC1. Another member from the ALMT family, ALMT4, was recently shown to function as an ion channel mediating malate^{2-} release from the vacuole during stomatal closure (Eisenach et al., 2017). Plants deficient in ALMT4 function had impaired stomatal closure in response to ABA and were more susceptible to drought.

Anion efflux from S-type anion channels results in depolarization of guard cell plasma membranes, which in turn activates outward-rectifying potassium channel, mediating K^+ efflux. The K^+ outward-rectifying channel of the guard cell membrane is encoded by *GATED OUTWARDLY-RECTIFYING K^+ CHANNEL* (*GORK*), and plants deficient in GORK exhibited increased transpiration and impaired stomatal closure in response to darkness (Hosy et al., 2003). Guard cell protoplasts from loss-of-function GORK mutants displayed similar inward K^+ currents as did wildtype protoplasts but lacked outward K^+ currents (Hosy et al., 2003), showing the importance of GORK in mediating K^+ efflux from guard cells. Recent evidence suggests that K^+ UPTAKE TRANSPORTERs (KUPs) KUP6 and KUP8 are also involved in guard cell K^+ efflux during stomatal closure (Osakabe et al., 2013).

1.2. Stomatal opening

Stomatal opening is induced by light, including blue and red light, low CO_2 concentration and increased air humidity. During stomatal opening, activation of plasma membrane H^+-ATPase leads to the efflux of H^+, and the plasma membrane becomes hyperpolarized. Hyperpolarization activates voltage-dependent K^+ inward-rectifying channels (Schroeder et al., 1987; Roelfsema et al., 2001) such as POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1), KAT2 and ARABIDOPSIS THALIANA K^+ TRANSPORTER 1 (AKT1) leading to K^+ uptake (Schachtman et al., 1992; Pilot et al., 2001; Szyroki et al., 2001). The accumulation of K^+ ions is counterbalanced by the uptake of anions, mainly malate^{2-}, Cl^- and NO_3^-. During stomatal opening, accumulation of malate^{2-} is preferred over other anions and can be produced in guard cells by the breakdown of starch (Pandey et al., 2007) or imported from the apoplast by the malate transporter ABCB14 (ABC transporter B14) (Lee et al., 2008). The uptake of Cl^- is hypothesized to occur via H^+/anion symporter (Pandey et al., 2007), and the uptake of NO_3^- is mediated by the dual-affinity nitrate transporter NRT1.1 (CHL1) (Guo et al., 2003). Uptake of cations and anions results in an influx of water, increased guard cell turgor and stomatal opening i.e., a larger stomatal aperture width.

Blue light-induced stomatal opening is initiated by the activation of photoreceptors PHOTOTROPIN 1 (PHOT1) and PHOT2 via autophosphorylation (Kinoshita et al., 2001; Inoue et al., 2008). Activated phototropins subsequently

phosphorylate the protein kinase BLUE LIGHT SIGNALLING 1 (BLUS1) (Takemiya et al., 2013a). Signal from the phototropins and BLUS1 ultimately activate a H^+-ATPase via the phosphorylation of the C-terminus, with subsequent binding of a 14-3-3 protein (Kinoshita and Shimazaki, 1999). Type 1 protein phosphatase (PP1) and its regulatory subunit PRSL1 have been shown to be positive regulators of blue light-induced H^+-ATPase activation downstream of phototropins but upstream of H^+-ATPase (Takemiya et al., 2006; Takemiya et al., 2013b). Recently, BLUE LIGHT-DEPENDENT H+-ATPASE PHOSPHORYLATION (BHP) was identified as another signalling component in blue light-dependent stomatal opening (Hayashi et al., 2017).

Red light-induced stomatal opening requires higher light intensity compared to that of blue light. It has been suggested that the response of red light is likely mediated by a reduction in intercellular CO_2 (C_i) via red light-driven mesophyll photosynthesis (Roelfsema et al., 2002). This result is supported by experiments where chloroplast containing guard cells in albino leaf patches of *Chlorophytum comosum* did not open in response to red light (Roelfsema et al., 2006). In addition, treatment with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), a PSII inhibitor, abolishes the red light stomatal opening response (Olsen et al., 2002; Fujita et al., 2013). In contrast, stomata did open in response to red light when the C_i was kept constant (Messinger et al., 2006), indicating that the red light response not only is a response to C_i but also results from a direct signal from the guard cells themselves. Furthermore, red light did not induce phosphorylation of H^+-ATPase under low CO_2 conditions, suggesting that a reduction in C_i is not sufficient for the stomatal opening response (Ando and Kinoshita, 2018).

1.3. Regulation of ABA-mediated signalling

ABA was first discovered as a growth inhibitor that accumulates during fruit abscission, which was later shown to happen via ethylene biosynthesis (Cracker and Abeles, 1969). ABA plays an important role in various physiological processes during plant growth and development, such as seed dormancy and germination, postgerminative growth, stomatal regulation and responses to various abiotic and biotic stresses, including drought and pathogen attack (Cutler et al., 2010). Regulation of cellular ABA levels through *de novo* biosynthesis, catabolism and transport within the plant enables adjustment to these various physiological and environmental conditions (Nambara and Marion-Poll, 2005; Merilo et al., 2015) (Figure 1). ABA levels are increased by *de novo* biosynthesis and recycling from inactive conjugates and are reduced by catabolic pathways including ABA degradation by hydroxylation and conjugation to inactive ABA glycose ester (ABA-GE) (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005).

1.3.1. ABA homeostasis – biosynthesis, catabolism and transport

ABA is synthesized from a C_{40} carotenoid precursor originating from the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Hirai et al., 2000; Kasahara et al., 2004). The initial steps of *de novo* ABA biosynthesis occur in plastids; the final two steps, which involve conversion of xanthoxin to ABA, take place in the cytoplasm (Seo and Koshiba, 2002) (Figure 1.). The first step in *de novo* biosynthesis is the conversion of zeaxanthin via intermediate antheraxanthin to violaxanthin by zeaxanthin epoxidase (ZEP), which was first identified in tobacco (*NpABA2*) (Marin et al., 1996) and is encoded by *ABA1* in *Arabidopsis* (Xiong et al., 2002). Violaxanthin is then converted to neoxanthin by the ABA4 protein (North et al., 2007). The proteins responsible for the isomerization of *trans*-viola/neoxanthin into 9-cis isomers have not yet been identified. The oxidative cleavage reaction of 9-cis-viola/neoxanthin to xanthoxin is catalysed by 9-cis-epoxycarotenoid dioxygenases (NCEDs). In *Arabidopsis*, nine *NCED* genes have been identified, and five of them (*NCED2*, *3*, *5*, *6* and *9*) are probably involved in ABA biosynthesis (Iuchi et al., 2001; Schwartz et al., 2003). Xanthoxin is translocated from plastids to the cytosol and catalysed to abscisic aldehyde by a short-chain dehydrogenase/reductase (SDR) encoded by *ABA2* (Gonzalez-Guzman et al., 2002; Cheng et al., 2002). The final step in the ABA biosynthesis pathway is the oxidation of abscisic aldehyde to ABA by an abscisic aldehyde oxidase (AAO) protein. There are four genes of AAO in *Arabidopsis*, among which AAO3 effectively converts abscisic aldehyde to ABA (Seo et al., 2000). The activity of AAO requires a molybdenum cofactor (MoCo), and mutants defective in MoCo synthesis, such as *Arabidopsis aba3* (Xiong et al., 2001) and tomato *flacca* (Sagi et al., 2002), are ABA deficient. In the tomato *flacca* and *sitiens* mutants, exogenously applied abscisic aldehyde was converted to abscisic alcohol and then oxidized to ABA via a shunt pathway (Taylor et al., 1988; Rock et al., 1991), which might be important in mutants impaired in aldehyde oxidation and appears to be a minor source of ABA in wildtype plants (Rock et al., 1991).

There are three pathways of ABA catabolism via hydroxylation that oxidize one of the methyl groups of the ring structure (C-7′, C-8′ and C-9′); the C-8′ position is the predominant position (Nambara and Marion-Poll, 2005). ABA hydroxylation at the 8′position yields the unstable intermediate 8′-hydroxy-ABA, which is spontaneously isomerized to phaseic acid (PA) and is then sometimes further reduced to dihydrophaseic acid (DPA) (Cutler and Krochko, 1999). ABA 8′-hydroxylation is mediated by a four-member CYP707A (CYP707A1 to CYP707A4) subfamily of cytochrome P450 monooxygenases (Saito et al., 2004; Kushiro et al., 2004). All four *CYP707A* genes were shown to be upregulated after drought stress and ABA treatment (Saito et al., 2004; Kushiro et al., 2004). CYP707A1 and CYP707A3 are important for post-germination growth because seedling growth by exogenous ABA was inhibited more effectively in *cyp707a1* and *cyp707a3* mutants (Okamoto et al., 2006), while CYP707A2 plays a key role in the rapid decrease in ABA levels prior to

seed germination, as the *CYP707A2* gene was upregulated after seed imbibition and the *cyp707a2* mutant was shown to over-accumulate ABA in dry and imbibed seeds (Kushiro et al., 2004). CYP707A3 is preferentially expressed in vascular tissue, while CYP707A1 is expressed in guard cells (Okamoto et al., 2009). In addition, it has been shown that CYP707As catalyse 9´-hydroxylation as a side reaction; neophaseic acid (neoPA) is spontaneously isomerized from 9´-hydroxyABA (Okamoto et al., 2011).

ABA and hydroxylated catabolites of ABA are conjugated with glucose for inactivation, with ABA-GE being the predominant form of conjugate (Cutler and Krochko, 1999) (Figure 1). Conjugation to ABA-GE is performed by ABA uridine diphosphate glycosyltransferase (UGT) (Xu et al., 2002; Priest et al., 2006). In *Arabidopsis*, UGT71B6 has been shown to convert active ABA to inactive ABA-GE (Priest et al., 2006), and its two homologues, UGT71B7 and UGT71B8, function in a redundant manner (Dong et al., 2014). In addition, another UGT, UGT71C5, glycosylates ABA to ABA-GE *in vitro* and *in vivo* (Liu et al., 2015). ABA is released from ABA-GE in a one-step hydrolysis reaction catalysed by the β-glycosidase homologues BG1 and BG2, which are localized to the ER and vacuole, respectively (Lee et al., 2006; Xu et al., 2012). A loss-of-function BG1 mutant showed increased transpiration, high drought sensitivity and defective stomatal closure in the dark, while overexpression of BG1 in rescued *bg1* plants enhanced tolerance to dehydration stress (Lee et al., 2006). The β-glycosidase BG2, which is from the same subfamily, showed *in vivo* ABA-GE hydrolysis activity, and similar to *bg1* mutant, the *bg2* mutant displayed increased sensitivity to dehydration stress (Xu et al., 2012).

Previous studies have shown that, in response to water stress, ABA is synthesized in the roots and transported to the shoots via long-distance transport (Zhang et al., 1987; Davies and Zhang, 1991). However, reciprocal grafting studies with wildtype plants and ABA-deficient mutants have revealed that stomatal aperture is predominantly regulated by leaf-sourced ABA (Holbrook et al., 2002; Christmann et al., 2007). Indeed, genes involved in ABA synthesis tend to show higher expression levels in the shoots, and several ABA biosynthetic enzymes, including NCED3, ABA2 and AAO3, are expressed in vascular tissues under both drought stress and well-watered growth conditions (Koiwai et al., 2004; Endo et al., 2008; Kuromori et al., 2014). Recent work has suggested that the peptide CLE25 (CLAVATA3/ EMBRYO-SURROUNDING REGION-RELATED 25) produced in the roots is a long-distance signal that moves from the roots to the shoots to modulate ABA accumulation in leaves by activating *NCED3* expression in response to drought (Takahashi et al., 2018). However, ABA levels in excised leaves can increase without a signal coming from the roots (McAdam and Brodribb, 2016; Sussmilch et al., 2017). By isolating mesophyll from the vascular tissue, McAdam and Brodribb (2018) showed that mesophyll cells are the main site of ABA biosynthesis. Furthermore, guard cells were shown to autonomously synthesize ABA (Bauer et al., 2013).

ABA is a weak acid (pK of 4.8) and exists either in an anionic (ABA^-) or protonated (ABA-H) forms. The latter form is uncharged and able to diffuse freely through the plasma membrane (Kaiser and Hartung, 1981). Under normal conditions, the apoplastic pH is more acidic than the cytoplasmic pH, and the ABA-H form prevails in the apoplast, resulting in diffusion into surrounding cells (Figure 1). Most of the diffused ABA-H shifts to the charged ABA^- form and becomes trapped on its way to guard cells as a less permeating anion, ABA^- (Boursiac et al., 2013). Under stress conditions, the apoplastic pH increases, and most of the ABA pools are in the ABA^- form, leading to decreased ABA diffusion into cells, including guard cells (Boursiac et al., 2013). Therefore, active ABA transport is thought to be important in stress conditions.

Several members of the G subfamily of ATP-binding cassette (ABC) transporters have been identified as ABA transporters (Kang et al., 2010; Kuromori et al., 2010) (Figure 1). ABCG25, which is localized to the plasma membrane in vascular tissue, exports ABA from the vasculature (Kuromori et al., 2010). Overexpression of *ABCG25* led to more closed stomata, whereas no aerial phenotype in *abcg25* knockout mutant was observed (Kuromori et al., 2010). ABCG40, which is expressed in guard cells, was identified as a plasma membrane ABA uptake transporter (Kang et al., 2010). Mutants defective in ABCG40 showed impaired stomatal closure in response to ABA and osmotic stress (Kang et al., 2010). Although both transporters are members of the ABCG subfamily, ABCG25 belongs to the branch of half-sized transporters (ABCG1-28); ABCG40, to full-size transporters (ABCG29-43) (Verrier et al., 2008). Therefore, functional redundancy might explain why the *abcg25* knockout mutant had no discernible aerial phenotype. Another half-size ABCG transporter, ABCG22, localizes to the guard cell plasma membrane and its knockout mutants presented larger stomatal apertures and increased transpiration and were more susceptible to drought stress compared to wildtype (Kuromori et al., 2011). However, the compound transported by ABCG22 is probably not ABA (Kuromori et al., 2011; Kuromori et al., 2017). Another type of ABA transporter, NPF4.6 (originally named AIT1), which is a member of the *Arabidopsis* NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY (NPF), was identified as a protein that could mediate ABA uptake into cells (Kanno et al., 2012). Recently, a member of the multidrug and toxin efflux (MATE) transporter family, DTX50, was shown to localize to the plasma membrane and mediate ABA efflux from the cytosol of both vascular and guard cells (Zhang et al., 2014). To obtain better understanding of ABA dynamics at the cellular level, FRET-based reporters of ABA were developed (Jones et al., 2014a; Waadt et al., 2014). These ABA-specific reporters enable the direct and noninvasive monitoring of ABA transport and changes in cytosolic ABA concentrations in a dynamic mode.

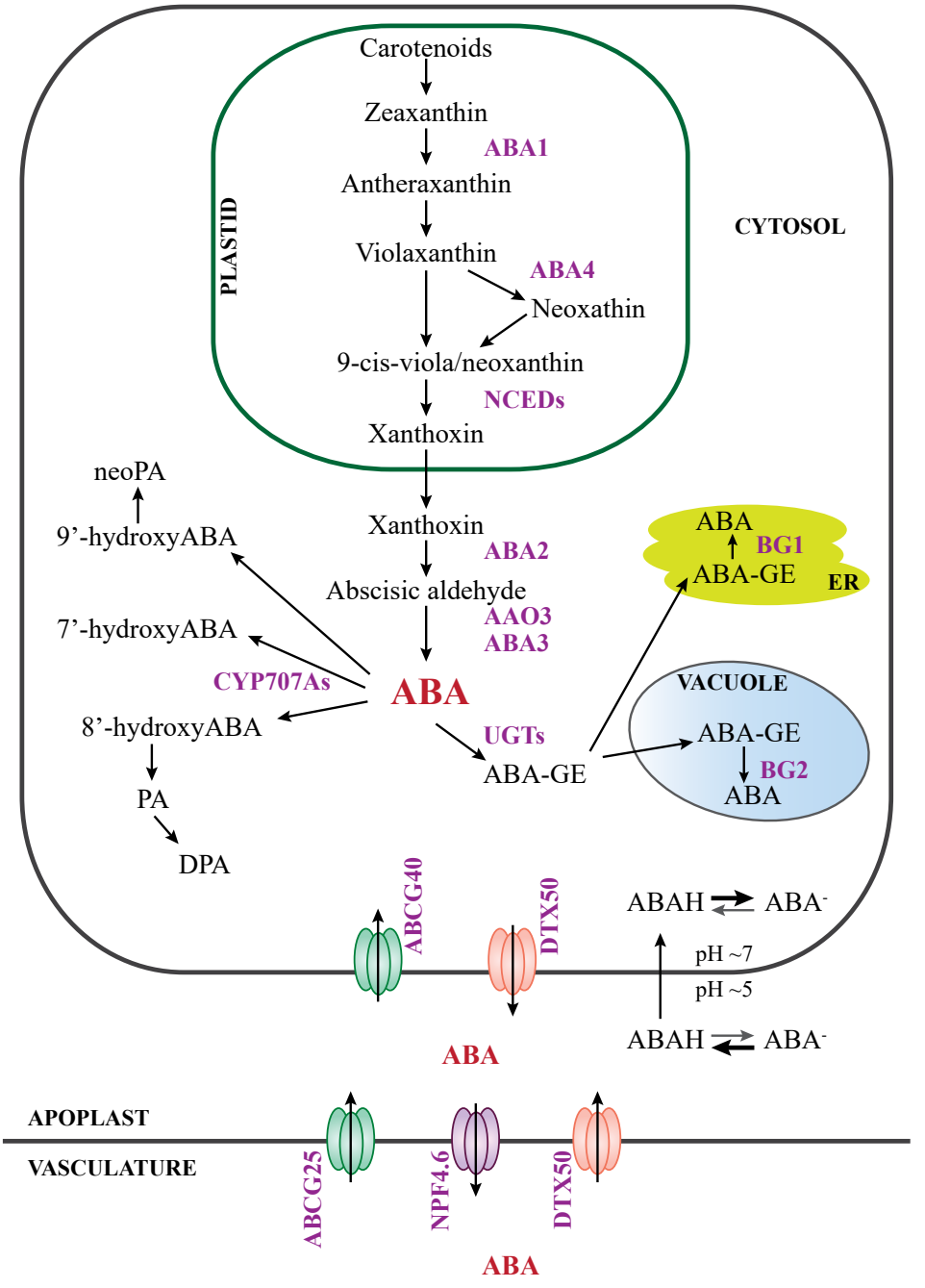

Figure 1. Overview of ABA biosynthetic, catabolic and transport pathways. ABA is synthesized from a C_{40} carotenoid precursor in a series of reactions in plastids and cytoplasm, and recycled from inactive conjugates. ABA is catabolized via hydroxylation and conjugated with glycose for inactivation.

1.3.2. ABA perception and signalling

The ABA-mediated signalling pathway is initiated by the perception of ABA by the ABA receptors PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/ REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) (Ma et al., 2009; Park et al., 2009) (Figure 2). In *Arabidopsis*, the PYR/RCAR family consists of 14 members divided into two subclasses depending on their oligomeric state: dimers (e.g., PYR1, PYL1, PYL2) and monomers (e.g., PYL4, PYL5, PYL6, PYL9, PYL10) (Yin et al., 2009; Hao et al., 2011). It has been found that the ABA affinity of dimeric receptors is lower than that of monomeric receptors, possibly because the dimer needs to dissociate for activity (Dupeux et al., 2011). However, the details of functional differentiation between these different receptors are not yet clear. Single PYR/PYL mutants do not show a distinct ABA-related phenotype (Park et al., 2009); however, the highest genetic impairment was observed in the *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* sextuple mutant, which presented the strongest ABA insensitivity and highest steady-state stomatal conductance (Gonzalez-Guzman et al., 2012). This result indicates that there is functional redundancy among PYR/RCAR proteins. Several of the PYR/RCARs were shown to interact with members of type 2C protein phosphatases (PP2Cs) and inhibit PP2C activity in an ABA-dependent manner (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). PYL5-PYL10 also inhibited PP2Cs to a certain extent in the absence of ABA (Hao et al., 2011). ABA binding to PYL/RCAR receptors leads to the formation of the ABA-PYR/RCAR-PP2C complex and inhibition of the PP2C active site (Melcher et al., 2009).

Members of clade A of the PP2C family, including ABI1, ABI2, HAB1, HAB2 and PP2CA/AHG3, act as negative regulators of ABA signalling (Gosti et al., 1999; Merlot et al., 2001; Saez et al., 2004; Kuhn et al., 2006). Plants carrying dominant mutations in *abi1-1* ($ABI1^{G180D}$) or *abi2-1* ($ABI2^{G168D}$) are highly insensitive to ABA (Leung et al., 1997), whereas loss-of-function mutants of ABI1 and ABI2 present enhanced ABA responses (Gosti et al., 1999; Merlot et al., 2001). In addition, it has been shown that overexpression of HAB1 or PP2CA leads to reduced sensitivity to ABA, while loss-of-function *hab1-1* and *pp2ca* mutants are hypersensitive to ABA (Saez et al., 2004; Kuhn et al., 2006). Compared to the single mutants, the triple mutants *hab1-1abi1-2abi2-2* and *hab1-1abi1-2pp2ca-1* showed stronger responses to ABA (Rubio et al., 2009). Inhibition of PP2Cs enables activation of the protein kinase Open Stomata 1 (OST1/SRK2E/SnRK2.6), an important positive regulator of ABA signalling (Vlad et al., 2009), via autophosphorylation (Belin et al., 2006). Furthermore, PP2Cs have been shown to directly dephosphorylate and deactivate SLAC1 (Brandt et al., 2012; Brandt et al., 2015). Additional targets of PP2Cs include Ca^{2+}-dependent protein kinases CPKs, calcineurin B-like proteins (CBLs) and CBL-binding protein kinases (CIPKs) (Geiger et al., 2010; Brandt et al., 2012; Maierhofer et al., 2014).

OST1 belongs to subclass III of the Sucrose Nonfermenting1-Related Protein Kinase 2 (SnRK2)-type family, which contains 10 members (Kobayashi et al., 2004). Two other subclass III protein kinases, SnRK2.2 and SnRK2.3, are also activated by ABA and regulate the transcriptional response to ABA (Fujii and Zhu, 2009; Fujita et al., 2009). SnRK2.2 and SnRK2.3 are predominantly expressed in seeds or vegetative tissue (Fujii and Zhu, 2009), whereas OST1 is expressed in guard cells and vascular tissue (Mustilli et al., 2002). The importance of subclass III SnRK2s was demonstrated by the triple *snrk2.2/2.3/2.6* knockout mutant that lacks most ABA responses and shows an ABA-insensitive phenotype in terms of seed germination and gene expression (Fujita et al., 2009). These three SnRK2 kinases control gene expression by the phosphorylation of ABA-responsive transcription factors (ABF/AREB) (Fujii and Zhu, 2009). In the absence of ABA, PP2Cs inactivate OST1 by dephosphorylating serine 175 in the activation loop of OST1 (Vlad et al., 2009; Umezawa et al., 2009). In the presence of ABA, PYR1 inhibited ABI1-mediated inactivation of OST1, whereas ABA-dependent activation of OST1 was repressed in the *abi1-1* mutant, which was unable to bind PYR/RCAR proteins (Umezawa et al., 2009). Furthermore, PP2Cs were shown to interact with the other subclass III SnRKs (Umezawa et al., 2009). Downstream targets of SnRK2s include SLAC1 (Geiger et al., 2009; Lee et al., 2009), QUAC1 (Imes et al., 2013) and KAT1 (Sato et al., 2009).

The SLAC1 anion channel is activated via direct phosphorylation by two calcium-independent kinases: OST1 (Vlad et al., 2009; Umezawa et al., 2009) and the receptor-like kinase GUARD CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1) (Hua et al., 2012). OST1 co-expression with SLAC1 in *Xenopus* oocytes activated SLAC1 anion channels, while the presence of ABI1 prevented SLAC1 activation (Geiger et al., 2009; Lee et al., 2009). *In vitro* studies have shown that OST1 can phosphorylate serine (S) sites at positions 59, 86, 113 and 120 within the N-terminal region of SLAC1 (Vahisalu et al., 2010). GHR1, which is involved in stomatal regulation in response to ABA and hydrogen peroxide, has also been shown to phosphorylate the SLAC1 N-terminal region (Hua et al., 2012). When expressed in *Xenopus* oocytes, GHR1 was shown to activate SLAC1 and this activation was inhibited by ABI2 but not ABI1 (Hua et al., 2012). However, recent evidence suggests that GHR1 may act as a scaffold for other regulatory kinases to trigger SLAC1 activation (Sierla et al., 2018). One possible target kinase is CPK3, which has been shown to interact with GHR1 in yeast (Jones et al., 2014b), *in vitro* and *in planta* (Sierla et al., 2018). Moreover, CPK3 is one of the Ca^{2+}-dependent kinases shown to activate SLAC1 by phosphorylating its N-terminus (Scherzer et al., 2012). In addition to CPK3, other Ca^{2+}-regulated kinases have been shown to participate in SLAC1 activation. CPK6, CPK21, CPK23 and CIPK23 in complex with CBL1 or CBL9 activate SLAC1 in *Xenopus* oocytes (Geiger et al., 2010; Brandt et al., 2012; Maierhofer et al., 2014), and CPK6 was found to phosphorylate site S59 in SLAC1 (Brandt et al., 2012). While the activities of CPK3 and CPK21 were dependent on Ca^{2+}, CPK6 and CPK23 showed kinase activity even at low Ca^{2+}

concentrations (Geiger et al., 2010; Scherzer et al., 2012), indicating that CPKs vary in their Ca^{2+} affinity. The activation of SLAC1 by CPKs and CBL1/9+CIPK23 was found to be inhibited by ABI1 and ABI2 (Geiger et al., 2010; Brandt et al., 2012). In addition to SLAC1, CPKs and CBL1/9+CIPK23 activate another S-type channel, SLAH3 (Geiger et al., 2011; Maierhofer et al., 2014). Furthermore, CPK9 is suggested to be involved in ABA-mediated signalling because the *cpk9* loss-of-function mutant exhibited a hypersensitive response to ABA (Chen et al., 2019).

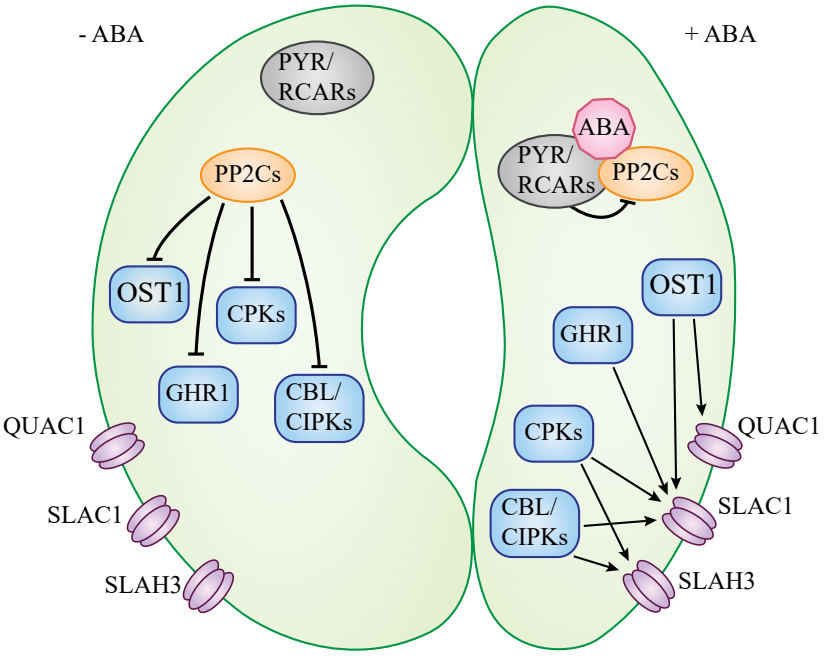

Figure 2. ABA signalling pathway in guard cells.
(Left) In the absence of ABA, ABA receptors PYR/RCARs are not bound to PP2Cs and PP2Cs are active, inhibiting their downstream target kinases OST1, GHR1, CPKs and CBL/CIPKs. Inactive kinases are unable to phosphorylate anion channels SLAC1, SLAH3 and QUAC1 and stomata remain open.
(Right) In the presence of ABA, ABA receptors PYR/RCARs bind to ABA and interact with PP2Cs to inhibit their phosphatase activity. This leads to the activation of downstream target kinases, which in turn activate anion channels and stomata close.

The pathways of stomatal closure induced by darkness, elevated CO_2 concentration and reduced air humidity (i.e., increased VPD, the difference between the vapour pressures of the leaf and the atmosphere) have been suggested to converge with the ABA signalling pathway at some point (Webb and Hetherington, 1997; Xie et al., 2006; Xue et al., 2011; Merilo et al., 2013). Recent evidence suggests that signalling induced by elevated CO_2 may be more ABA-independent than previously thought. A model for ABA-independent regulation of SLAC1 during CO_2-induced stomatal closure has been suggested, where the proteins HT1

(HIGH LEAF TEMPERATURE 1), MPK12, MPK4 and GHR1 have important roles (Hõrak et al., 2016; Jakobson et al., 2016; Tõldsepp et al., 2018). Regarding the role of ABA in the stomatal VPD response, several papers show either increased foliar ABA concentration within minutes or increased expression of NCED in response to high VPD (Bauer et al., 2013; McAdam and Brodribb, 2015), supporting the hypothesis that ABA mediates the VPD response. McAdam and Brodribb (2015) showed that in lycophytes, ferns and gymnosperms, VPD-induced stomatal closure is an ABA-independent passive hydraulic response due to reduced leaf water potential, whereas in angiosperms, the stomatal VPD response is mediated by ABA (McAdam and Brodribb, 2015).

1.4. OST1, an important positive regulator of ABA signalling

Screening of drought-stressed EMS mutant plants by thermal imaging led to the identification of *ost1-1* and *ost1-2* lines based on their low leaf temperature that pointed to a high transpiration rate. Further analysis revealed that these two mutants corresponded to two independent recessive alleles of *OST1* (Merlot et al., 2002). Initially, OST1 was thought to be involved specifically in guard cell responses to ABA, since loss of OST1 function led to insensitivity both to ABA-induced stomatal closure and ABA inhibition of stomatal opening (Mustilli et al., 2002), whereas seed dormancy and seed germination in the presence of ABA were not affected (Mustilli et al., 2002). By contrast, overexpression of OST1 led to ABA hypersensitivity in ABA promotion of stomatal closure and inhibition of stomatal opening (Acharya et al., 2013). Mutations in OST1 did not affect low CO_2- induced stomatal opening, suggesting that OST1 is not involved in CO_2 signalling (Mustilli et al., 2002). However, later, it was shown that OST1 loss-of-function mutants were strongly impaired in both CO_2-induced stomatal closure and activation of S-type anion channels (Xue et al., 2011). More recent evidence suggests that the CO_2 signalling pathway merges with the core ABA signalling pathway downstream of OST1 because OST1 kinase activity in guard cells is not enhanced by CO_2 elevation; however, a basal level of OST1 activity is still necessary for CO_2-induced stomatal closure (Hsu et al., 2018). Furthermore, mutation in *ost1* led to impaired stomatal closure responses to reduced air humidity, darkness and O_3 (Merilo et al., 2013). The *ost1* loss-of-function mutant was also impaired in the production of reactive oxygen species (ROS) in response to ABA; however, H_2O_2- and extracellular Ca^{2+}-induced stomatal closure was not affected, indicating that *OST1* acts upstream of ROS signalling (Mustilli et al., 2002). Supporting the role of OST1 in ABA-induced ROS production, Acharya et al. (2013) showed that the ABA-induced production of ROS in the guard cells of *ost1* mutants was impaired but was enhanced in the guard cells of OST1 overexpression lines.

Cloning of OST1 revealed that it is an ABA-activated protein kinase similar to *Vicia faba* AAPK (ABA-activated serine-threonine protein kinase), which is a guard cell-specific positive regulator of stomatal ABA signalling (Li et al.,

2000; Mustilli et al., 2002). OST1 is also similar to wheat *PKABA1*, which is upregulated in seeds by drought and ABA (Anderberg and Walker-Simmons, 1992). Similar to *Arabidopsis*, rice harbours 10 SnRK2 genes designated SAPK1-SAPK10 and SAPK8/9/10, which are homologous to SnRK2.2/OST1/ SnRK2.3 and were shown to be activated by ABA in a protoplast system (Kobayashi et al., 2004). In maize, ZmOST1 was shown to be homologous to OST1, and *ZmOST1* loss-of-function mutant had an impaired stomatal ABA response and was less tolerant to drought stress (Wu et al., 2019), indicating that ZmOST1 and OST1 have similar roles in guard cell function. Furthermore, evolutionary studies based on sequence data revealed that the function of OST1 has been conserved in mosses, lycophytes and seed plants (Chater et al., 2011; Ruszala et al., 2011). These findings were supported by another study, that showed that OST1 kinases from mosses, lycophytes and seed plants were able to activate *Arabidopsis* SLAC1 in *Xenopus* oocytes (Lind et al., 2015).

Several substrates of OST1 have been identified. Two basic leucine zipper transcription factors from the AREB/ABF subfamily that regulate the transcription of ABA-induced genes are suggested to be OST1 substrates (Furihata et al., 2006; Sirichandra et al., 2010). Via an in-gel kinase assay, OST1 was shown to phosphorylate AREB1/ABF2 (Furihata et al., 2006). Bimolecular fluorescence complementation experiments with OST1 and ABF3 revealed that ABF3 interacts with OST1 in the nucleus of guard cells and ABF3 was shown to be phosphorylated by OST1 *in vitro* (Sirichandra et al., 2010). Moreover, transcriptome analysis of the triple *snrk2.2/2.3/2.6* and quadruple *areb1 areb2 abf3 abf1* mutants revealed that the majority of AREB/ABF target genes downregulated in the triple mutant of SnRk2 were also downregulated in the quadruple AREB/ ABF mutant (Yoshida et al., 2015). In addition, phosphoproteomic analysis has been used to identify potential targets of SnRK2s. Comparative analysis between Col-0 wildtype plants and triple *snrk2.2/2.3/2.6* mutants to identify proteins that were differentially phosphorylated in response to dehydration stress or ABA revealed a previously unknown protein, SnRK2-substrate 1 (SNS1), that accumulated in response to both ABA and dehydration treatments (Umezawa et al., 2013). An *in vitro* kinase assay showed that SNS1 was phosphorylated by OST1, and a *sns1* loss-of-function mutant was hypersensitive to ABA-induced post-germination growth (Umezawa et al., 2013), indicating that SNS1 is involved in ABA signalling. OST1 also targets NADPH oxidase respiratory burst oxidase homologue F (RbohF) and RbohD, which generate ROS in response to ABA (Sirichandra et al., 2009; Acharya et al., 2013).

1.5. ENHANCED RESPONSE TO ABA 1 (ERA1)

In *Arabidopsis*, the *ERA1* gene encodes the β-subunit of farnesyltransferase (FT) (Cutler et al., 1996). FT mediates protein farnesylation, a post-translational protein modification by which a 15-carbon farnesyl isoprenoid moiety is attached

to the C-terminal region of target proteins with a CaaX motif (C = cysteine, a = aliphatic amino acid, X = typically serine, cysteine, glutamine, methionine or alanine) (Crowell and Huizinga, 2009). In a similar process, geranylgeranyl-transferase type I (GGT I) attaches a 20-carbon geranylgeranyl isoprenoid moiety to a target protein with a CaaX motif, where 'X' is leucine (Crowell and Huizinga, 2009). Both FT and GGT I are heterodimeric enzymes that have a common α-subunit but distinct β-subunits, which determine substrate specificity (Zhang and Casey, 1996). A third enzyme mediating protein prenylation in addition FT and GGT I, Rab geranylgeranyltransferase (Rab-GGT), adds two geranylgeranyl groups to RAB proteins bound to RAB Escort Protein (REP) (Leung et al., 2006). After prenylation by FT or GGT I, modified proteins undergo additional processing in the ER. The last three amino acids from the CaaX motif are removed via proteolysis by one of two CaaX endoproteases encoded by *STE24* (*CaaX PRENYL PROTEASE 1*) and *FACE2* (*FARNE-SYLATED PROTEIN-CONVERTING ENZYME 2*) (Bracha et al., 2002; Cadiñanos et al., 2003), and the prenylated cysteine is methylated either by either *STE14A* or *STE14B* (*ISOPRENYL CYSTEINE METHYLTRANSFERASE 14A; 14B*) (Narasimha Chary et al., 2002). Prenylation and subsequent pro-cessing increases the hydrophobicity of the modified proteins and promotes their membrane association and interactions with other proteins (Zhang and Casey, 1996).

An *era1* mutant was isolated in a screen for plants with a hypersensitive response to ABA-induced inhibition of seed germination (Cutler et al., 1996). Plants lacking functional ERA1 show increased seed dormancy and stomatal closure in response to ABA and increased tolerance to drought stress (Cutler et al., 1996; Pei et al., 1998). Moreover, guard cells of the *era1* mutant displayed hypersensitive S-type anion current activation in response to ABA (Pei et al., 1998). These results suggest that one or more negative regulators of ABA signalling are targets for farnesylation. In addition, a lack of FT activity in *era1* plants affects growth and development, including delayed growth, enlarged meristems, flowers with an increased number of petals and sepals, and late flowering (Yalovsky et al., 2000). Disruption within the *PLP* (*PLURIPETALA*) gene, which encodes the common α-subunit of FT and GGT I, leads to a more severe developmental phenotype compared to that of *era1* plants, suggesting that GGT I is partially able to compensate for the loss of FT in *era1* plants (Running et al., 2004). Furthermore, ERA1 was shown to regulate the response to pathogens because *era1* plants exhibited enhanced susceptibility towards the virulent pathogens *Pseudomonas syringae* pv *maculicola* and *Hyaloperono-spora parasitica* (Goritschnig et al., 2008).

There are approximately 700 proteins identified as potential targets with the 'CaaX' motif in *Arabidopsis* (Northey et al., 2016), making it difficult to discern the different roles and elucidate the physiological significance of farnesylation in plant growth and development, and so far, only a few of the target proteins have been functionally characterized. One of the target proteins characterized is the cytochrome P450 enzyme CYP85A2, which mediates the conversion of

castasterone to brassinolide at the last step in brassinosteroid biosynthesis. Similar to plants lacking functional ERA1, *cyp85a2* loss-of-function plants displayed round-shaped rosettes and flowers with protruding carpels; moreover, they were more tolerant to drought, and their seeds were more sensitive to ABA (Northey et al., 2016). Another target protein is ALTERED SEED GERMINATION2 (ASG2) whose CaaX motif was shown to be farnesylated *in vitro* (Dutilleul et al., 2016). Plants deficient in ASG2 and *era1* mutants both show a similar ABA-hypersensitive seed germination phenotype (Dutilleul et al., 2016).

1.6. Stomatal development in *Arabidopsis*

1.6.1. Stomatal development and patterning

Plants regulate their water use by opening and closing stomata as a fast and short-term response, and by the number of stomata on the surface of leaves (i.e., stomatal density) as a long-term response. The molecular mechanism of stomatal development is best understood in *Arabidopsis*. Interestingly, the basic module of the complex stomatal development mechanism described below for *Arabidopsis* may also be functional in non-vascular land plants that have stomata, pointing at a single ancient genetic origin of stomata (Chater et al., 2017).

Steps to the formation of guard cells are known as the stomatal lineage, where meristemoid mother cells (MMCs) undergo a series of asymmetric cell divisions and a single symmetric cell division to form a pair of guard cells (Bergmann and Sack, 2007; Vatén and Bergmann, 2012; Zoulias et al., 2018) (Figure 3). In the first step, an MMC asymmetrically divides into a smaller meristemoid and a larger cell, which differentiates into an epidermal pavement cell. This step is controlled by the basic helix-loop-helix (bHLH) transcription factor SPEECHLESS (SPCH) (MacAlister et al., 2007). Mutants lacking functional SPCH are unable to commence the stomatal lineage and develop an epidermis consisting of only pavement cells (MacAlister et al., 2007; Lau et al., 2014). Next, each meristemoid undergoes up to three asymmetric divisions before differentiating into a guard mother cell (GMC), and this transition is regulated by the bHLH transcription factor MUTE (Pillitteri et al., 2007; Pillitteri and Torii, 2007). In *mute* loss-of-function mutants, meristemoids undergo asymmetric division before arresting without forming a GMC, while overexpression of MUTE in the wildtype leads to an epidermis formed mostly of stomata (Pillitteri et al., 2007). The final step in the stomatal lineage is the symmetric division of the GMC to form a stoma of a pair of guard cells, which is regulated by a third bHLH transcription factor, FAMA (Ohashi-Ito and Bergmann, 2006). Each of the bHLH transcription factors, SPCH, MUTE and FAMA, are expressed at specific time points in stomatal development, and two other bHLH transcription factors expressed throughout the stomatal lineage encoded by *INDUCER OF CBF EXPRESSION1/SCREAM* (*ICE1/SCRM*) and *SCRM2* form heterodimers with SPCH, MUTE and FAMA (Kanaoka et al., 2008).

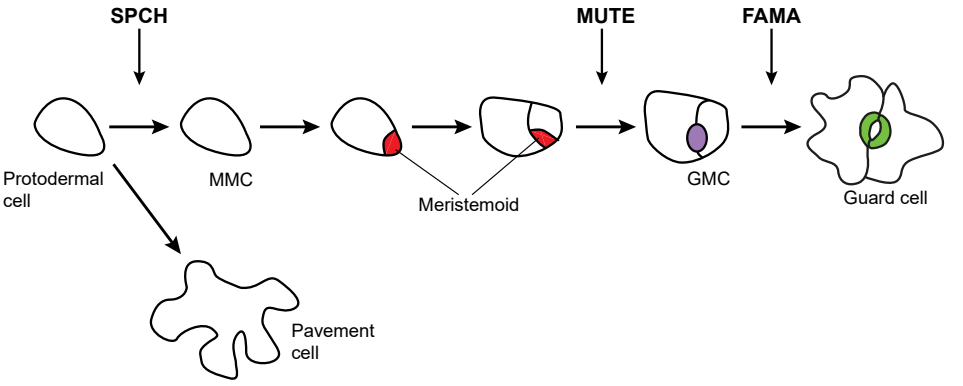

Figure 3. Stomatal development in *Arabidopsis*. Three bHLH transcription factors, SPCH, MUTE and FAMA, regulate cell transitions during stomatal development. Stomatal lineage is initiated when a protodermal cell transforms to a meristemoid mother cell (MMC). MMC asymmetrically divides into a smaller meristemoid cell (red) and a larger epidermal pavement cell. Meristemoids may undergo further asymmetric divisions before differentiating into a guard mother cell (GMC; purple). GMC divides symmetrically to form a pair of guard cells (green).

Stomata follow a specific developmental pattern called the one-cell spacing rule: each stoma is separated from others by at least one pavement cell (Larkin et al., 1997). This one-cell spacing rule is ensured by key regulators of the asymmetric cell division identified via several patterning mutants. These key regulators include the secreted peptides EPIDERMAL PATTERNING FACTOR (EPF) and EPF-LIKE (EPFL); three leucine-rich-repeat receptor-like kinases (LRR-RLKs), ERECTA (ER), ER-LIKE1 (ERL1) and ERL2; LRR receptor-like protein (RLP) TOO MANY MOUTHS (TMM); and components of the mitogen-activated protein kinase (MAPK) cascade (Zoulias et al., 2018). Among the EPF/EPFL family genes, EPF1 and EPF2 expressed in stomatal lineage cells (Hara et al., 2007; Hunt and Gray, 2009; Hara et al., 2009) and STOMAGEN (STOM/EPFL9) expressed in mesophyll cells (Sugano et al., 2009; Hunt et al., 2010) regulate stomatal development. Although EPF1 and EPF2 have high similarity in sequence and structure, these two peptides have distinct functions: EPF2 inhibits entry into the asymmetric cell division of the stomatal lineage, whereas EPF1 controls stomatal patterning via the asymmetric cell division (Hara et al., 2007; Hunt and Gray, 2009; Hara et al., 2009). By contrast, STOM promotes stomatal production and overexpression of STOM increases stomatal density and clustering (Sugano et al., 2009; Hunt et al., 2010). The perception of EPF1 and EPF2 occurs via a plasma membrane receptor complex formed by TMM binding to ER or ERL1 (Lee et al., 2012; Lin et al., 2017). The ER-TMM receptor complex was shown to be a predominant receptor for EPF2 (Lee et al., 2012), whereas EPF1 signals are perceived by the ERL1-TMM receptor complex (Lin et al., 2017). On the other hand, STOM was shown to compete with EPF1 as well as with EPF2 for binding to the ERL1- or ER-containing

complex, respectively (Lin et al., 2017). Downstream of the receptors are components of the MAPK signalling cascade, including YODA (MPKKK), MKK4/5 and MPK3/6 and loss of function of these kinases results in increased clustering of stomata (Bergmann et al., 2004; Wang et al., 2007). The downstream target of the YODA-MKK4/5-MPK3/6 module has been shown to be SPCH (Lampard et al., 2008).

Rice plants overexpressing EPF1 showed reduced stomatal density and conductance, higher drought tolerance and, importantly, no grain yield penalty (Caine et al., 2019). Thus, manipulating stomatal density could be a viable option to generate crops that perform better in future climates.

1.6.2. Stomatal development control by environmental signals

Environmental signals such as light intensity, atmospheric CO_2 concentration and endogenous plant hormone stimuli have been shown to regulate stomatal development (Hetherington and Woodward, 2003). A study of 100 species revealed that more that 70% of the analysed species showed a reduction in stomatal density when grown under elevated CO_2 levels (Woodward and Kelly, 1995). Moreover, many accessions of *Arabidopsis* showed a decrease in stomatal density and the stomatal index (the ratio of stomata to epidermal cells) under high CO_2 concentrations (Woodward et al., 2002). However, no change or increase in stomatal density under CO_2 enrichment has been observed both within and across species (Woodward and Kelly, 1995; Woodward et al., 2002). Additionally, mature leaves are able to detect environmental signals, which are then signalled systemically to developing leaves (Lake et al., 2001). By using a cuvette system, Lake and co-authors (2001) were able to separate developing leaves from mature leaves and treat isolated leaves with different CO_2 concentrations. The stomatal density of developing leaves decreased when the mature leaves were subjected to elevated CO_2 levels, and the stomatal density increased in the developing leaves when the mature leaves were under ambient CO_2 levels and when expanding leaves were under elevated CO_2 levels (Lake et al., 2001). Similarly, shading of mature *Arabidopsis* or tobacco leaves led to a decrease of stomatal density in untreated developing leaves compared with those of control plants (Lake et al., 2001; Thomas et al., 2004). Thus, these data provide evidence of a long-distance systemic signalling response from mature leaves to developing leaves in response to elevated CO_2 and shading.

Among several phytohormones affecting stomatal development, brassinosteroid treatment was shown to reduce stomatal density (Kim et al., 2012), whereas plants grown in the presence of the exogenous ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) showed increased stomatal density (Serna and Fenoll, 1996). Plants treated with gibberellin showed increased stomatal density in hypocotyls, which was enhanced by co-application of ethylene and auxin (Saibo et al., 2003). Moreover, stomatal density negatively correlates with leaf ABA concentration, since ABA biosynthesis mutants

display high stomatal density (Tanaka et al., 2013; Chater et al., 2015), whereas ABA catabolism mutants have fewer stomata compared to wildtype (Tanaka et al., 2013).

At present, little is known about the molecular mechanism by which environmental cues control stomatal development. The *HIGH CARBON DIOXIDE* (*HIC*) gene was found to be involved in the CO_2-controlled stomatal development response since *hic* loss-of-function mutants present increased numbers of stomata when exposed to elevated CO_2 levels without disrupting the one-cell spacing rule (Gray et al., 2000). Mutations in the β-carbonic anhydrase genes *CA1* and *CA4*, whose products bind CO_2 and catalyse its conversion to HCO_3^- and H^+, led to increased stomatal density under elevated CO_2 levels compared to those of plants kept under low CO_2 (Engineer et al., 2014). In wildtype plants, elevated CO_2 levels induce the upregulation of *EPF2* and *CO_2 RESPONSE SECRETED PROTEASE* (*CRSP*) transcripts, and this upregulation was impaired in the *ca1 ca4* mutant (Engineer et al., 2014). Recently, a novel allele of the *Arabidopsis BIG* locus named *CO_2 insensitive 1* (*cis1*) was identified via infrared thermography undertaken to identify new genes required for stomatal CO_2 responses. Mutation in *cis1* resulted in increased stomatal density when plants were grown under elevated levels of CO_2 (He et al., 2018). In terms of light-mediated regulation of stomatal development, the blue light receptors CRYPTOCHROME1 (CRY1) and CRY2 and red/far-red light photoreceptors phytochrome B (PHYB) and PHYA are involved (Kang et al., 2009). In the *cry1 cry2* double mutant and in the *phyB* loss-of-function mutant, stomatal development was inhibited under blue and red light, respectively (Kang et al., 2009). Moreover, mutants lacking functional *phyA* had very few stomata in the epidermis under far-red light (Kang et al., 2009). In the same paper, CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) was shown to act parallel with TMM to positively regulate YODA.

2. AIMS OF THE STUDY

Rapid changes in stomatal aperture width and changes in stomatal density on a longer timescale affect stomatal conductance, one of the most important leaf physiological traits. Thus, manipulations of stomatal aperture width and density affect plant water use and stress tolerance. The phytohormone ABA is an important endogenous regulatory molecule that affects both of these responses. In response to stress, ABA signalling is triggered by the induction of ABA synthesis and transport from the site of biosynthesis to target cells. ABA transporters from various protein families have been purported to be involved in this process; however, reports comparing the roles of different transporters are not available. When ABA signalling is initiated, a positive regulator of this pathway, OST1, activates anion channels to mediate stomatal closure in response to ABA and environmental factors. A negative regulator role of ABA signalling has been proposed for ERA1, a mediator of protein farnesylation. However, the role of protein farnesylation in regulating stomatal movement has yet to be elucidated: very few farnesylated proteins have been characterized in stomatal regulatory pathways. My thesis aims to study the fast stomatal regulation affected by exogenously applied ABA spraying, abiotic environmental factors and protein farnesylation and the long-term regulation of stomatal development affected by ABA and OST1.

The specific aims of the thesis were as follows:

1. To study the effects of leaf ABA concentration on steady-state stomatal conductance.
2. To study the effects of leaf ABA concentration on stomatal responses to an increase in VPD and other abiotic stimuli.
3. To investigate the importance of guard cell- and phloem companion cell-specific ABA biosynthesis in ABA responses.
4. To study the role of different ABA transporters in regulating steady-state stomatal conductance and fast stomatal responses to high VPD, elevated CO_2 concentration and exogenous ABA spraying.
5. To study the role of OST1, a positive regulator of the ABA signalling pathway, in fast stomatal responses to environmental factors and its role in determining stomatal density.
6. To study the role of ERA1 in stomatal closure in response to high VPD, darkness, elevated CO_2 concentration and exogenous ABA spraying.
7. To study the role of ERA1 in stomatal opening in response to light.

3. MATERIALS AND METHODS

The details of the plant growth conditions and experimental procedures are described in the materials and method sections of manuscripts I–IV. The principle of whole-plant gas exchange measurements and calculations of stomatal conductance are provided below.

3.1. Stomatal conductance measurements

The stomatal conductance of intact plants was measured with a rapid-response gas exchange measurement device, like that described by Kollist et al. (2007) and a modified device consisting of eight thermostated flow-through whole-rosette cuvettes. These gas exchange measurement systems enable continuous measurements of water vapour and CO_2 in the air entering the plant cuvette and coming out of the cuvette. Furthermore, the air temperature inside the cuvettes was also measured to calculate the leaf temperature.

The air entering the cuvette (reference channel) and air coming from the cuvette (measurement channel) were analysed with an infrared gas analyser (Li-700; Li-Cor), and the net assimilation rate (A_{net}; µmol CO_2 m^{-2} s^{-1}) and transpiration (E; mmol H_2O m^{-2} s^{-1}) were calculated as follows:

$$A_{net} = \frac{\text{air flow rate}*(CO_2\text{in-}CO_2\text{out})}{\text{rosette area}}$$

$$E = \frac{\text{air flow rate}*(H_2O\text{out-}H_2O\text{in})}{\text{rosette area}}$$

The total leaf conductance (G_t; mmol m^{-2} s^{-1}) to water vapour was calculated as (von Caemmerer and Farquhar, 1981):

$$G_t = \frac{E}{(H_2O\text{leaf-}H_2O\text{air})}$$

The leaf temperature, which is needed to determine the leaf-to-air humidity gradient (H_2O_{leaf}-H_2O_{air}), was calculated using the energy budget equation (Parkinson, 1985). Stomatal conductance (mmol H_2O m^{-2} s^{-1}) was calculated from G_t by subtracting the values of the boundary layer and cuticular conductance.

In my thesis, steady-state stomatal conductance is the value of stomatal conductance of well-watered plants measured after a stabilization period of at least 60 min at ambient CO_2 concentration, a relative air humidity of 60–70% and 150 µmol m^{-2} s^{-1} of photosynthetically active radiation.

3.2. Plant material

Crossing of *Arabidopsis* was performed with plants at the early stage of flowering. From an unopened bud on a female parent, all flower parts were removed except for the pistil. From the male parent, a fully open flower was removed, and the female parent was pollinated by brushing the stigma with the removed flower multiple times, so that the pollen is visible on the stigma. A label was attached to the stem under the bud to mark the cross. Seeds were harvested from the developed siliques and the progeny resulting from the crosses were genotyped with PCR-based markers. All studied single and crossed mutants from publications I–IV are presented in Table 1.

Table 1. *Arabidopsis*, tomato and pea mutants used in publications I–IV. All *Arabidopsis* mutants are in Col-0 background except for *aba1-1*.

Mutant	Suggested role	Publication	Reference
ABA homeostasis and transport			
aba1-1 (Ler)	Encodes a zeaxanthin epoxidase that functions in the first step of ABA biosynthesis	I	(Xiong et al., 2002)
aba4-3	Protein involved in neoxanthin synthesis from violaxanthin	I	(North et al., 2007)
nced3 nced5	NCED's catalyse the cleavage reaction of neo- and violaxanthin to xanthoxin	I	(Iuchi et al., 2001; Frey et al., 2012)
aba2-11	Catalyses the conversion of xanthoxin to abscisic aldehyde	I	(Schwartz et al., 1997)
Pea *wilty*	Catalyses the conversion of xanthoxin to abscisic aldehyde	I	(McAdam et al., 2015)
aao3-2	Catalyses the oxidation of abscisic aldehyde to ABA	I	(Seo et al., 2000)
aba3-1	Encodes MoCo sulfurase required by aldehyde oxidase for its catalytic activity	I, II, III	(Xiong et al., 2001)
Tomato *flacca*	Encodes MoCo sulfurase required by aldehyde oxidase for its catalytic activity	I	(Sagi et al., 2002)
cyp707a1 cyp707a3	Double mutant of ABA 8´-hydroxylases	II	(Saito et al., 2004; Kushiro et al., 2004)
abcg22	Role in ABA transport is not yet identified	III	(Kuromori et al., 2011)
abcg25	ABA exporter from vascular tissue	III	(Kuromori et al., 2010)

Mutant	Suggested role	Publication	Reference
abcg27	Shows increased expression in response to reduced humidity and ABA	III	(Merilo et al., 2015)
abcg40	ABA importer in guard cells	III	(Kang et al., 2010)
ait1	ABA importer	III	(Kanno et al., 2012)
bg1	β-glycosidase that hydrolyzes ABA-GE to ABA in the ER	III	(Lee et al., 2006)
bg2	β-glycosidase that hydrolyzes ABA-GE to ABA in the vacuole	III	(Xu et al., 2012)
ABA signalling pathway			
112458	Sextuple mutant of ABA receptors	I	(Ma et al., 2009; Park et al., 2009)
abi1-1	Point mutation in ABI1 leading to the loss of PYR/RCAR binding and ABA insensitivity	IV	(Umezawa et al., 2009)
ost1-3	ABA activated protein kinase	I, II, III, IV	(Mustilli et al., 2002)
Other			
era1-2	Encodes the β-subunit of farnesyltransferase	IV	(Cutler et al., 1996)
asg2-1	Farnesylated protein that is suggested to participate in ABA signalling	IV	(Dutilleul et al., 2016)
asg2-2			
cyp85a2-2	Mediates the last step in brassinosteroids biosynthesis	IV	(Northey et al., 2016)
rop10 rop11	ROP10 and ROP11 are members of the plant-specific ROP small GTPases family and negative regulators of ABA responses	IV	(Zheng et al., 2002; Li et al., 2012)

4. RESULTS AND DISCUSSION

4.1. ABA biosynthesis and signalling affect steady-state stomatal conductance

To address the role of ABA in determining steady-state stomatal conductance, we studied plants defective in proteins of different steps in the ABA biosynthesis pathway. We found that the values of stomatal conductance of well-watered plants correlated significantly with the leaf ABA concentration in our dataset consisting of *Arabidopsis* ABA-deficient lines and the respective wildtype: steady-state stomatal conductance was highest in plants with the lowest ABA concentration (I). In *aba4-3* and *aao3-2*, the concentration of ABA was approximately 72% of wildtype ABA, whereas in *aba3-1*, *aba2-11* and *nced3 nced5*, it was 42%, 32% and 31% of wildtype ABA, respectively (I, Fig. 2B). In addition, ABA-deficient mutants of pea and tomato displayed higher stomatal conductance compared to their respective wildtypes (I, Fig. 2A). Thus, these results suggest that ABA is involved in determining the steady-state stomatal conductance, which is related to the effects of leaf ABA concentration on stomatal density (I, Fig. 4D) and stomatal aperture width (Figure 4B).

To gain further insight into the contribution of different ABA-related pathways in determining steady-state stomatal conductance, we crossed *ost1-3* into *aba3-1*, a line defective in MoCo activity and thus ABA synthesis. We also crossed *ost1-3* to *cyp707a1 cyp707a3* (abbreviated as *cyp707a1/a3*), which lacks two proteins involved in ABA catabolism (II). These plants combined defective ABA biosynthesis or breakdown with strong ABA insensitivity caused by impairment of OST1. As observed in a previous study (I), the *aba3-1* mutant had higher stomatal conductance, however, *cyp707a1/a3* displayed lower stomatal conductance compared to wildtype (Figure 4A). These findings are also consistent with previously described results for *aba3-1* and *cyp707a1/a3* (Okamoto et al., 2009; Merilo et al., 2013). Furthermore, the *ost1 aba3* double mutant had increased stomatal conductance compared to either parent. In contrast, *ost1 cyp707a1/a3* had reduced stomatal conductance compared with *ost1-3* (Figure 4A). Thus, reduced or increased ABA concentration was able to further change the stomatal conductance of the strongly ABA-insensitive *ost1-3* (Figure 4A), indicating that changes in ABA concentration affected some other trait related to stomatal conductance, which was unaffected in *ost1-3*.

To study this possibility, we measured the stomatal aperture width and stomatal density of these mutants, as both aperture width and density are involved in determining steady-state stomatal conductance. We found that *aba3-1* and *ost1-3* showed significantly wider apertures compared with wildtype, whereas *cyp707a1/a3* had aperture widths similar to those of wildtype. There was no difference in aperture width between *ost1 cyp707a1/a3* and *ost1-3*, while the aperture of *ost1 aba3* was significantly wider compared to that of either single mutant (Figure 4B). These results suggest that defects in ABA signalling and

biosynthesis affect stomatal pore width. Indeed, other ABA signalling mutants such as *112458* and *snrk2.2/2.3/2.6*, are reported to have stomata that are more open than wildtype (Gonzalez-Guzman et al., 2012). However, defective ABA catabolism appears to have no effect on aperture width.

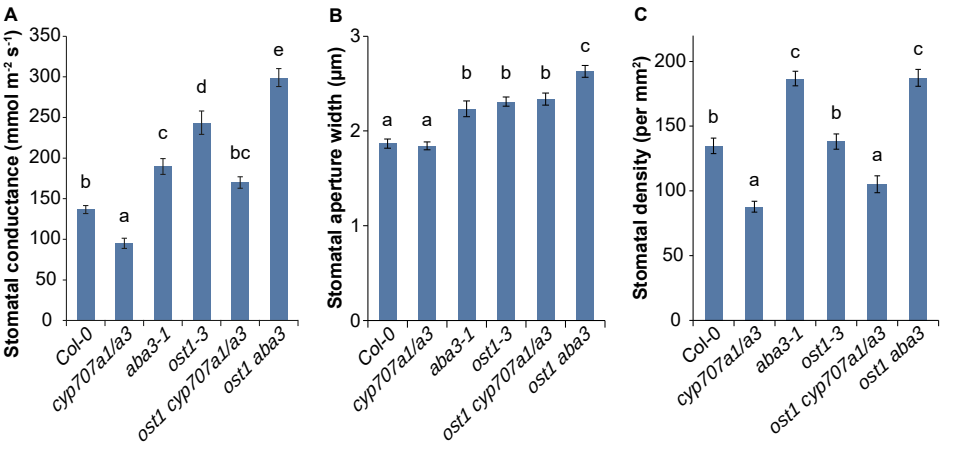

Figure 4. (A) Whole-plant steady-state stomatal conductance of three- to four-week-old plants (n=8–13). (B) Stomatal aperture measured on epidermal peels of four-week-old plants (n=6). (C) Stomatal density of five-week-old plants (n=24). Small letters (in A, B and C) denote statistically significant differences according to one-way ANOVA with Tukey's HSD post hoc test equal (B, C) and unequal sample size (A).The figure is modified from II, Fig. 2A, 2B and 3C.

The effect of ABA concentration on stomatal density has been reported in the literature as ABA deficiency leads to increased stomatal density, while over-accumulation of ABA leads to decreased stomatal density (Tanaka et al., 2013; Chater et al., 2015). We measured stomatal density from stomatal impressions to determine whether this characteristic would explain the lower stomatal conductance of *cyp707a1/a3* and *ost1 cyp707a1/a3* compared to wildtype and *ost1-3*, respectively (Figure 4A). The higher stomatal density of *aba3-1* and lower stomatal density of *cyp707a1/a3* compared to wildtype (Figure 4C) corresponds with previous findings (Tanaka et al., 2013; Chater et al., 2015). There was no difference in stomatal density between *ost1-3* and wildtype. However, when changes in ABA concentration were combined with ABA insensitivity, the stomatal density was significantly changed: it was increased in the *ost1 aba3* mutant but reduced in the *ost1 cyp707a1/a3* mutant (Figure 4C). Overall, the increased stomatal conductance in *ost1-3* was due to a wider stomatal pore width, as the stomatal density was similar to that of the wildtype, whereas ABA deficiency affected stomatal pore width as well as stomatal density. Thus, leaf ABA concentration is an important signal for stomatal development and is regulated independently of OST1. However, it is plausible that a canonical ABA signalling pathway determines stomatal development. In support of this, we found that a line lacking six ABA receptors, *112458*, showed increased stomatal

density (I), and other studies have shown that the PP2C mutants *abi1-1* and *abi2-1* have higher stomatal densities than wildtype (Tanaka et al., 2013). Other SnRKs from the same subclass as OST1 such as SnRK2.2 and SnRK2.3 may be involved in stomatal development. The extremely ABA-insensitive phenotype of the *snrk2.2/2.3/2.6* triple mutant suggests that SnRK2.2, SnRK2.3 and OST1 function redundantly in seed germination, seedling development and stomatal regulation (Fujii and Zhu, 2009). Therefore, it is possible that these three SnRKs also function redundantly in stomatal development.

4.2. Guard cells and phloem companion cells as ABA biosynthesis sites

There has been much debate on the main sites of ABA synthesis, and transgenic lines with restored guard cell- or phloem companion cell-specific ABA synthesis have been generated. ABA2 tagged with sGFP was introduced under the control of the guard cell-specific *GC1* promoter or the phloem-specific *SUC2* promoter into the *aba2-11* background (I). The visual phenotype of *aba2-11*, which has narrow leaves and stunted growth, was rescued in plants expressing ABA2-sGFP under the control of either the *GC1* or *SUC2* promoter (Figure 5A). The high stomatal conductance of *aba2-11* was also reduced to wildtype level in the transgenic lines (Figure 5B). Furthermore, ABA measurements showed that transgenic lines with ABA synthesis restored in guard cells or the vasculature showed wildtype-like rosette ABA concentrations (Figure 5C). Thus, this finding indicates that both guard cells and phloem companion cells are capable of synthesizing ABA and exporting it to the rest of the plant. On the other hand, the results of a recent study indicate that mesophyll cells are the most important ABA biosynthesis site (McAdam and Brodribb, 2018). McAdam and Brodribb (2018) pointed out that guard cells make up only a small fraction of the total number of leaf cells and thus, it is unlikely that guard cell ABA synthesis could provide ABA to the whole leaf. However, if the rest of the plant is devoid of ABA, guard cells as the only sources probably produce and export more ABA according to the principle that sources and sinks operate in a coordinated manner. Our results do not rule out the conclusion of McAdam and Brodribb (2018) that ABA is synthesized mainly in mesophyll cells. ABA is probably synthesized in multiple cell types because it is involved in many plant processes. Given the importance of ABA in stomatal regulation, it seems very plausible that guard cells are capable of synthesizing ABA. In support of this, Bauer et al. (2013) showed that guard cells express all of the ABA biosythetic genes and that these genes are upregulated after ABA treatment, representing a positive feedback loop.

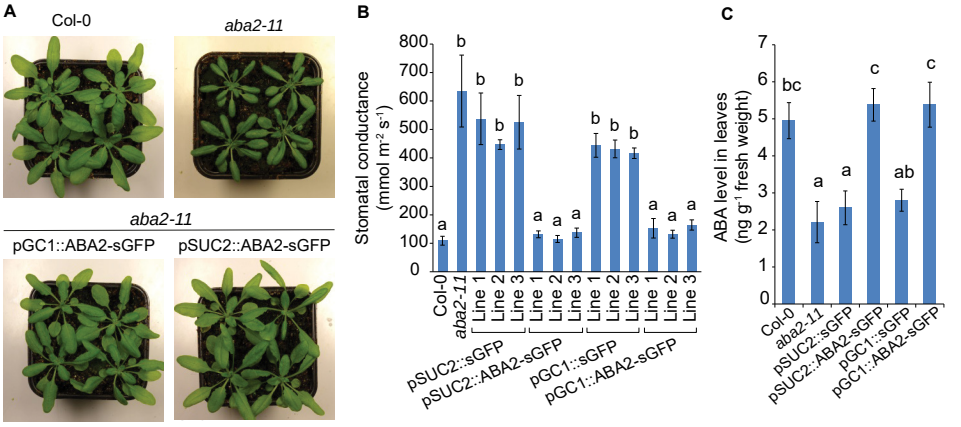

Figure 5. Transgenic plants expressing ABA2-sGFP under the control of either *GC1* or *SUC2* promoters in the *aba2-11* background. (A) Representative photographs of each genotype of four-week-old plants, showing that wildtype-like phenotype was restored in transgenic plants. (B) Whole-plant steady-state stomatal conductance (n=3–4). (C) ABA levels measured in four-week-old plants. The results were pooled for each genotype (n=12–7). The figure is modified from I, Fig. 4A, 4B and 4F.

4.3. The role of ABA transporters and recycling

Our aim was to study the role of active ABA transport and the recycling of ABA from the inactive conjugate ABA-GE in rapid guard cell responses to changes in the abiotic environment and ABA. We studied stomatal responses to high VPD, elevated CO_2, and exogenous ABA in plants defective in ABA transporter and recycling proteins. Several known ABA transporter mutants, *abcg25*, *abcg40* and *ait1*, were tested, and loss-of-function mutants of two β-glucosidases, BG1 and BG2, were also tested. We included plants lacking functional ABCG22 because of its hypothetical role in ABA transport and generated *abcg22-2 abcg25 abcg40* triple mutants (abbreviated as *abcg22/25/40*). Furthermore, another loss-of-function mutant from the ABCG transporter family, *abcg27*, was included due to its high expression in guard cells and its increased expression in response to low humidity and ABA (Merilo et al., 2015).

All studied lines with defective transporters had steady-state stomatal conductance similar to that of wildtype, and their responses to high VPD, elevated CO_2 and ABA spraying were also wildtype-like (III, Supplemental Fig. 1, Fig. 1A and B). Loss-of-function plants of ABCG22 specifically showed delayed high-VPD response and longer response half-times (Figure 6A and III, Fig. 1A), indicating that ABCG22 is involved in initiating stomatal closure in response to reduced air humidity. However, at the end of the VPD experiment, the stomata of the *abcg22* loss-of-function plants were as closed as those of Col-0 (Figure 6A). Both *abcg22* and *ost1-3* showed and impaired high-VPD response (Figure 6A), and considering that 1) OST1 activates the ultimate targets of the

ABA signalling pathway and that 2) it is not known how the activity of ABCG transporters is regulated, although pH is suggested to be involved (Boursiac et al., 2013), it is tempting to hypothesize that OST1 is able to activate ABCG22. However, this does not fully explain the delayed but still functional VPD response of *abcg22*, since *ost1-3* is VPD insensitive (Figure 6A). In addition, our results suggest that ABCG22 is not involved in importing ABA into guard cells, as its response to ABA spraying was intact. Kuromori *et al*. (2017) were also unable to show that the molecule transported by ABCG22 is ABA.

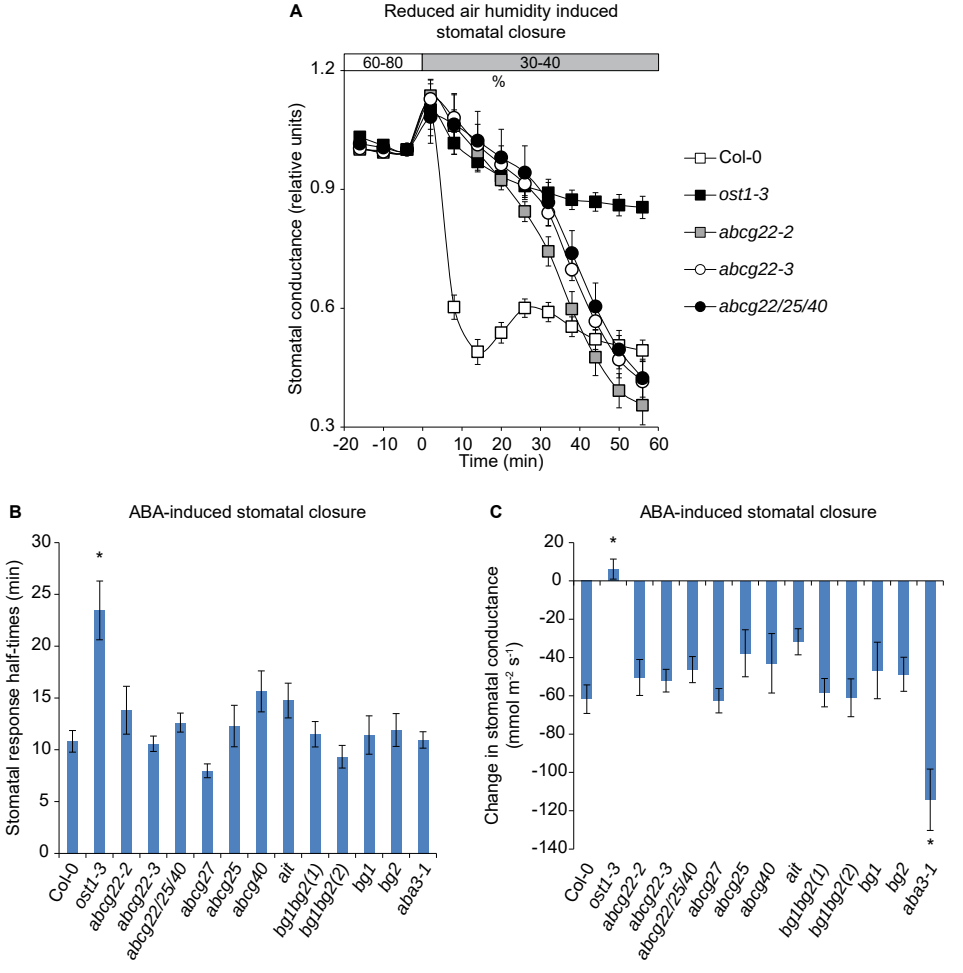

Figure 6. (A) Stomatal response to reduced air humidity. Relative stomatal conductance values (normalized to pre-treatment steady-state values) ± SEM are shown. (B) Stomatal response half-times to ABA treatment. (C) Changes in stomatal conductance values during the first 16 minutes of ABA treatment. Star denotes a significant difference between mutant and Col-0 (ANOVA & Tukey HSD test; $P<0.05$; n=5–9). The figure is modified from III, Fig. 1A and 1B.

Stomatal responses to exogenous ABA in ABA transporter mutants were intact; however, ABA-induced closures were reduced, and response half-times were longer, though non-significantly, in *abcg40* and *abcg22/25/40* plants (Figure 6B and C). Thus, ABCG40 probably participates in guard cell ABA import as reported previously (Kang et al., 2010). There are probably other proteins with redundant functions facilitating ABA transport into guard cells. In addition to AIT1/NPF4.6, several other *Arabidopsis* NPFs, such as NPF4.5/AIT2, NPF4.1/AIT3 and NPF4.2/AIT4, have been found to display ABA import activity in yeast, of which NPF4.1 showed relatively high ABA import activity compared with that of the others (Kanno et al., 2012). In addition, DTX50 was shown to mediate ABA efflux when expressed in *E. coli* and *Xenopus* oocyte cells (Zhang et al., 2014). Therefore, it is plausible that additional guard cell ABA transport proteins will be identified in the future. ABCG17, 21, 29 and 31 are potential candidates due to their high expression in guard cells, whereas ABCG1 showed increased expression in response to ABA (Merilo et al., 2015). It is also possible that active ABA transport to guard cells is not required for fast stomatal responses, since ABA synthesized in guard cells (I; Bauer et al., 2013) is enough to elicit stomatal closure or increasing ABA concentration by recycling initiates the signalling pathway. Regarding the stomatal response to ABA spraying, as was done in our experiment, the transport of sprayed ABA into guard cells still seems the most plausible explanation for the stomatal closure response; thus, transporters are needed, or inactive uptake into guard cells (Boursiac et al., 2013) takes place under these no-stress conditions.

The studied ABA-recycling mutants *bg1*, *bg2* and double mutant *bg1 bg2* had steady-state stomatal conductances similar to those of wildtype (III, Supplemental Fig. 1) and showed intact stomatal responses to all studied stimuli (III, Fig. 1C). Thus, either there are other β-glucosidases with redundant function in converting ABA-GE to ABA or the *de novo* synthesis of ABA is more important for ABA homeostasis, at least under well-watered conditions in our experiment. McAdam *et al.* (2016) did not detect any significant changes in foliar ABA-GE concentrations in several angiosperm species in response to doubling of the VPD, indicating that the role of ABA-GE hydrolysis to increase ABA concentration was irrelevant under stress. However, ABA-GE hydrolysis could be important in response to prolonged dehydration stress, since in plants treated with fluridone, a carotenoid and ABA biosynthesis inhibitor, the ABA concentration increased considerably when plants were subjected to low air humidity for 10 hr (Lee et al., 2006). Thus, contribution to the active ABA pool by recycling from inactive conjugates is probably dependent on the stress conditions and duration.

4.4. The role of ABA in VPD responses

To study stomatal responses to reduced air humidity, mutants defective in different steps of ABA biosynthesis and/or signalling have been used with the assumption that if ABA is involved in this process, the impaired VPD responses of these lines serve to reveal it. We subjected ABA-deficient and ABA signalling mutants to a reversible sequence of VPD transitions from 1.2 to 2.2 kPa and back to 1.2 kPa, which corresponds to a change in air humidity from ~70% to ~30% and back to ~70%. The lack of OST1 resulted in the strongest impairment in the high VPD-induced stomatal closure (I, Fig. 5B). Even after 60 min in low air humidity, a decrease in stomatal conductance of *ost1-3* plants was not evident, and thus, OST1 is crucial for VPD responses. In contrast, a plant line defective in six PYR/RCAR receptors, *112458*, showed a VPD-induced decrease in stomatal conductance (I, Fig. 5A) and increased 40-min closure compared with Col-0 (Figure 7B); the response half-time was, however, longer in *112458* (Figure 7A). Interestingly, all *Arabidopsis* ABA-deficient lines showed stomatal closure to increased VPD (I, Fig. 5A and B). In some lines, this closure was even more pronounced than that of wildtype: *aba2-11* and *nced3 nced5* showed significantly larger 40-min closures and initial closure rates (Figure 7B and C). Tomato *flacca* plants showed increased 40-min closure and initial closure rate compared with the corresponding wildtype (Figure 7B and C), whereas there was no significant difference between the pea *wilty* mutant and its wildtype in terms of high-VPD-induced stomatal closure (Figure 7; I, Fig. 5C). These results contrast with previous findings, where the VPD response was evident but reduced in ABA synthesis and signalling mutants (Xie et al., 2006), while in another study, the VPD response was absent in ABA-deficient pea and tomato mutants (McAdam et al., 2016). On the other hand, Assmann et al. (2000) showed that in ABA-deficient and ABA-insensitive *Arabidopsis* mutants, the VPD response is wildtype-like, which is more in line with our results. Hence, it is still not clear whether ABA is involved in the stomatal VPD response. Although ABA-deficient mutants have reduced leaf ABA concentrations, they are still capable of synthesizing it under normal conditions (I) and in response to stress (references in I, Fig. 1). It is thus possible that they produce enough ABA to initiate its signalling under high-VPD conditions.

When the air humidity was increased back to the initial level, stomatal opening was induced in all the studied mutants, except *ost1-3* (I, Fig. 5). *112458* showed similar closure and opening half-response times and fully rescued the pretreatment stomatal conductance values (I, Fig. 5A and 6A). This symmetrical VPD response points at a passive hydraulic stomatal response, as observed in lycophytes, ferns and conifers (McAdam and Brodribb, 2015). We found that if the stomatal conductance of *ost1-3* plants was further increased by CO_2 withdrawal or additional blue light, those plants also showed VPD-induced stomatal closure (I, Fig. 8). Thus, we speculate that under the well-watered conditions in our experiment, the passive hydraulic stomatal response may be conditional and depend on initial stomatal conductance, which in turn determines the ability of plant hydraulic conduits to maintain unchanged leaf water potential.

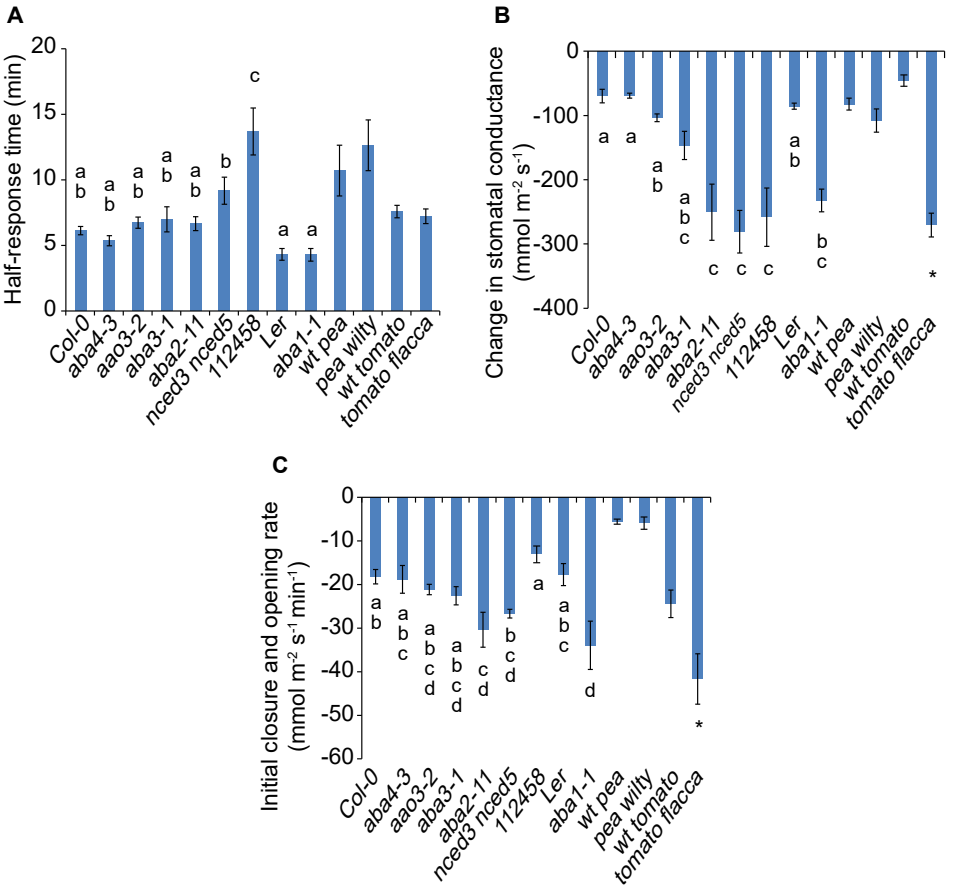

Figure 7. Characteristics describing high-VPD-induced stomatal closure. (A) Stomatal closure half-response time indicates the time when half of the measured VPD response was completed. (B) High-VPD-induced stomatal closure within the initial 40 min of VPD change. (C) Initial closure rate calculated by fitting a second-order polynomial on the time series of stomatal conductance values after the change in VPD. Significant differences are denoted with different letters in *Arabidopsis* and with asterisks in tomato (ANOVA and Tukey's post hoc test). The figure is modified from I, Fig. 6A, 6B and 6C.

As the sextuple ABA receptor mutant showed clear stomatal closure in response to high VPD, it is possible that some of the PYR/RCAR receptors remain functional in the mutant and initiate ABA signalling under high VPD. To rule out this possibility, we measured the response to exogenously applied ABA in *112458* and *ost1-3* mutants and both showed ABA-insensitivity (I, Fig. 7). In contrast, the response to exogenous ABA in ABA-deficient mutants was intact and even hypersensitive in some of the lines (I, Fig.7).

4.5. The function of ERA1 in stomatal responses

The enhanced response to ABA in ERA1 loss-of-function plants indicates that a farnesylated protein functions as a regulator in ABA signalling (Cutler et al., 1996). To address the role of ERA1 in guard cell regulation and to investigate whether a target protein for farnesylation is involved in the ABA signalling pathway, a double mutant analysis was carried out. We crossed *era1* with *ost1-3* and with plants carrying a dominant *abi1-1* mutation, which abolishes the inhibition of ABI1 by PYR/RCAR receptors, resulting in reduced ABA sensitivity.

We observed that plants defective in ERA1 had reduced stomatal conductance compared to wildtype plants and the *era1* mutation significantly lowered the high stomatal conductance of *abi1-1* and *ost1-3* in the *era1 abi1-1* and *era1 ost1* double mutants, respectively (IV, Fig. 1). Thus, ERA1 plays a role in determining steady-state stomatal conductance. It is possible that ERA1 functions in the ABA-independent stomatal signalling pathway, since the components of the ABA pathway do not have the CaaX motif at the C-terminus and therefore are not direct targets for farnesylation. Another possible explanation would be altered ABA metabolism: increased ABA biosynthesis or reduced ABA catabolism. However, we did not detect higher expression levels of the ABA biosynthetic enzymes NCED3 and AAO3 or lower expression levels of the ABA catabolic enzyme CYP707A3 in *era1* guard cells compared with wildtype guard cells under well-watered conditions (IV). Moreover, no changes in ABA concentration of *era1-2* plants compared with wildtype have been reported (Ghassemian et al., 2000). Thus, altered ABA metabolism probably does not explain the reduced stomatal conductance of *era1* and the suppression of the high stomatal conductance of *abi1-1* and *ost1* by *era1* in the studied double mutants.

Stomatal closure responses to the studied stimuli were intact in plants lacking functional ERA1 (IV, Fig. 2). The initial stomatal closure of *abi1-1* in response to high VPD, darkness and CO_2 was similar to that of wildtype, whereas the response to ABA was reduced (IV, Fig. 2E-H). Similar to the single *abi1-1* mutant, the *era1 abi1-1* double mutant displayed partially impaired ABA-induced stomatal closure. Except for the ABA response, which was slightly larger in the *era1 ost1* plants compared with *ost1-3* (IV, Fig. 2H), all other responses were strongly impaired in the double mutant *era1 ost1* (IV, Fig. 2). In addition, the known ERA1 targets ASG2 and CYP85A2 were not involved in stomatal closure responses, since lack-of-function mutants of these proteins show wildtype-like stomatal closure responses (IV). Thus, these results indicate that ERA1 is not required for stomatal closure in response to abiotic factors or ABA. Overall, the lack of functional ERA1 had no effect on rapid stomatal sensitivity, whereas the lack of OST1 resulted in strongly reduced stomatal sensitivity. The importance of OST1 was further confirmed in the lines combining altered ABA concentration and ABA insensitivity due to the absence of OST1. Stomatal closure induced by high VPD, darkness and elevated CO_2 was strongly impaired in the *ost1 aba3* and *ost1 cyp707a1/a3* mutants (II, Fig. 4). Generally, in all double mutants with *ost1-3*, rapid stomatal responses were impaired.

Next, we tested whether ERA1 is involved in stomatal opening. No differences in stomatal conductance values were detected between *era1* and wildtype in darkness. We observed similar initial opening rates in *era1* and wildtype after application of light. However, *era1* plants displayed lower stomatal conductance compared to wildtype plants at the end of the experiment (IV, Fig. 3A). It was reported that the inward-rectifying K^+ currents were significantly reduced in *era1-2* guard cells (Allen et al., 2002). Furthermore, Allen et al. (2002) observed a narrower aperture width in *era1-2* compared with wildtype at high extracellular K^+. Therefore, it is possible that reduced inward-rectifying K^+ channel activity explains the lower stomatal conductance observed in *era1* at the end of the opening experiment and also the lower steady-state stomatal conductance observed throughout the experiments

We further tested the stomatal opening response of *era1* and wildtype to blue and red light. The *era1* plants had an impaired stomatal opening response to blue light, while red light-induced stomatal opening was not impaired (IV, Fig. 3). Blue light-activated H^+-ATPase results in hyperpolarization of the plasma membrane, further activating the inward-rectifying K^+ channel to induce K^+ influx, which is reduced in *era1-2* plants (Allen et al., 2002). It is therefore likely that the impaired blue light opening in *era1-2* was due to limited K^+ influx. Similar delayed opening responses as those in the *era1-2* mutants were reported for plants lacking functional PATROL1 (PROTON ATPase TRANSLOCATION CONTROL 1) or SYP121 (SYNTAXIN OF PLANTS 121) (Eisenach et al., 2012; Hashimoto-Sugimoto et al., 2013). Plants lacking functional SYP121 also showed reduced activity of inward-rectifying K^+ currents, similar to *era1-2* (Eisenach et al., 2012). PATROL1 controls the translocation of H^+-ATPase AHA1 to the plasma membrane (Hashimoto-Sugimoto et al., 2013), whereas SYP121 is a vesicle-trafficking protein involved in the transport of K^+ channels (Eisenach et al., 2012). Thus, it is possible that ERA1 is associated with the transport of H^+-ATPases or K^+ channels to the plasma membrane. However, at which point farnesylation acts and the relevant proteins farnesylated in the signalling pathway have yet to be elucidated.

5. CONCLUSIONS

1. Leaf ABA concentrations were significantly correlated with the values of steady-state stomatal conductance: plants with reduced ABA concentrations had high stomatal conductance, and this was associated with increased stomatal density and larger aperture width. On the other hand, plants with increased ABA concentrations had reduced stomatal conductance associated with reduced stomatal density.
2. Stomatal responses to high VPD and elevated CO_2 concentration in ABA-deficient mutants were wildtype-like, indicating that either those plants synthesize enough ABA to initiate signalling or that ABA is not involved in those responses.
3. Restoring ABA synthesis in the ABA-deficient *aba2-11* mutant's guard cells or phloem companion cells resulted in a wildtype-like leaf ABA concentration, plant phenotype and steady-state stomatal conductance. Thus, these cell types are capable of synthesizing ABA and providing it to the rest of the plant. Considering how important ABA is in stomatal regulation, it is not surprising that guard cells are independent in managing their own ABA homeostasis.
4. Lack of functional ABCG22, a transporter from the ABCG protein family that includes several ABA transporters, resulted in delayed stomatal closure to high VPD. The intact stomatal closure to exogenous ABA spraying in *abcg22* plants suggests that the molecule transported by ABCG22 is not ABA. Other studied ABA transporter mutants showed intact stomatal responses to high VPD and ABA spraying. This suggests that there is likely functional redundancy between different ABA transporter proteins to ensure the stress-induced transport of ABA.
5. OST1 protein kinase was crucially important for an intact VPD response. However, it is not clear whether its activation under high VPD was ABA dependent. In all the double mutants with *ost1-3* as one parent, stomatal responsiveness to abiotic factors and ABA was impaired. This stresses the role of OST1 in rapid stomatal sensitivity. Since *ost1-3* plants had wildtype-like stomatal density, OST1 is not involved in stomatal development.
6. ERA1 is important in determining steady-state stomatal conductance because a lack of ERA1 function led to a decrease in stomatal conductance. However, an intact stomatal closure response to abiotic factors and ABA spraying in era1 plants suggests that ERA1 is not required for stomatal closure.
7. The role of ERA1 in stomatal regulation is associated with the opening process, as plants deficient in farnesylation showed reduced blue light-induced opening.

Stomatal regulation is important for plant productivity and stress tolerance, even more so under climate change conditions. This is why understanding the fundamental principles of stomatal development, opening and closure is important: it serves to modify the water economy of model plants and crops according to the prevailing conditions in their geographical area.

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SUMMARY IN ESTONIAN

Õhulõhede juhtivust ja tundlikkust mõjutavad signalisatsioonirajad

Taimelehtede pinnal asuvad õhulõhed, kahest sulgrakust ning nendevahelisest õhupilust moodustunud struktuurid. Õhulõhed reguleerivad taimede gaasivahetust – fotosünteesiks vajaliku CO_2 sisenemist lehte ning veeauru väljumist transpiratsioonis. Õhupilu avatust reguleeritakse sulgrakkude turgorrõhu muutustega, mis on tingitud ioonide ning nende järel vee sisenemisest või väljumisest sulgrakkudest. Õhupilu avatusest sõltub õhulõhede juhtivus, mis väljendab veeauru väljumise kiirust lehe pinnaühiku kohta ning mis arvutatakse, lähtudes transpiratsioonist. Peale õhulõhede avatuse mõjutab õhulõhede juhtivust ka õhulõhede arv lehepinnal ehk õhulõhede tihedus. Õigeagne ning täpne õhupilu avatuse regulatsioon taimesiseste signaalide ja keskkonnatingimuste toimel on taimedele eluliselt oluline.

Oma doktoritöös uurisin õhulõhede juhtivuse muutumist keskkonnas toimuvate muutuste ja taimse stressihormooni abstsiishappe (ABA) toimel. Õhulõhede kiire regulatsioon toimub õhupilu laiuse muutmise kaudu, pikemas ajaskaalas kontrollib taim õhulõhede juhtivust ka õhulõhede tiheduse muutumise kaudu. Töös uurisin ka ABA signaalraja positiivse regulaatori, kinaasi OST1 rolli õhulõhede kiires regulatsioonis keskkonnafaktorite, eelkõige madala õhuniiskuse, toimel ning õhulõhede tiheduse määramises.

Esmalt uurisime lehe ABA kontsentratsiooni mõju õhulõhede juhtivusele ning õhulõhede sulgumisele keskkonnafaktorite toimel, samuti ABA rolli õhulõhede tiheduse määramises. Selleks kasutasime taimeliine, milles puuduvad ABA sünteesiraja individuaalsed ensüümid või ABA katabolismi läbi viivad ensüümid. Leidsime, et lehe ABA kontsentratsioon on negatiivses korrelatsioonis õhulõhede juhtivusega: mida väiksem oli ABA kontsentratsioon lehes, seda kõrgem oli õhulõhede juhtivus. Negatiivne korrelatsioon lehe ABA sisalduse ning õhulõhede juhtivuse vahel tulenes sellest, et ABA-defitsiitsetel taimeliinidel oli õhupilu laiem ning õhulõhede tihedus suurem. Taimedes, kus ABA katabolismi läbiviivad ensüümid olid vigased ning lehe ABA kontsentratsioon seetõttu tõenäoliselt kõrgem, mõõtsime õhulõhede tiheduse vähenemisest põhjustatud juhtivuse languse. Uuritud ABA sünteesi- ja katabolismiradade mutantide õhulõhede vastused keskkonnafaktoritele olid metsiktüübiga sarnased. Seega võib järeldada, et ABA-defitsiitsed taimed sünteesivad piisavalt ABA, et käivitada signaalrada või ei ole ABA neis vastustes oluline.

ABA süntees toimub erinevates taimerakkudes, kuid siiani ei ole selge, millistel rakkudel on ABA sünteesi põhiroll. Käesoleva töö eesmärgiks oli selgitada õhulõhe sulgrakkude ja floeemi saaterakkude rolle ABA sünteesis ja taime ABA-ga varustamises. Kui taimedes, milles puudus ABA sünteesil osalev ensüüm ABA2, taastati ABA süntees ainult sulgrakkudes või floeemi saaterakkudes, siis taastusid nii lehe ABA kontsentratsioon, taimede visuaalne välimus kui ka õhulõhede juhtivus. Seega, saadud tulemused viitavad, et mõlemad nimetatud rakutüübid on suutelised tootma ABA sellisel määral, et varustada

kogu taime maapealset osa. Sulgrakkude sõltumatus oma ABA homöostaasi hoidmisel on loogiline, arvestades ABA olulist rolli õhulõhede regulatsioonis.

Järgnevalt selgitasime erinevate ABA transporterite rolle õhulõhede juhtivuse määramisel ning kiiretes reaktsioonides keskkonnafaktorite (madal õhuniiskus ja kõrge CO2 kontsentratsioon) ning ABA toimel. ABA-toimeliste reaktsioonide uurimiseks pihustasime taimi ABA lahusega. Leidsime, et individuaalsete ABA transporterite puudumisel on vastavate taimeliinide õhulõhede juhtivus ning nende sulgumine ABA ning keskkonnafaktorite toimel sarnased metsiktüübiga. Seega, ilmselt on taimerakkude membraanides erinevad ABA transporterid, mis tagavad ABA liikumise rakkudesse ja neist välja ka üksikute individuaalsete transporterite puudumisel. Lisaks leidsime, et taimedel, kel puudub ABCG22, valk ABCG transporterite hulgast, oli õhulõhede sulgumine madala õhuniiskuse tõttu oluliselt hilinenud, samas vastused kõrgele CO_2 kontsentratsioonile ja ABA-ga pihustamisele olid sarnased metsiktüübiga. Hetkel pole teada, millist molekuli ABCG22 transpordib, kuid normaalne ABA sulgumine nendes taimeliinides viitab, et see molekul ei ole ABA. Edasist selgitamist vajab ka ABCG22 roll niiskusvastuse initsiatsioonis.

Doktoritöös uurisime ka kinaas OST1 mõju õhulõhede sulgumisele madala õhuniiskuse ja teiste keskkonnafaktorite toimel ning õhulõhede tiheduse määramises. Leidsime, et vigase OST1-ga taimeliinides, oli õhulõhede sulgumine madalas õhuniiskuses sisuliselt puudu. Seega, OST1 osaleb õhulõhede vastustes õhuniiskusele, kuid pole selge, kas OST1 aktivatsioon toimub siis ABA-st sõltuvalt või mitte. Teisalt leidsime, et OST1 ei oma rolli õhulõhede arengus, kuna *ost1* mutantides oli õhulõhede tihedus metsiktüübiga sarnane. Kõigis taimeliinides, kus OST1 oli defektne, olid õhulõhede kiired reaktsioonid keskkonnafaktoritele häiritud, mis kinnitab selle kinaasi olulist rolli õhulõhede tundlikkuse määramisel.

On teada, et valk ERA1, mis kodeerib farnesüültransferaasi β-subühikut ning osaleb valkude transkriptsioonijärgses farnesüleerimises, võib osaleda õhulõhede regulatsioonis. Käesolevas töös uurisime ERA1 valgu mõju õhulõhede kiiretele reaktsioonidele keskkonnafaktorite mõjul. Leidsime, et vigase ERA1-ga taimedes oli õhulõhede juhtivus madalam, võrreldes metsiktüübiga. Sama taimeliini õhulõhede sulgumine keskkonnafaktorite mõjul oli normaalne, kuid õhulõhede avanemine sinise valguse toimel häiritud. Seega, ERA1 valgul on tähtis roll õhulõhede juhtivuse määramisel ning õhulõhede avanemise reguleerimisel.

Õhulõhede täpne ja kiire regulatsioon tagab looduslike ning põllumajanduslike taimede konkurentsivõimelisuse, tootlikkuse ning stressitaluvuse. Kliimamuutuste käigus muutuvad keskkonnatingimused paljudes piirkondades pigem põllumajandusele ebasoodsamaks. Seega on oluline mõista õhulõhede arengut ning avanemise ja sulgumise regulatsioone, et tagada tuleviku kliimaks sobivate põllumajanduslike taimede tulemuslik aretustöö ning mõista ja võimalusel suunata protsesse looduslikes kooslustes.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisors, Ebe Merilo and Mikael Brosché, for their guidance, support and patience during my studies. I have learned a lot from the both of you.

I also thank Hannes Kollist for the opportunity to work in his group.

I would also like to thank all the past and present members of the Plant Signal Research Group. I have been very fortunate to work with the nicest people. Special thanks to Hanna Hõrak for all the insightful discussions and motivation; Liina Jakobson, Maris Nuhkat and Kristiina Laanemets for great company and encouragement; Karin Johansson and Olena Zamora for the fun trips we have had. I am grateful for all of you and the time we shared together.

I am grateful to my friends who have supported me along the way. Special thanks to Anu Rätsep, Merit Annamaa, Helena Haav, Angela Koort and Merilin Hallik. Over the years we have had some great times and memorable moments.

Last but not least, I would like to thank my family for being always there for me. Thank you for your unconditional love and support.

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