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OLENA ZAMORA

Impacts of plant hormones on controlling stomatal conductance

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Supervisors: Dmitry Yarmolinsky, PhD
Associate Professor of Molecular Plant Biology
Institute of Bioengineering, University of Tartu, Estonia

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

Reviewer: Pirko Jalakas, PhD
Research Fellow in Plant Biology
Institute of Technology, University of Tartu, Estonia

Opponent: Professor Heribert Hirt
King Abdullah University of Science & Technology (KAUST)
Saudi Arabia

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Author's contribution

I. The author performed a part of the experiments, analyzed the data, visualized the results, and participated in the editing of the manuscript.

II. The author participated in the planning and designing of the study, performed most of the experiments, analyzed the data, visualized the results, and participated in the writing and editing of the manuscript.

III. The author performed a part of the experiments, analyzed the data, visualized the results, and participated in the editing of the manuscript.

ABBREVIATIONS

ABA – abscisic acid
ABCB14 – ATP-BINDING CASSETTE B14
ABCG25 – ATP-BINDING CASSETTE G25
ACC – 1-aminocyclopropane-1-carboxylate
ALMT – ALUMINUM-ACTIVATED MALATE TRANSPORTER
AMY3 – α-AMYLASE3
ATP – adenosine triphosphate
BAM1 – β-AMYLASE1
BLUS1 – BLUE LIGHT SIGNALING1
CBC1 and CBC2 – CONVERGENCE OF BLUE LIGHT AND CO_2 1 and 2
CCD7/MAX3 and CCD8/MAX4 – CAROTENOID CLEAVAGE DIOXYGENASE 7 and 8/MORE AXILLARY GROWTH 3 and 4
CEND – carboxyl end of EIN2
C_i – intercellular CO_2 concentration
COI1 – CORONATINE INSENSITIVE 1
COP1 – CONSTITUTIVE PHOTOMORPHOGENIC 1
CPK – CALCIUM-DEPENDENT PROTEIN KINASE
CRY1 and CRY2 – CRYPTOCHROMES 1 and 2
CTR1 – CONSTITUTIVE TRIPLE RESPONSE 1
EBF1 and EBF2 – EIN3-BINDING F-BOX PROTEIN 1 and 2
EDS5 – ENHANCED DISEASE SUSCEPTIBILITY 5
EIL1 – EIN3-LIKE 1
EIN2 – ETHYLENE INSENSITIVE 2
EMS – ethylmethane sulfonate
ERF1 – ETHYLENE-RESPONSE-FACTOR 1
ET – ethylene
ETP1 and ETP2 – EIN2 TARGETING PROTEIN 1 and 2
ETR1 and ETR2 – ETHYLENE RESPONSE 1 AND 2
GHR1 – GUARD CELL HYDROGEN PEROXIDE RESISTANT 1
GORK – GATED OUTWARDLY-RECTIFYING K^+ CHANNEL,
GRL3.3 – GLUTAMATE RECEPTOR-LIKE 3.3
HDA6 and HDA19 – HISTONE DEACETYLASE 6 and 19
HPCA1 – HYDROGEN-PEROXIDE-INDUCED CA^{2+} INCREASES 1
HT1 – HIGH LEAF TEMPERATURE 1
ICS – isochorismate synthetase
JA – jasmonic acid
JA-Ile – JA-isoleucine

JAT1, JAT3 and JAT4 – JA transfer protein 1, 3 and 4
JAZ – JASMONATE ZIM DOMAIN
KAT1 and KAT2 – potassium channel in *Arabidopsis thaliana* 1 and 2
MAX2 – MORE AXILLARY GROWTH 2
MeJA – methyl jasmonate
MPK – MITOGEN-ACTIVATED PROTEIN KINASE
NINJA – NOVEL INTERACTOR OF JAZ
NPR1 – NONEXPRESSOR OF PATHOGENESIS-RELATED GENE 1
OPDA – (9S,13S)-12-oxo-phytodienoic acid
OST1/SnRK2.6 – OPEN STOMATA 1/SUCROSE NON-FERMENTING 1-RELATED PROTEIN KINASE 2.6
OST2/AHA1 – OPEN STOMATA 1/H^+-ATPase 1
PAL – phenylalanine ammonia lyase
PATROL1 – PROTON ATPASE TRANSLOCATION CONTROL 1
PBS3 – avrPphB SUSCEPTIBLE 3
PHOT1 and PHOT2 – PHOTOTROPINS 1 and 2
PIP2;1 – PLASMA MEMBRANE INTRINSIC PROTEIN 2;1
PP2C – TYPE 2C PROTEIN PHOSPHATASE
Pst – *Pseudomonas syringae pv tomato*
PYR/PYL/RCAR – PYRABACTIN RESISTANCE/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR
QUAC1 – QUICK-ACTIVATING ANION CHANNEL 1
RBOH – Respiratory Burst Oxidase Homolog
RHC1 – RESISTANT TO HIGH CO_2
ROS – reactive oxygen species
R-type – rapid-type
SA – salicylic acid
SAR – systemic acquired resistance
SAUR – SMALL AUXIN UP RNA
SCF – SKP1-CULLIN1-F-box protein
SHAM – salicylhydroxamic acid
SL – strigolactone
SLAC1 – SLOW ANION CHANNEL 1
SMAX6, SMAX7, and SMAX8 – SUPPRESSOR OF MAX2 6,7 and 8
ßCA1 and ßCA4 – ß-carbonic anhydrases 1 and 4
STP1 and STP4 – SUGAR TRANSPORTER PROTEIN 1 and 2
S-type – slow-type
TF – transcription factor
TPL – TOPLESS
VPD – vapor pressure deficit

INTRODUCTION

Plants are the basis of terrestrial life, by breaking down water in photosynthesis they produce atmospheric oxygen for aerobic organisms. Furthermore, by converting carbon dioxide (CO_2) and solar energy into biomass, plants provide energy-rich carbohydrates for most food chains. They also play global roles in the carbon and water cycles being crucial for sustaining our world for future generations. Humankind grows plants for food, oil, spices, timber, paper, rubber, etc. Various plant species are widely used in medicine, textile production, and chemical industry. Moreover, fossil organic fuels composed mostly of plant remnants are currently the major energy sources for modern civilization, highlighting the central roles of plants in human life.

During the colonization of the land, higher plants developed a gas-impermeable lipid barrier, cuticle, which prevents excessive desiccation in dry air. Since CO_2 is required for photosynthesis in mesophyll, microscopic pores, stomata, evolved to ensure gas exchange between plants and the atmosphere. Thus, water absorbed by roots and transported into leaves is evaporated through stomata in exchange for CO_2 taken up from the atmosphere and converted into carbohydrates during photosynthesis. Stomata are formed by pairs of specialized guard cells with changeable volumes and shapes. Stomatal movements, which are opening and closing of the stomatal pores, depend on transport of osmotically active solutes across the guard cell membranes, followed by uptake/release of water. Guard cells have a complex signaling network that recognizes inner and environmental stimuli and controls the guard cell volume and turgor pressure by activating and deactivating the ion channels and transporters in the guard cell membranes. The fast recognition of environmental factors and switching on and off protein activities in guard cells made stomata an attractive object for studies of plant signaling. The regulation of stomatal opening and closure has been extensively studied, however, there are still many gaps in understanding how stomata regulate gas exchange in plants. Thus, further studies are required to discover missing components in the regulation of stomatal functioning.

Plant hormones (or phytohormones) are chemical compounds produced by plants to regulate numerous processes in plants. They are involved in many aspects of a plant's life starting from germination, growth, flowering, and senescence and death. Classical phytohormones can be grouped into classes by their chemical structures: auxins, gibberellins, cytokinins, ethylene (ET), and abscisic acid (ABA). Relatively recently, researchers extended the list of phytohormones with brassinosteroids, salicylic acid (SA), jasmonic acid (JA), and strigolactones (SLs).

Since stomata should be coordinated throughout the whole plant life cycle, guard cells recognize long-distance endogenous stimuli of different natures, including hormones. ABA is best known for its roles in drought resistance and stomatal regulation. Since stomata are the main entry sites for pathogens, plant hormones regulating defense processes also control stomatal apertures. Several

studies have pointed to JA and SA to trigger fast stomatal closure for plant protection. SLs also regulate stomata in addition to their role in controlling plant interactions with microorganisms and plant growth. ET is involved in stress adaptation and interacts with other hormones, in particular ABA, SA, and JA. Although numerous studies clearly demonstrated the role of ABA as a key regulator of stomatal closure, stomatal regulation by other hormones has been studied significantly less.

In this thesis, I focused on the roles of phytohormones, including SLs, JA, SA, and ET, in stomatal functioning. In contrast to several previous studies based on stomatal assays, I measured whole-rosette stomatal conductance in hormonal mutants exposed to various stimuli. Such an approach allowed me to get time-resolved data for stomatal movements in intact plants and estimate impacts of phytohormones on stomatal regulation.

1. REVIEW OF THE LITERATURE

1.1 Structure of stomata and their functions in plants

Stomata are specialized microscopic pores on leaf surfaces, whose opening and closure regulate plant gas exchange, balancing CO_2 uptake into the leaves with water loss through evaporation. Accordingly, stomata close in response to scarce water availability and enhanced evaporative demand during drought and low air humidity to retain water in plant tissues. Guard cells that form stomata are able to recognize decreased or increased CO_2 levels in the air via changes in intercellular CO_2 (C_i) concentrations, leading to stomatal opening and stomatal closure, respectively (Mott, 1990; Engineer et al., 2016; Zhang et al., 2018a). Moreover, stomata have a dynamic barrier function. Stomatal closure is induced by pathogen-associated molecular patterns and elicitors, preventing an invasion of pathogenic microorganisms into plant leaves (Melotto et al., 2006; Sawinski et al., 2013). An uptake of air pollutants, e.g. ozone, depends on stomatal pores, which recognize and close in response to some of these gases (Merilo et al., 2013; Kollist et al., 2014; Gupta et al., 2016; Sierla et al., 2016).

During stomatal closure or stomatal opening, transportation of osmotically-active ions and substances occurs into or out of guard cells, resulting in influx or efflux of water and altering turgor pressure. Apparently, guard cell walls, which have different thicknesses, play an important role in the mechanism of stomatal movements. In guard cells the dorsal cell walls, facing away from the pore, are significantly thinner and more elastic than the ventral cell walls, surrounding the airway – stomatal pore. The difference in thickness of the cell walls prevents a bulging toward the pore, while the thin dorsal outer wall protrudes outward and pulls the inner cell wall, leading to the opening of the pore. Cellulose microfibrils in the cell walls of guard cells are located in parallel to each other, preventing a deformation during a turgor pressure increase (Carter et al., 2017; Rui et al., 2018). Flexibility of the guard cell walls is important, as alterations in the composition of the cell walls in guard cells might affect an amplitude of stomatal opening or closing (Amsbury et al., 2016; Merced & Renzaglia, 2019; Chen et al., 2021b; Waszczak et al., 2023).

Eudicots, such as *Arabidopsis thaliana*, have kidney-shaped guard cells, whereas, monocots typically have dumbbell-shaped guard cells (Figure 1) (Franks & Farquhar, 2007; Harrison et al., 2020). Monocot stomata are considered to be more efficient as their opening or closure requires relatively small changes in turgor pressure. Moreover, dumbbell-shaped stomata open and close faster than kidney-shaped guard cells and can be called “speedy” (Drake et al., 2013; McAusland et al., 2016; Chen et al., 2017).

To sum up, stomata function as an interface between plants and the environment. Stomatal apertures, which are pores between two guard cells, are regulated in accordance with environmental and endogenous factors, providing one of the means of plant adaptation to the ever-changing environment.

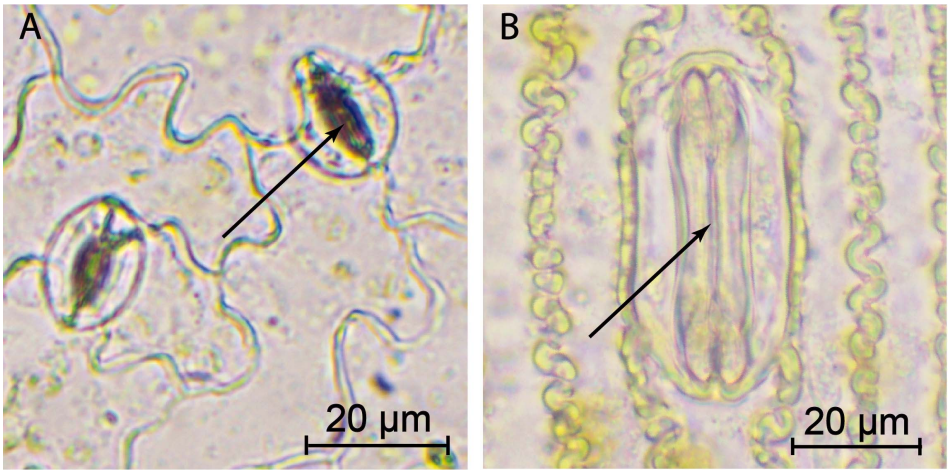

Figure 1. Stomatal shapes in epidermal peels from various plant species. (A) Kidney-shaped stomata in *Arabidopsis thaliana*. (B) Dumbbell-shaped stomata in *Hordeum vulgare*. The inner thick cell walls are shown by the arrows.

1.2 Stomatal movements

1.2.1 Stomatal opening

Plasma membrane H^+-ATPases use adenosine triphosphate (ATP) to pump H^+ out from guard cells, leading to the hyperpolarization of the guard cell plasma membrane. In Arabidopsis, there are 11 isoforms of H^+-ATPases (AHAs) which share similar structures and sequence homology. All AHAs are expressed in guard cells, while three of them, AHA1, AHA2 and AHA5, are the most abundant in these cells (Ueno et al., 2005; Yamauchi et al., 2016). Among the members of this family, AHA1 is considered to be the most important for stomatal opening (Ando & Kinoshita, 2018). Plants defective in AHA1 have strong stomatal insensitivity to blue and red light, while the T-DNA insertion mutants of AHA2 and AHA5 do not demonstrate any defects in stomatal response (Yamauchi et al., 2016). The constitutive activation of H^+-ATPase AHA1 by the dominant *open stomata 2* mutations (*ost2-1D* and *ost2-2D)* induces uncontrolled ion and water uptake by guard cells and results in constantly open stomata. Experiments with epidermal peels demonstrated that the *ost2-1D* and *ost2-2D* stomata did not respond to blue and red lights, ABA, and darkness (Merlot et al., 2007; Costa et al., 2015; Yamauchi et al., 2016). However, another report showed that stomatal conductance in *ost2-1D* and *ost2-2D* decreased after ABA treatments (Pantin et al., 2013).

Activation of AHAs is a key step in stomatal opening and requires phosphorylation of the regulatory domain at the C-terminus of the protein. The phosphorylated plasma membrane H^+-ATPase binds 14-3-3 proteins, preventing the self-inhibition of the proton pump by the C-terminal R-domain (Merlot et al., 2007; Falhof et al., 2016; Ando et al., 2022). The SMALL AUXIN UP RNA

(SAUR) proteins are involved in the activation of AHA by inhibiting PP2C.D phosphatases and by promoting stomatal opening (Spartz et al., 2014; Wong et al., 2021; Akiyama et al., 2022). The *pp2c.d2/5/6* triple knockout mutant exhibits enhanced stomatal aperture and conductance, the *pp2c.d6/8/9* triple mutant demonstrates more open stomata and enhanced plasma membrane H^+-ATPase phosphorylation under blue light, and delayed dephosphorylation of H^+-ATPase in guard cells. The *saur56 saur60* double mutant has reduced stomatal aperture and stomatal conductance (Wong et al., 2021; Akiyama et al., 2022). Recently, it was demonstrated that AHAs activation and deactivation can occur very fast, as the level of the plasma membrane H^+-ATPase phosphorylation was significantly decreased in Arabidopsis leaves under elevated CO_2 within 1 min (Ando et al., 2022).

Activation of AHAs results in hyperpolarization of the guard cell plasma membrane and K^+ influx due to the negative electric charge on the inner side of the plasma membrane. The K^+ uptake during stomatal opening is mediated by the inward rectifying K^+ channels, such as POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 and 2 (KAT1 and KAT2), and ARABIDOPSIS THALIANA K^+ TRANSPORTER 1 (Schachtman et al., 1992; Pilot et al., 2001; Szyroki et al., 2001). The increased concentration of positively charged K^+ is balanced by the accumulation of anions in guard cells, including $Cl^–$, malate$^–$, and $NO_3^–$. The uptake of $NO_3^–$ is mediated by the nitrate transporter NRT1.1 (Guo et al., 2003a). Malate is either produced from starch or imported from the apoplast by the malate transporter ATP-BINDING CASSETTE B14 (ABCB14) (Lee et al., 2008; Horrer et al., 2016; Daloso et al., 2017; Santelia & Lunn, 2017). Main hexose sugars, such as glucose and fructose, are transported into guard cells by SUGAR TRANSPORTER PROTEIN 1 and 4 (STP1 and STP4) (Flütsch et al., 2020a).

Stomatal opening is an energy-demanding process and requires a considerable amount of ATP, in contrast to stomatal closure, which is a more passive process (Matthews et al., 2020). A recent report demonstrated limited photosynthetic activity in guard cells chloroplasts and suggested mitochondria as a primary source of ATP for stomatal opening (Lim et al., 2022). The NUCLEOTIDE TRANSPORTER proteins mediate the import of ATP into guard cell chloroplasts, which is required for starch biosynthesis and stomatal opening (Lim et al., 2022).

1.2.2 Stomatal closure

Activation of the ion channels in guard cells plays a central role in stomatal closure. The patch-clamp studies on guard cell protoplast demonstrated that guard cells have two types of anion channels: rapid-type (R-type) and slow-type (S-type). The electrophysiological characteristics of these channels suggested their different roles in regulating stomatal movements (Schroeder & Keller, 1992). Upon guard cell membrane depolarization, the R-type anion channels activate in ~50 ms and further rapidly deactivate. R-type anion channels are highly permeable to $NO_3^–$, $SO_4^{2–}$, $Cl^–$, $CO_3^{2–}$, and malate$^–$ (Frachisse et al., 1999; Eugene et al.,

2010). In contrast to R-type anion channels, S-type anion channels show a weak voltage dependence, are activated much slower (for several tens of seconds), and stay open longer (Schroeder & Keller, 1992). S-type anion channels are permeable for Cl^- and NO_3^- but not for SO_4^{2-} (Schmidt & Schroeder, 1994; Vahisalu et al., 2008; Chen et al., 2010; Geiger et al., 2009, 2011; Stange et al., 2010).

Forward genetic screens using ozone sensitivity and thermal imaging under elevated CO_2 led to the identification of the S-type anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) that plays a central role in stomatal closure (Vahisalu et al., 2008; Negi et al., 2008). The plants lacking SLAC1 are impaired in high CO_2-induced stomatal closure, as well as in stomatal response to ozone, low air humidity, and ABA (Vahisalu et al., 2010; Merilo et al., 2013). Among SLAC1 homologs (SLAC1 HOMOLOGUEs 1 to 4), SLAH3 is also involved in stomatal closure (Geiger et al., 2011). SLAC1 mediates the efflux of Cl^- and NO_3^- from guard cells, while SLAH3 is more important for releasing NO_3^-(Geiger et al., 2009, 2011).

The family of ALUMINUM-ACTIVATED MALATE TRANSPORTERS (ALMTs) was discovered in the studies of aluminum toxicity and were shown to function as transporters for malate and fumarate as well as some inorganic anions, such as Cl^-, $NO_3^-$and SO_4^{2-}(Sasaki et al., 2010). A member of this family in Arabidopsis, ALMT12 was found to be strongly expressed in guard cells (Meyer et al., 2010). The *almt12* mutants displayed impaired stomatal responsiveness to darkness, CO_2, and ABA treatments (Meyer et al., 2010; Sasaki et al., 2010). Further studies showed that ALMT12 is an R-type anion channel, whose activation depends on the concentration of extracellular malate. Since ALMT12 is not induced by exogenous Al^{3+} in contrast to other genes in the ALMT family, it was renamed to QUICK-ACTIVATING ANION CHANNEL 1 (QUAC1) in analogy to the S-type anion channel of guard cells, SLAC1 (Negi et al., 2008; Vahisalu et al., 2008; Meyer et al., 2010; Malcheska et al., 2017). Two other channels of this family ALMT6 and ALMT9 are expressed in the tonoplast of the guard cell, which proposes their role in malate and Cl^- transport (Meyer et al., 2011; De Angeli et al., 2013; Sharma et al., 2016).

The K^+ efflux during stomatal closure is mediated by a single K^+_{out} channel, named GATED OUTWARDLY-RECTIFYING K^+ CHANNEL (GORK) (Ache et al., 2000; Hosy et al., 2003; Kollist et al., 2014). GORK is a voltage-dependent channel and is activated by depolarization of the guard cell plasma membrane, resulting from the efflux of anions. The *gork1* loss-of-function mutants demonstrate impaired stomatal closure in response to ABA and darkness in addition to elevated water-loss phenotype (Hosy et al., 2003; Osakabe et al., 2013).

Starch biosynthesis seemingly is involved in stomatal closure. Guard cells contain fewer chloroplasts smaller in size in comparison with mesophyll cells, indicating that the role of guard cell photosynthesis is limited (Lawson, 2009; Iwai et al., 2019). At the same time, starch biosynthesis in guard cell chloroplasts contributes to stomatal closure by reducing the amount of malate and decreasing turgor pressure (Azoulay-Shemer et al., 2016; Flütsch et al., 2022).

1.3 Roles of hormones in stomatal regulation

Plant hormones regulate various aspects of a plant's life. In the following chapter, I describe phytohormones that contribute to stomatal regulation, particularly ABA, JA, SA, SLs, and ET.

1.3.1 Abscisic acid

ABA controls seed dormancy, plant growth, development, and stress responses to biotic and abiotic factors. It was discovered in the 1960s and named after leaf abscission, although the role of ABA in this process is limited by modulating the ET pathway, which regulates leaf abscission. Importantly, ABA controls plant adaptation to drought by promoting water retention in water-stressed plants and triggering stomatal closure (Lim et al., 2015; Sah et al., 2016; Chen et al., 2020).

ABA synthesis occurs sequentially in plastids and cytosol with the first steps of this process starting from carotenoids (C40) in plastids (Xiong & Zhu, 2003). The conversion of zeaxanthin to violaxanthin is catalyzed by zeaxanthin epoxidase. Violaxanthin is then oxidized to 9-cis-epoxycarotenoid and later to the C15 compound xanthoxin. This step is catalyzed by 9-cis-epoxycarotenoid dioxygenases (NCEDs) and is considered as limiting in ABA biosynthesis. The next stages of ABA biosynthesis occur in cytosol, where xanthoxin is converted to ABA-aldehyde. The final step of ABA synthesis is performed by ABA aldehyde oxidase (Xiong & Zhu, 2003; Finkelstein, 2013).

ABA is synthesized directly in guard cells (Bauer et al., 2013; Zhang et al., 2021a), or it can be imported into them (Kang et al., 2010). ABA transport is mediated by specific transporters, for instance ATP-BINDING CASSETTE G25 (ABCG25). This protein is expressed in the vascular tissue, where ABA is also synthesized (Kuromori et al., 2010). ABA transportation is facilitated by DETOXIFICATION EFFLUX CARRIER 50, which is mainly expressed in the vascular tissue and mediates both import and export (Zhang et al., 2014; Merilo et al., 2015). ABCG40 is an ABA importer that is localized in the plasma membranes of plant cells, including guard cells (Kang et al., 2010).

The mechanism of ABA-triggered stomatal closure has been thoroughly characterized. The main components of ABA signaling include ABA receptors PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR), TYPE 2C PROTEIN PHOSPHATASES (PP2Cs), SUCROSE NON-FERMENTING 1-RELATED PROTEIN KINASE 2.6/ OPEN STOMATA 1 (SnRK2.6/OST1), and CALCIUM-DEPENDENT PROTEIN KINASES (CPKs) (Figure 2). In the absence of ABA, the dephosphorylation of OST1 and CPKs by PP2Cs prevents their activation. Binding of ABA by the PYR/PYL/RCAR proteins inactivates PP2C via formation of complexes between the ABA receptors, ABA, and PP2Cs. It results in the activation of OST1 and CPKs that phosphorylate SLAC1. Activation of anion channels eventually leads to stomatal closure. Activation of SLAC1 by ABA depends

on the receptor-like pseudokinase GUARD CELL HYDROGEN PEROXIDE RESISTANT 1 (GHR1) (Sierla et al., 2018).

The PYR/PYL/RCAR family consists of 14 members, which function as monomers (PYL4, PYL5, PYL6, PYL8, PYL9, PYL10) or dimers (PYR1, PYL1, PYL2) or are able to form both monomeric and dimeric forms (PYL3) (Dupeux et al., 2011; Hao et al., 2011). Six members of the PYR/PYL/RCAR family (PYR1/RCAR11, PYL1/RCAR12, PYL2/RCAR14, PYL3/RCAR13, PYL8/RCAR3, PYL9/RCAR1) are expressed in guard cells (Ma et al., 2009; Park et al., 2009). Seed germination and root growth are not inhibited by ABA in the *pyr1 pyl1 pyl2 pyl4* quadruple mutant (Park et al., 2009). In addition, this mutant exhibits strong stomatal insensitivity to ABA in epidermal peel assay (Nishimura et al., 2010).

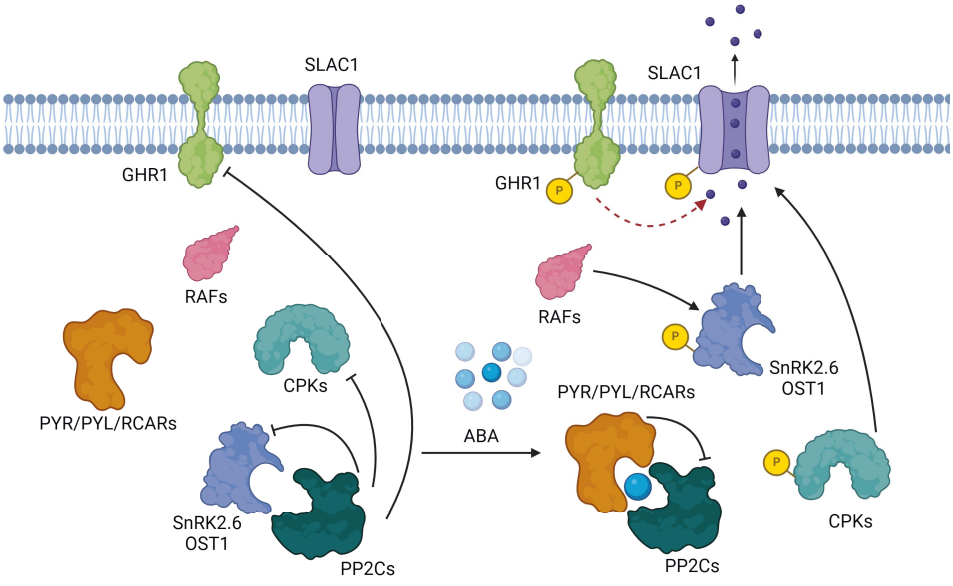

Figure 2. The model of ABA-dependent SLAC1 activation in guard cells. In the absence of ABA, PYR/PYL/RCARs are not bound to PP2Cs which activity prevents an activation of SNRK2.6/OST1, GHR1, and CPKs. The inactive kinases are unable to phosphorylate SLAC1. In the presence of ABA, PYR/PYL/RCARs form a complex with ABA and PP2Cs, resulting in the inhibition of PP2Cs. This leads to the activation of the protein kinases that phosphorylate SLAC1, inducing stomatal closure. GHR1 acts as a scaffolding component and does not directly phosphorylate SLAC1 (shown by dashed red arrow). Created with BioRender.com.

Group A of PP2Cs includes 9 members, with 6 of them functioning as negative regulators in ABA signaling (ABI1, ABI2, HAB1, HAB2, AHG1, PP2CA/AHG3) (Schweighofer et al., 2004). Overexpression and/or upregulation of PP2Cs result in insensitivity to ABA, while their deficiency increases sensitivity to ABA (Merilo et al., 2013). Dominant mutations in ABI1 and ABI2 (*abi1-1D* and *abi2-*

1D), which disrupt the interactions of these proteins with PYR/RCAR, cause strong insensitivity to ABA. The *abi1-1D* and *abi2-1D* mutants display ABA-resistant germination of their seeds, altered seedling morphology, and high water-loss from leaves (Leung et al., 1997). The single loss-of-function mutations in ABI1 and ABI2 do not affect plant's phenotype, while the *abi1-1R4 abi2-1R1* double mutant is more responsive to ABA than the single ones (Merlot et al., 2001). Arabidopsis plants over-expressing HAB1 or PP2CA have ABA-insensitive phenotypes, while the T-DNA insertional mutants for these proteins are hypersensitive to ABA (Saez et al., 2004; Kuhn et al., 2006).

The SnRK2-type family consists of 10 genes in Arabidopsis (Hrabak et al., 2003). The role of SnRK2.6/OST1 in stomatal regulation was identified in a mutant screen using thermal imaging (Merlot et al., 2002). OST1 is highly expressed in guard cells (Mustilli et al., 2002) and plays a central role in ABA-triggered stomatal closure. Interestingly, in addition to stomatal ABA insensitivity, the *ost1-3* mutant demonstrates impaired stomatal responses to environmental cues such as elevated CO_2, changes in air humidity, light-to-dark transitions, and ozone pulse. It indicates that OST1 functions as a convergence point in many signaling pathways (Merilo et al., 2013; Sierla et al., 2018). In addition to ABA signaling, RAF-like kinases can directly phosphorylate and activate OST1 (Fàbregas et al., 2020; Lin et al., 2020; Wang et al., 2023). The SnRK2.2 and SnRK2.3 are homologous and closely related to SnRK2.6/OST1 (Boudsocq et al., 2004). The *snrk2.2 snrk2.3* double mutant demonstrates the lack of ABA suppression in seed germination and root growth (Fujii et al., 2007).

There are 34 CPKs in Arabidopsis, several of which are involved in stomatal closure by ABA (Hrabak et al., 2003). Functions of CPKs in stomatal regulation are highly redundant and therefore it is necessary to generate high order *cpk* mutants for researching their functions. Ca^{2+} activation of S-type anion channels is impaired in the *cpk3 cpk6* double mutant (Mori et al., 2006). The *cpk4 cpk11* double mutant shows an ABA-insensitive phenotype in seed germination and stomatal closure (Zhu et al., 2007). The *cpk5 cpk6 cpk11 cpk23* quadruple mutant exhibits impaired S-type anion currents and stomatal closure in response to ABA and Ca^{2+} (Brandt et al., 2015). Another study demonstrated that the *cpk3 cpk5 cpk6 cpk11 cpk23* quintuple mutant had a slightly impaired stomatal response to ABA and exhibited defects in stomatal opening and closure in response to shifts in CO_2 concentrations (Schulze et al., 2021).

Hua et al. suggested that GHR1 is a leucine-rich repeat receptor-like protein kinase and directly phosphorylates SLAC1 (Hua et al., 2012). However, Sierla et al. demonstrated that GHR1 is a pseudokinase and activates SLAC1 indirectly (Sierla et al., 2018). It is possible that the GHR1 protein kinase activity was observed by Hua et al. due to a copurification of protein kinases responsible for GHR1-mediated activation of SLAC1 (Hua et al., 2012; Sierla et al., 2018). It was suggested that GHR1 acts as a scaffolding component that mediates the activation of SLAC1 by other proteins, such as CPKs (Sierla et al., 2018). The *ghr1* mutants demonstrate impaired stomatal responses to ABA, ozone pulse, elevated CO_2 changes, and darkness (Hua et al., 2012; Sierla et al., 2018).

1.3.2 Strigolactones

SLs stimulate seed development in the parasitic root plants belonging to the genera *Striga, Orobanche, Alectra*, and *Phelipanche* (Cook et al., 1966; Cardoso et al., 2011). These substances are exuded from roots in various plant species. Further, it was demonstrated that SLs coordinate plant growth and architecture. SLs enhance root hair elongation and the growth of primary roots, suppress shoot branching, increase stem thickness and its secondary growth, and speed up leaf senescence (Snowden et al., 2005; Kapulnik et al., 2011; Brewer et al., 2013; Ruyter-Spira et al., 2013).

All SLs have similar chemical structures consisting of a tricyclic lactone (ABC-ring) and a hydroxymethyl butenolide (D-ring) (Umehara et al., 2014). D-ring part is a common part in all SLs, suggesting that this part is essential for the SL biological activity. Up to now, over 20 SLs have been characterized (Xie et al., 2010; Wang & Bouwmeester, 2018).

The biosynthesis of SLs starts in plastids and continues in the cytosol. In plastids, all-trans-β-carotene is converted by DWARF27 (D27) to 9-cys-β-carotene. Then, this substance is turned into 9-cys-β-apo-10-carotene and further to carlactone by CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7)/ MORE AXILLARY GROWTH 3 (MAX3) and CCD8/MAX4, respectively (López-Ráez et al., 2010; Abe et al., 2014; Wang & Bouwmeester, 2018). Carlactone is the biological precursor for all SLs. Carlactone is transported to the cytosol, where it is modified to SLs by CYTOCHROME P450 monooxygenase (MAX1) and by other yet-to-be-identified enzymes (Saeed et al., 2017).

Similar to auxin, JA, and gibberellin, SLs signaling is based on hormone-activated proteolysis, which includes protein ubiquitination and degradation of ubiquitinated proteins by 26S proteasomes (Tai & Schuman, 2008; Waters et al., 2017). In the ubiquitin-proteasome system, proteins for degradation are initially labelled with the small proteins ubiquitins (Figure 3A). SKP1-CULLIN1-F-box (SCF) is the largest and the most characterized class of ubiquitin ligases in plants (Tai & Schuman, 2008; Waters et al., 2017; Wang, et al., 2020b). Studies demonstrated that, in many hormonal signaling pathways, the F-box proteins that target ubiquitination toward specific proteins are hormone receptors (Stefanowicz et al., 2015; Zhang et al., 2019a). SL signaling depends on MAX2 which is an F-box protein and acts as a ubiquitin-ligase enzyme. In addition, SL perception and signaling require D14, which upon binding SLs forms a complex with SCF^{MAX2} to initiate ubiquitination and degradation of downstream signaling components (De Saint Germain et al., 2016; Marzec, 2016; Morffy et al., 2016; Yao et al., 2016). These components include SUPPRESSOR OF MAX2 6 (SMAX6), SMXL7, and SMXL8 (Soundappan et al., 2015; Wang, et al., 2020c).

Only a few studies have addressed the role of SLs in guard cell signaling. The stomatal aperture in SL biosynthesis (*max1-1*, *max3-9,* and *max4-1*) and signaling (*d14-5* and *max2-1*) mutants is significantly wider compared with that in wild-type plants (Lv et al., 2018). Stomatal conductance in the *max2* plants is significantly higher in comparison with wild-type plants (Piisilä et al., 2015). The

treatment of epidermal peels with the synthetic SL analog GR24 induced stomatal closure in wild-type plants and in SL biosynthesis (*max1-1*, *max3-9,* and *max4-1*) mutants. The same SL treatments did not lead to significant stomatal closure in the signaling (*d14-5* and *max2-1*) mutants (Lv et al., 2018). The *max2* plants demonstrate ozone-sensitivity phenotype as after the 6-h exposure to 300 ppb ozone they developed pronounced leaf lesions and showed enhanced ion leakage (Piisilä et al., 2015). Stomatal closure in response to bacterial infections can depend on SL signaling. Stomatal aperture of wild-type plants decreased after infection with *Pseudomonas syringae pv tomato* (*Pst*) stain DC3000, while in the *max2-1* and *max2-4* plants, no stomatal closure was detected (Piisilä et al., 2015).

1.3.3 Jasmonic acid

Plant hormone JA is well known as a biotic stress hormone. JA is involved in the regulation of the plant defense responses induced by some pathogens and pests as well as wounding by biting and chewing insects. Moreover, JA regulates flower development, seed formation, root growth, trichome formation, and stomatal signaling (Acharya & Assmann, 2009; Wasternack & Hause, 2013; Kim et al., 2015; Huang et al., 2017).

Jasmonates include JA and its derivatives, such as methyl jasmonate (MeJA) and JA-isoleucine conjugate (JA-Ile) (Ruan et al., 2019). MeJA can be converted into JA and JA-Ile (Wasternack & Strnad, 2016). MeJA was first isolated in 1962 from the essential oil of *Jasminum grandiflorum* (Demole et al., 1962; Wasternack, 2007).

JA biosynthesis occurs in plastids, peroxisomes, and cytosol. JA biosynthesis starts from α-linolenic acid (18:3), which is converted to (13S)-hydroperoxyoctadecatrienoic acid by 13-lipoxygenase. (13S)-hydroperoxyoctadecatrienoic acid is modified to 12,13(S)-epoxyoctadecatrienoic acid by ALLENE OXIDE SYNTHASE and further to (9S,13S)-12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase (Huang et al., 2017; Wang et al., 2021). OPDA is transported to peroxisomes, where it is transformed to 3-oxo-2-(cis-2′-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) and OPC-8:0 CoA by OPDA reductase and OPC-8:0 CoA ligase, respectively. The following steps leading to JA synthesis are performed by acyl-CoA oxidase, MULTIFUNCTIONAL PROTEINs, and 3-ketoacyl-CoA thiolase. JA is transported to the cytoplasm, where it is modified to JA-Ile or MeJA (Wasternack, 2007; Huang et al., 2017; Ruan et al., 2019).

Several G-subfamily ABC transporters regulate JA transport. JA TRANSFER PROTEIN 1 (JAT1 or ABCG16) is localized in the plasma membrane and nuclear membrane and mediates both cellular efflux and nuclear influx of JA and JA-lle (Li et al., 2017). In 2020, Li et al. reported about other JA transporters, such as JAT3 (or ABCG6) and JAT4 (or ABCG20) (Li et al., 2020a). The JAT3 and JAT4 proteins regulate the long-distance cell-to-cell JA transport in wounded leaves (Li et al., 2020a). The GLUTAMATE RECEPTOR-LIKE 3.3 (GRL3.3) mediates JA production in distal leaves by transmitting wound-induced Ca^{2+} waves. It has

been demonstrated that GRL3.3 works synergistically in core phloem cells with JAT3 and JAT4, presumably also through cell-to-cell transportation (Li et al., 2020a; Liu & Timko, 2021).

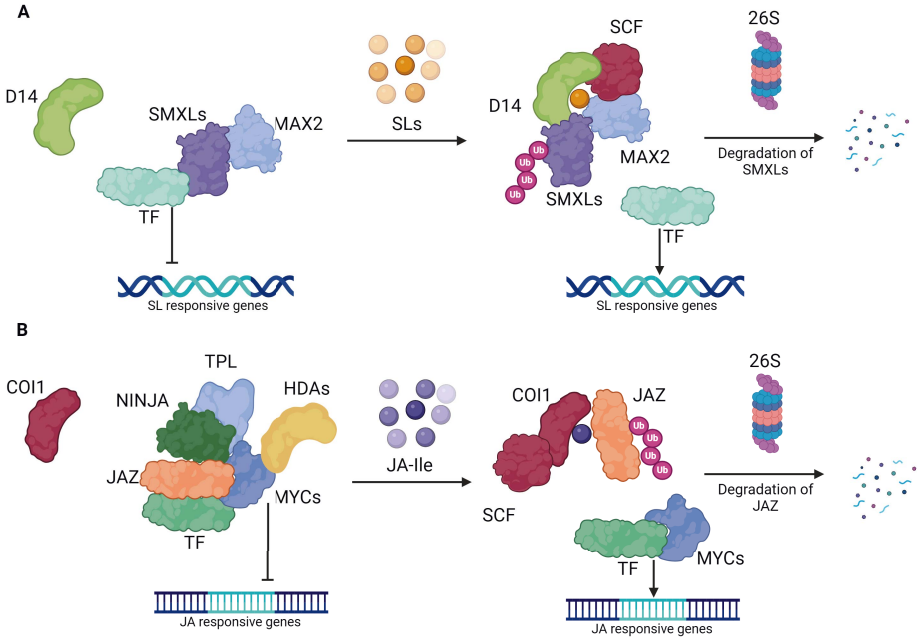

Figure 3. The scheme showis canonical SLs and JA signaling. (A) The model of the SLs signaling pathway. In the absence of SLs, D14 is inactive, and SMXLs repress transcription factors (TF) for the expression of SL-responsive genes. SLs bind to D14, triggering the formation of the D14-SCF MAX2 complex binding SMXLs. It leads to the ubiquitination and degradation of SMXLs, releasing SL-responsive genes from the repression. (B) The model of the JA signaling pathway. Without JA, JAZ proteins repress the TF, preventing the activation of the promoters of JA-responsive genes. JAZ interacts with NINJA, TPL and HDAs proteins forming the active transcriptional repression complex that inhibits the MYC TF. JA-Ile promotes the interaction of JAZ with the F-box protein COI1 within the SCF complex, leading to the proteasomal degradation of JAZ proteins. The degradation of JAZ leads to the de-repression of the JA-responsive genes. Created with BioRender.com.

JA signaling is mediated by ubiquitination and degradation of proteins in 26S proteasomes. CORONATINE INSENSITIVE 1 (COI1) was identified as a receptor for JA-Ile. This protein plays a central role in JA signaling as *coi1* mutants lose all responses to JA (Feys et al., 1994; Xie et al., 1998). COI1 is an F-box protein, which functions in the SCF^{COI1} ubiquitination complex (Xie et al., 1998). In 2007, it was found that JASMONATE ZIM DOMAIN (JAZ) proteins are JA co-receptors acting as negative regulators in the JA signaling pathway (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). By now,13 JAZ proteins (JAZ1-JAZ13) have been identified in Arabidopsis (Thireault et al., 2015). In the

absence of JA, the JAZ proteins repress the MYC (MYC2/3/4/5) transcription factors (TFs) and expression of JA-responsive genes. This process involves other co-repressors, such as NOVEL INTERACTOR OF JAZ (NINJA), TOPLESS (TPL), and HISTONE DEACETYLASE 6 and 19 (HDA6 and 19) required to form the active transcriptional repression complex (Figure 3B) (Chini et al., 2007; Yan et al., 2009; Sheard et al., 2010; Thireault et al., 2015; Liu & Timko, 2021). Upon an increase in the JA level, JA-Ile interacts with JAZs and COI1, which leads to the formation of the SCF^{COI1} -JAZ complex and JAZ degradation by 26S proteasomes (Sheard et al., 2010). Further, MYC TFs are activated, which initiates JA response by expression of numerous downstream TFs genes (Chini et al., 2007; Thines et al., 2007; Yan et al., 2009; Sheard et al., 2010; Liu & Timko, 2021).

Several independent research groups reported that JA biosynthesis and signaling participate in stomatal regulation. Treatments with MeJA led to a decrease in stomatal aperture in wild-type plants (Hossain et al., 2011; Yin et al., 2016; Khokon et al., 2017; Munemasa et al., 2007, 2019). However, in the JA-insensitive mutant *coi1*, the stomata did not respond to treatments with MeJA in epidermal peels, unlike in wild-type plants (Munemasa et al., 2007). Contradictory results have been obtained by other research groups. Thus, no stomatal closure in epidermal peels was observed after the treatments with MeJA in the concentration range from 1 nM to 100 μM (Montillet et al., 2013). Other researchers found that MeJA weakly induced stomatal closure or could even be involved in stomatal opening (Savchenko et al., 2014; Zhu et al., 2020). In some studies, OPDA, a JA precursor, was even more efficient regarding triggering stomatal closure than MeJA (Montillet et al., 2013; Savchenko et al., 2014; Melotto et al., 2017; Zhu et al., 2020). Moreover, in 2016, it was reported that stomata of the JA biosynthesis and signaling mutants *coi1, jasmonate resistant 1 (jar1), jasmonate insensitive 1 (jin1)* were insensitive to elevated CO_2 treatment (Geng et al., 2016).

JA and ABA can share common components in their signaling pathways (Munemasa et al., 2007; Hossain et al., 2011; Yin et al., 2016). Experiments with epidermal peels showed that MeJA-induced stomatal closure, H_2O_2 and NO production were impaired in *ost1-2*, *srk2e,* and *aba2-2* mutants. At the same time, ABA receptors were not required for stomatal reactions in response to MeJA, as shown using the *pyr1 pyl1 pyl2 pyl4* quadruple mutant. This quadruple mutant demonstrated the same stomatal phenotype as wild-type plants in response to MeJA treatment (Yin et al., 2016). By using a genetically encoded reporter for OST1 activity (SNACS), it was shown that MeJA does not enhance OST1 activity (Zhang et al., 2020). The participation of CPKs in JA-induced stomatal closure was shown in the experiments with the *cpk6-1* single and *cpk3-1 cpk6-1* double mutants. These mutants demonstrated no stomatal closure after applying exogenous MeJA (Munemasa et al., 2011). Reactive oxygen species (ROS) and NO production were increased by MeJA in both *cpk6-1* and wild-type plants (Munemasa et al., 2011).

Wounding induces local and systemic stomatal closure by activating JA signaling (Förster et al., 2019). Upon wounding and triggering JA-mediated stomatal

closure, JA promotes K^+ efflux in guard cells by activation of the GORK channel through its phosphorylation by Ca^{2+} sensor-kinase complex CBL1-CIPK5. Accordingly, the *gork1-2* and *cipk5-2* single mutants, and the *cbl1/9* double mutant demonstrate no stomatal closure in response to MeJA (Förster et al., 2019).

1.3.4 Salicylic acid

SA was first isolated in the mid-19th century from the bark of white willow (*Salix alba*) (White, 1979). Salicylates are widely used in medicine in the form of acetylsalicylic acid to treat pain and inflammation (Vlot et al., 2009). SA plays an essential role in the defense responses against pathogens and triggers stomatal closure to prevent microorganism entry into plants (Melotto et al., 2006; Zeng & He, 2010). The bacterial pathogen *Pst* DC3000 induces stomatal closure in wild-type Arabidopsis plants 1 h after inoculation, however, the closed stomata reopen 3 to 4h after the treatment starts (Melotto et al., 2006). Stomatal closure in response to *Pst* DC3000 is absent in SA-deficient *nahG* transgenic plants, expressing the peroxidase degrading SA to catechol, and SA-biosynthetic mutants (Melotto et al., 2006).

Pathogen attacks and some abiotic stresses induce SA synthesis in plants (Huang et al., 2020b). There are two biosynthesis pathways in plants, which lead to SA production. In Arabidopsis, more than 90% of SA is synthesized in chloroplasts via the isochorismate synthetase (ICS) pathway. The rest of SA is synthesized in the cytoplasm by phenylalanine ammonia lyase (PAL) pathway (Chen et al., 2009). In the ICS pathway in chloroplasts, chorismate is converted to isochorismate by ICS. The ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) regulates the transport of isochorismate from the chloroplasts to the cytosol. There, avrPphB SUSCEPTIBLE 3 (PBS3) conjugates isochorismate and glutamate to isochorismate-9-glutamate. Later, isochorismate-9-glutamate can be modified to SA and 2-hydroxy-acryloyl-N-glutamate spontaneously or by ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (Rekhter et al., 2019; Torrens-Spence et al., 2019). The PAL pathway starts from the modification of chorismate to prephenate by chorismate mutase. Prephenate after several steps of modifications is converted to phenylalanine. Phenylalanine by PAL is converted to trans-cinnamic acid (Huang et al., 2010). After several reactions catalyzed by abnormal inflorescence meristem 1, trans-cinnamic acid is converted to benzoic acid (Bussell et al., 2014). The last step of SA synthesis from benzoic acid is catalyzed by an unknown enzyme (Lefevere et al., 2020; Mishra & Baek, 2021). Some steps of SA biosynthesis are not fully understood, and more research is needed to determine the missing components of SA synthesis (Zhang & Li, 2019b; Lefevere et al., 2020).

Several forward genetic screens were performed to uncover components in SA signaling. These screens led to the identification of NONEXPRESSOR OF PATOGENESIS-RELATED GENES 1 (NPR1) protein which acts as SA receptor (Cao et al., 1994; Delaney et al., 1995; Canet et al., 2010; Peng et al., 2021). Its

close homologs, NPR3 and NPR4, have a similar domain structure and in addition have a transcriptional repression domain (Ohta et al., 2001; Yuli Ding et al., 2018). NPR1 functions as a transcriptional activator (Rochon et al., 2006), while NPR3 and NPR4 are transcription repressors (Zhang et al., 2006; Fu et al., 2012).

The NPR1 and NPR3 bind SA with similar affinity, while the affinity of NPR4 to SA is 5-10-fold higher (Li & Zhang, 2020b; Wang et al., 2020d). It has been hypothesized that as-yet-unidentified proteins are involved in the functioning of NPR1 and NPR4 (Li & Zhang, 2020b). In the absence of SA, NPR4 interacts with NPR1 to keep it inactive (Li & Zhang, 2020b; Wang et al., 2020d). When the SA level is low, NPR1 is inactive, and NPR3 and NPR4 repress the expression of SA-induced genes. When the concentration of SA is high, this hormone interacts with NPR1, leading to the activation of SA target genes. When SA binds NPR3 and NPR4, it results in the de-repression of SA target genes (Figure 4). The *npr4-4D* mutant, where NPR4 cannot bind SA, demonstrates the constitutive repression of SA-dependent immune response (Ding et al., 2018). NPR1 interacts with multiple TFs, including positive (Zhou et al., 2000; Kim & Delaney, 2002) and negative (Weigel et al., 2001) transcription regulators, as well as histone modifiers (Jin et al., 2018), and other proteins (Chen et al., 2019; Chen et al., 2021a).

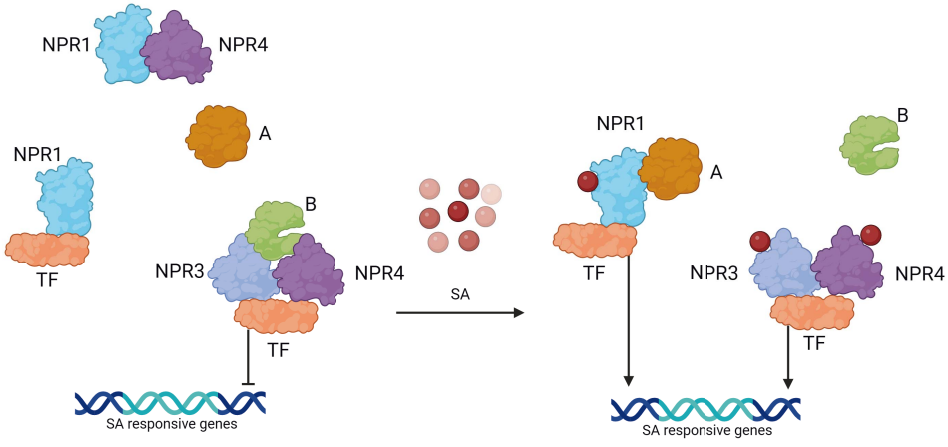

Figure 4. The model of SA signaling in plant cells. In the absence of SA, NPR4 binds NPR1 leading to its repression and degradation. NPR3 and NPR4 inhibit the expression of SA-dependent genes with participation of hypothetical protein partner B. When SA concentration increases, it binds to NPR3 and NPR4, which leads to their dissociation from protein B, and their inactivation, resulting in the de-repression of the target SA-dependent genes. SA also binds to NPR1 leading to its interaction with not-identified partner A and induction of SA-responsive genes. Created with BioRender.com.

NPR1 can bind to many TF complexes and potentially it can crosstalk with other hormonal signaling pathways (Zhou et al., 2023). NPR1 can interact with the JA-induced MYC2 transcriptional factor, inhibiting the JA-dependent transcriptional activation (Nomoto et al., 2021). It also represses the ET signaling components ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1) (Huang et al.,

2020a). On the contrary, the SA receptors NPR3 and NPR4 activate JA signaling, instead of the canonical JA receptor COI1, at the early stages of effector-triggered immunity (Liu et al., 2016).

SA induces systemic acquired resistance (SAR) that provides whole-plant prolonged protection against a broad spectrum of pathogens (Chern et al., 2008). Activation of SAR requires a cascade of transcriptional events induced by NPR1. This protein controls the activation of PATHOGENESIS-RELATED GENEs, which mediate plant immunity (Cao et al., 1997). In addition, it has been shown that the expression of NPR1 is regulated by numerous WRKY TFs (Wang et al., 2006). Plants express large numbers of various WRKYs, for example Arabidopsis and rice genomes contain 74 and 109 WRKY genes, respectively (Phukan et al., 2016). More than half of WRKY TFs respond to exogenous SA treatments (Dong et al., 2003). Several studies demonstrated that WRKY TFs can act as positive or negative regulators of defense gene expression. The overexpression of WRKY28 and WRKY46 results in an increased ICS1 expression and SA biosynthesis (van Verk et al., 2011). The WRKY18 and WRKY40 negatively affect activities of ICS1, EDS5, and PBS3 (Birkenbihl et al., 2017). Two other WRKY TFs, WRKY70 and WRKY54, are also involved in the negative regulation of SA biosynthesis (Wang et al., 2006). In addition, WRKY70 mediates the crosstalk between SA and JA signaling pathways (Li et al., 2004a). The WRKY TFs can regulate diverse plant reactions and contribute to the flexibility and specificity of plant defense responses.

Several researchers demonstrated that SA is involved in stomatal regulation (Mori et al., 2001; Khokon et al., 2011). It was reported that SA induces stomatal closure in epidermal peels of wild-type plants (Mori et al., 2001; Khokon et al., 2011; Panchal et al., 2016). The *npr1-1* and *npr1-2* mutants displayed impaired stomatal closure after SA treatments (Wang et al., 2020a). The *npr1-1* mutant also demonstrated reduced stomatal closure after inoculation with *Pst* DC 3000 (Zeng & He, 2010). SA-triggered stomatal closure depends on calcium signaling as the *cpk3-2 cpk6-1* double mutant did not demonstrate a reduction of stomatal aperture in response to SA (Prodhan et al., 2018). SA activates S-type anion channels in wild-type plants and in the *ost1-3* mutant, leading to stomatal closure, in contrast to the *cpk3-2 cpk6-1* mutant (Prodhan et al., 2018). Stomatal closure triggered by SA requires ROS and NO as second messengers. In guard cells, SA signaling activates ROS production by salicylhydroxamic acid (SHAM) – sensitive peroxidases (Mori et al., 2001; Khokon et al., 2011). Experiments with epidermal peels demonstrated that a pre-treatment with SHAM leads to suppression of SA-induced stomatal closure (Khokon et al., 2011). These results indicate that cell wall peroxidases are involved in ROS production during SA stomatal closure. The involvement of MITOGEN-ACTIVATED PROTEIN KINASES (MPKs) in SA-induced stomatal closure was studied. SA induces stomatal closure in the *mpk9* and *mpk12* single mutants but not in the *mpk9 mpk12* double mutant (Khokon et al., 2017). The *mpk9 mpk12* mutations do not impair ROS production in response to SA, despite the lack of SA-triggered S-type anion channel activation in this mutant (Khokon et al., 2017).

1.3.5 Ethylene

ET is a gaseous compound that functions as a hormone. It is synthesized in plants and is involved in numerous processes, including germination, senescence, fruit ripening, abscission, and adaptation to various stresses (Guzmán & Ecker, 1990; Arraes et al., 2015).

ET biosynthesis has been intensively studied and well-characterized (Wang et al., 2002; Sun et al., 2017; Pattyn et al., 2021). This process is quite simple and occurs via two enzymatic reactions. In the first step, S-adenosyl-methionine is converted to 1-aminocyclopropane-1-carboxylate (ACC) by the ACC synthase. Then, ACC is oxidized by the ACC oxidase into ET, CO_2, and cyanide. ET, as a gaseous molecule, can quickly diffuse to nearby cells, mediating a local reaction at the site of reactions.

ET signaling pathway includes its receptors, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), ETHYLENE INSENSITIVE 2 (EIN2), and a number of TFs, such as EIN3, EIL1, and ETHYLENE-RESPONSE-FACTOR 1 (ERF1) (Wang et al., 2002; Ju & Chang, 2015; Liu et al., 2015; Binder, 2020). In Arabidopsis, five ET receptor isoforms have been identified: ETHYLENE RESPONSE 1 and 2 (ETR1 and ETR2), ETHYLENE RESPONSE SENSOR 1 and 2 (ERS1 and ERS2), and EIN4 (Hua & Meyerowitz, 1998). These receptors are localized in the endoplasmic reticulum and the Golgi apparatus. The ET receptors are negative regulators of ET response. In the absence of ET, the receptors keep the CTR1 kinase active, which subsequently phosphorylates the C-terminal domain of EIN2, called carboxyl end of EIN2 (CEND). It leads to the EIN2 ubiquitination and degradation, preventing the activation of the ET-dependent TFs. The EIN2 and EIN3/EIL1 are regulated by proteasomal degradation through EIN2 TARGETING PROTEIN 1 and 2 (ETP1 and ETP2) and EIN3-BINDING F-BOX PROTEIN 1 and 2 (EBF1 and EBF2), respectively (Guo & Ecker, 2003b; Qiao et al., 2009; Yang et al., 2015). Binding ET to the receptors inhibits the CTR1 protein kinase activity. Non-phosphorylated EIN2 undergoes cleavage and the CEND domain is transported to the nucleus, where it activates the downstream EIN3/EIL1 transcriptional cascade, leading to the ET response (Figure 5) (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012).

Studying the role of ET in stomatal regulation has been controversial. Some studies suggested that ET induces stomatal closure (Desikan et al., 2006; Liu et al., 2010; Shi et al., 2015), while other researchers found that ET mediates stomatal opening (Merritt et al., 2001; Tanaka et al., 2005, 2006; Iqbal et al., 2011). ET-induced stomatal closure requires hydrogen peroxide synthesized by NADPH oxidases (Desikan et al., 2006; Ge et al., 2015). ET can affect signaling pathways activated by other hormones. Thus, ET functions as an inhibitor in ABA- and JA-induced stomatal closure. It was shown that ET delays stomatal closure by inhibiting ABA-signaling pathway in guard cells (Tanaka et al., 2005, 2006). Also, the *ethylene overproducer 1* (*eto1-1*) mutant with an elevated level of ET demonstrated a delayed stomatal response to ABA (Tanaka et al., 2005). The leaves preincubated in the ET precursor ACC demonstrated impaired ABA- and

MeJA-induced stomatal closure (Munemasa et al., 2019). This effect depended on ETR1 as the *etr1-1* mutant did not show affected stomatal closure triggered by ABA and JA after a preincubation with ACC. On the other hand, ET is required for SA-mediated stomatal closure. The exogenous application of ACC rescued defective SA-induced stomatal closure in the *npr1* mutants (Wang et al., 2020a).

MPKs, such as the MKK1/3-MPK3/6 cascade, mediate ethylene-induced stomatal closure (Zhang et al., 2021b). The *mkk1* and *mkk3* single mutants have partially inhibited ACC-induced stomatal closure, while the double mutant *mkk1 mkk3* demonstrates completely impaired stomatal closure after ACC treatment. Transgenic lines expressing $MPK6^{CA}$ (the constitutively active form of MPK6) in the wild type and *mkk1/3* genetic backgrounds demonstrated constitutive stomatal closure with or without ACC treatment. The MKK1/3-MPK3/6 signaling cascade was suggested to function downstream of ETR1 and CTR1, but upstream of EIN2 and EIN3 in ET signaling (Zhang et al., 2021b).

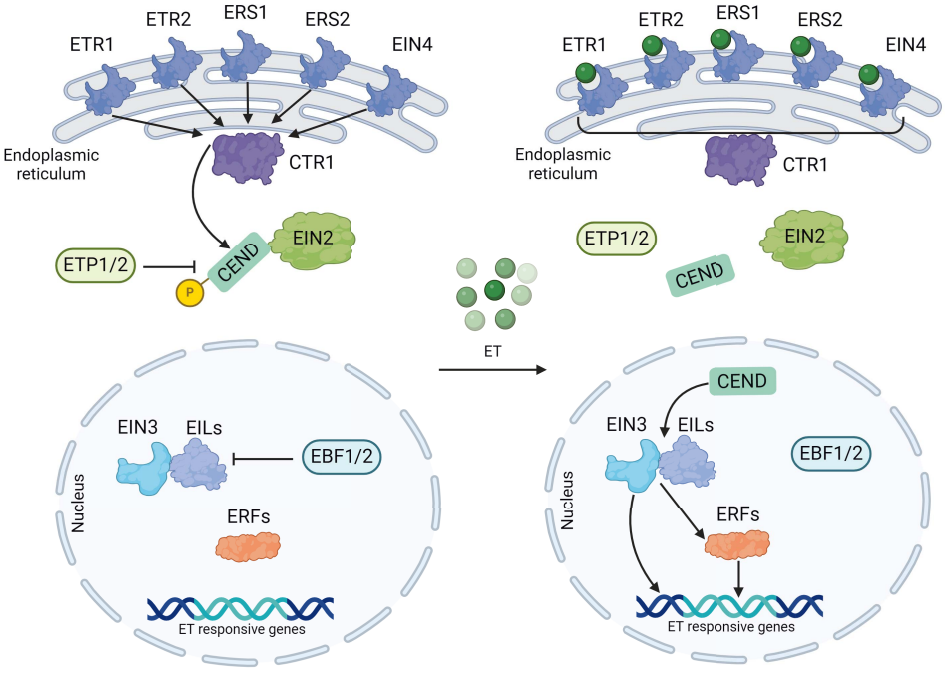

Figure 5. The model of ET signaling pathway. In the absence of ET, the ET receptors (ETR1, ETR2, ERS1, ERS2, EIN4) activate CTR1, suppressing the ET response. CTR1 directly phosphorylates the C-terminal domain of EIN2, which leads to the degradation of EIN2 involving EPT1/2 and the repression of ET-dependent gene expression. In the nucleus EIN3/EIL1 are degraded in the presence of EBF1/2. Upon an increase in ET level, it binds to the ET receptors, resulting in the inactivation of receptors and CTR1. The inactivation of CTR1 leads to the dephosphorylation of EIN2 and its cleavage. The C-terminal fragment of EIN2 (CEND) is transported into the nucleus, where it participates in stabilization of EIN3/EIL1, followed by the consequent transcription of ERF1 and other ET-responsive genes. Created with BioRender.com.

1.4 Stomatal regulation by environmental factors

Plant productivity can be limited by numerous unfavorable environmental factors, including drought, extremes in temperature, air pollution, leading to losses in crop productivity. Our understanding of stomatal adaptation to ever-changing environment can give us a clue for the development of better crops for agriculture.

In this part, I give an overview of the stomatal regulation mechanisms by CO_2, darkness, low humidity and ozone.

1.4.1 Stomatal signaling under changed CO_2

The atmospheric CO_2 concentration is rising on a global scale due to the active consumption of fossil fuels. It affects stomatal development – stomatal density and stomatal size decrease upon the elevation of the CO_2 concentration (Woodward, 1987; Luomala et al., 2005; Wall et al., 2023). Increasing the concentration of CO_2 induces fast stomatal closure and vice versa lowering CO_2 concentration provokes stomatal opening (Dubeaux et al., 2021). Guard cell CO_2 signaling plays a central role in balancing CO_2 uptake into the leaf with water evaporation by transpiration. Under light, CO_2 is assimilated in photosynthesis, resulting in a decrease of C_i and inducing stomatal opening. Changes in C_i are sensed by guard cells to regulate stomatal apertures in accordance with the photosynthesis rate (Mott & Peak, 2018). Experiments with epidermal peels demonstrated that mesophyll-free guard cells react to changes in CO_2 level, indicating that they have all the required signaling pathways to recognize CO_2 levels directly (Engineer et al., 2016).

CO_2 transportation into plants was shown to depend on aquaporins which define the internal CO_2 conductance and net CO_2 assimilation (Hanba et al., 2004; Flexas et al., 2006; Katsuhara & Hanba, 2008). In Arabidopsis, CO_2 enters guard cells via diffusion through the aquaporin PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1) (Katsuhara & Hanba, 2008; Wang et al., 2015). The T-DNA knockout mutant *pip2;1* demonstrates the wild-type stomatal responsiveness to elevated CO_2 (Wang et al., 2015), indicating a possible involvement of other aquaporins in CO_2 uptake by guard cells. In the cytosol, CO_2 is converted into bicarbonate (HCO_3^-) by ß-carbonic anhydrases ßCA4 and ßCA1 (Hu et al., 2010; Dubeaux et al., 2021). βCA4 is localized at the plasma membrane while βCA1 is expressed in chloroplasts (Hu et al., 2015). PIP2;1 physically interacts with ßCA4, potentially enhancing the conversion of CO_2 into HCO_3^- in guard cells. The single mutants *ca1* and *ca4* do not affect stomatal movements, while the *ca1 ca4* double mutant demonstrates impaired stomatal responsiveness to both elevated and low CO_2. The lack of the CO_2 to HCO_3^- conversion in the *ca1 ca4* mutant specifically affects CO_2 signaling in guard cells as this mutant displays unaffected stomatal responsiveness to blue light, darkness, and ABA (Hu et al., 2010).

The importance of ßCA4 and ßCA1 for CO_2 signaling indicates that HCO_3^- plays a significant role in activating CO_2 signaling in guard cells. The identi-

fication of the HCO_3^- sensor benefited from the discoveries of mutants with specific impairment of CO_2-driven stomatal movements. Thus, mutant screens and studies of natural variation highlighted a significant role for the MPK4/MPK12 – HIGH LEAF TEMPERATURE 1 (HT1) signaling hub in CO_2 signaling. Initially, the recessive *ht1-1* and *ht1-2* mutants, carrying mutations in the protein kinase HT1, were isolated in a thermal imaging CO_2 screen. These mutants demonstrated a constantly decreased level of stomatal conductance combined with strong insensitivity to elevated and low CO_2 (Hashimoto et al., 2006). Other stomatal responses remained intact in the mutants, suggesting that HT1 acts as a negative regulator in CO_2 signaling. In the recessive *ht1-1* and *ht1-2* mutants, HT1 is incapable of inhibiting the SLAC1 activation, which results in the constitutive SLAC1 activity, constitutively closed stomata, and low stomatal conductance (Hashimoto et al., 2006). The lack of the protein kinase MPK12, similarly led to a specific, although partial, impairment of stomatal responsiveness to CO_2, while stomatal closure after ABA and light-to-darkness treatments remained the same as in wild-type plants (Hõrak et al., 2016; Jakobson et al., 2016; Tõldsepp et al., 2018). The MPK4, which is expressed in various plant tissues, including guard cells, functions together with MPK12 during CO_2-induced stomatal regulation (Hõrak et al., 2016; Lin & Chen, 2018). The double mutant *mpk12 mpk4GC*, with silencing MPK4 in the guard cells, demonstrated the complete lack of CO_2-induced stomatal responses and impaired activation of S-type anion currents by elevated CO_2/HCO_3^- (Tõldsepp et al., 2018). Along with the loss-of-function *ht1* mutants, the dominant *ht1-3* and *ht1-8D* mutations with completely disrupted CO_2 responses were isolated. In *ht1-3* and *ht1-8D,* the interaction of HT1 with MPK12 and MPK4 is disrupted, leading to constitutive activation of HT1 and inhibition of SLAC1, resulting in open stomata and high stomatal conductance (Hashimoto-Sugimoto et al., 2016; Hõrak et al., 2016). In response to elevated CO_2 concentration, MPK4 and MPK12 bind to HT1, resulting in the inhibition of HT1 (Hõrak et al., 2016; Jakobson et al., 2016; Tõldsepp et al., 2018; Takahashi et al., 2022). It activates downstream signaling that eventually promotes the activity of anion channels, although the exact mechanism is still not fully understood (Xue et al., 2011; Sierla et al., 2018) (Figure 6).

Two related protein kinases CONVERGENCE OF BLUE LIGHT AND CO_2 1 and 2 (CBC1 and CBC2) were found to function in the same signaling pathway with HT1 in CO_2 signaling. These enzymes are involved in stomatal opening induced by low CO_2 concentrations and blue light (Hiyama et al., 2017). The double *cbc1 cbc2* mutant is insensitive to blue light and low CO_2, while the single *cbc1* and *cbc2* mutants demonstrate only slightly impaired stomatal responsiveness to the same stimuli. Interestingly, the double *cbc1 cbc2* mutant maintains ABA-induced stomatal closure. Further analysis demonstrated that CBCs physically interact with HT1 *in vivo* and HT1 phosphorylates CBC1 and CBC2. CBC protein kinases act downstream of HT1, which was demonstrated using *cbc1 cbc2 ht1-A109V* triple mutant. The dominant *ht1-A109V* mutant exhibits constitutively open stomatal phenotype, characterized by elevated stomatal conductance. However, *cbc1 cbc2 ht1-A109V* triple mutant demonstrates a closed

stomatal phenotype, which is similar to *cbc1 cbc2* (Takahashi et al., 2022). Under low CO_2, HT1 phosphorylates and activates the CBC protein kinases, inhibiting S-type anion channels and stomatal closure (Hiyama et al., 2017; Zhang et al., 2020) (Figure 6). It was previously suggested that the MATE-type transporter RESISTANT TO HIGH CO_2 (RHC1) could act as a HCO_3^- sensing protein and function upstream of HT1, as *rhc1* mutant demonstrated impaired stomatal closure in response to high CO_2 (Tian et al., 2015). However, another study showed that RHC1 alone was able to produce ionic currents in oocytes that are not affected by $NaHCO_3$ (Wang et al., 2015) and *rhc1* had a wild-type stomatal response to elevated CO_2 treatment (Tõldsepp et al., 2018), which indicate that a role of RHC1 in CO_2 signaling should still be confirmed (Tian et al., 2015; Tõldsepp et al., 2018).

As mentioned above, SLAC1 plays a central role in stomatal closure in response to various changing conditions, including elevated CO_2 (Negi et al., 2008; Vahisalu et al., 2008). Interestingly, in addition to its main function as the anion channel, SLAC1 was suggested to be a part of CO_2/HCO_3^- sensing in guard cells. Several possible sites in SLAC1, which might be involved in CO_2-dependent stomatal regulation, have been reported. To detect sites necessary for CO_2 stomatal response, transgenic plants expressing truncated SLAC1 proteins were generated, and computer-aided modelling was performed (Wang et al., 2015; Yamamoto et al., 2015; Zhang, et al., 2018b). It was shown that the R256 residue is important for the HCO_3^- activation of SLAC1 in oocytes and impairs high CO_2-induced stomatal closure, but not ABA-mediated stomatal closure (Zhang, et al., 2018b). High CO_2 induces apoplastic malate accumulation (Rainer Hedrich et al., 1994), which can directly activate SLAC1 and provide a potential link between mesophyll and stomata in CO_2 regulation (Mimata et al., 2022).

The mechanism of stomatal opening under decreased CO_2 concentration has been characterized less than high CO_2-triggered stomatal closure. The main gap in our knowledge is understanding the signaling pathways that control the activation of AHAs under low CO_2 (Engineer et al., 2016). As shown recently, an increase in C_i inactivates AHAs through their dephosphorylation, leading to the inhibition of stomatal opening (Ando et al., 2022). Moreover, stomata in the *ost2* mutants with constant AHA1 activity are insensitive to elevated CO_2 treatment (Costa et al., 2015). It was also reported that Munc13-like PROTON ATPASE TRANSLOCATION CONTROL 1 (PATROL1) is essential for stomatal opening under low CO_2 (Hashimoto-Sugimoto et al., 2013). PATROL1 is expressed in all plant cells, including guard cells and subsidiary cells, and controls the translocation of AHA1 from the inner membranes to the plasma membrane in the guard cells and subsidiary cells (Higaki et al., 2014). Some transporters also can contribute to low CO_2 stomatal opening by increasing osmotic pressure in the guard cell. The NRT1.1 modulates NO3- accumulation in the guard cells (Guo et al., 2003a), and ABCB14 imports malate from apoplasts (Lee et al., 2008).

Malate plays a dual role in the regulation of stomatal movement. It acts as an osmotically active ion involved in stomatal opening. During stomatal closure, malate which is a substrate for starch biosynthesis should be removed from the

guard cells to reduce osmotic pressure. Plants defective in starch biosynthesis demonstrate reduced stomatal responsiveness to elevated CO_2, indicating that the synthesis of carbonates is involved in the regulation of turgor pressure during stomatal closure (Azoulay-Shemer et al., 2016). At the same time, malate in the apoplast promotes stomatal closure. The stomatal closure under elevated CO_2 levels can be influenced by the production of malate in the mesophyll. Apoplastic malate promotes stomatal closure by activation of anion channels, such as ALMT12 (Hedrich & Marten, 1993; Lawson et al., 2014; Sasaki et al., 2022) and SLAC1 (Mimata et al., 2022).

1.4.2 Stomatal movements triggered by illumination conditions

Both blue light and red light induce stomatal opening, although through different mechanisms. Red light activates photosynthesis in mesophyll, leading to a decrease in C_i (Lawson et al., 2002; Roelfsema et al., 2002) and triggering low CO_2 signaling in guard cells (Matrosova et al., 2015). Potentially, photosynthesis in the guard cells could be involved in recognizing light conditions (Lawson et al., 2002). The importance of low CO_2 signaling for the stomatal reaction to red light is highlighted by the fact that HT1 is essential for red light-induced stomatal opening. The *ht1-2* mutant demonstrates strong stomatal insensitivity to red light; however, this mutant opens stomata in response to blue light (Matrosova et al., 2015). Low CO_2 and blue light signaling in guard cells converge at CBC1 and CBC2, promoting stomatal opening by inhibiting S-type anion channels (Hiyama et al., 2017).

Blue light-induced stomatal opening involves phosphorylation of several signaling cascades, which leads to the activation H^+-ATPase pump (Hiyama et al., 2017). Plants have multiple light receptors, including blue light receptors PHOTOTROPINS 1 and 2 (PHOT1 and 2), blue and ultraviolet-A receptors CRYPTOCHROMES 1 and 2 (CRY1 and 2), and red light receptors phytochromes (Phy A-E) (Franklin & Quail, 2010; Rai et al., 2020; Yang et al., 2020). The *phot1 phot2* and *cry1 cry2* double mutants demonstrate an attenuated response to blue light, however these mutants respond differently at different fluence rates. Stomata of the *cry1 cry2* double mutant exhibit impaired responsiveness to blue light at fluence rates exceeding 1 μmol m^{-2} s^{-1}, while the *phot1 phot2* double mutant is unresponsive to blue light at fluence rates lower than 5 μmol m^{-2} s^{-1} and shows impaired response at fluence rates higher than 5 μmol m^{-2} s^{-1} (Kinoshita et al., 2001; Mao et al., 2005; Takemiya & Shimazaki, 2016). In contrast, stomata of the *cry1 cry2 phot1 phot2* quadruple mutant hardly open under blue light at any fluence rates. This indicates that CRY1/2 and PHOT1/2 act additively in mediating blue light stomatal opening (Mao et al., 2005). In addition, phyB was shown to be involved in red light-mediated stomatal opening, while the roles of other phytochromes in this process are still unknown (Wang et al., 2010; Yang et al., 2020).

PHOT1 and PHOT2 are light-activated protein kinases that phosphorylate the protein kinase BLUE LIGHT SIGNALING1 (BLUS1). Phosphorylation of BLUS1 leads to the activation of type 1 PROTEIN PHOSPHATASE 1 (PP1) and PP1 REGULATORY SUBUNIT2-LIKE PROTEIN1 (PRSL1). These reactions eventually activate H^+-ATPase pumps, leading to stomatal opening (Elhaddad et al., 2014; Takemiya & Shimazaki, 2016; Matthews et al., 2020) (Figure 6). Stomata in the *ost2* mutants with constitutively active AHA1 do not respond to blue light and darkness (Costa et al., 2015; Yamauchi et al., 2016).

Increasing concentrations of sugars in guard cells contribute to the elevation of turgor pressure during stomatal opening under light. It was shown that starch degrades in guard cells illuminated with blue light (Santelia & Lawson, 2016; Flütsch & Santelia, 2021). Glucan hydrolases α-AMYLASE3 (AMY3) and β-AMYLASE1 (BAM1) mediate starch degradation upon illumination. The *amy3 bam1* double mutants open their stomata more slowly and to a lesser extent under blue light (Flütsch, et al., 2020b). STP1 and STP4, the main hexose sugar transporters in the guard cells, contribute to stomatal opening under light. In the *stp1 stp4* double mutants, light-induced stomatal opening is inhibited, leading to an impairment in CO_2 assimilation and biomass production (Flütsch, et al., 2020a). It should be noted that roles of organic metabolites, including glucose and sucrose, in stomatal opening are still not fully understood (Santelia & Lawson, 2016; Flütsch & Santelia, 2021).

The mechanism of stomatal closure induced by light-to-darkness transition is more complex. It might include unknown specific signaling events as well as deactivation of red and blue light signaling and an increase in C_i due to inactive photosynthesis and CO_2 production by respiration (Pridgeon & Hetherington, 2021). In 2015, the isolation of 5 mutants named *open all night long 1* to *5* (*opal 1* to 5) defective in stomatal closure during nighttime was reported (Costa et al., 2015). In contrast to other mutants with disrupted darkness-induced stomatal closure, the *opal* mutants close their stomata normally in response to high CO_2 and ABA. Later in 2017, it was reported that the phenotype of the *opal5* mutant was caused by a mutation in *At5g18410*, encoding the PIR/SRA1/KLK subunit of the Arabidopsis SCAR/WAVE complex that controls actin cytoskeleton reorganization (Isner et al., 2017). Other studies have also demonstrated that reorganization of guard cell actin filaments is involved in stomatal closure in response to darkness and other stimuli. The *high sugar responses 3* (*hsr3*) mutants demonstrate stomatal insensitivity to light-to-dark transition and ABA. The *hsr3* mutations affect ACTIN RELATED PROTEIN C2 (ARPC2), which is one of the subunits of the ARP2/3 regulating actin remodelling (Jiang et al., 2012). The *constitutive photomorphogenic 1* (*cop1*) mutants have constitutively open stomata in darkness, which are not responsive to blue and red light (Mao et al., 2005). COP1 is a RING-finger-type ubiquitin E3 ligase that functions downstream of CRY1/2 and PHOT1/2 and represses stomatal opening. COP1 is essential for the reorganization of tubulin, which is required for stomatal closure. The *cop1* mutation is impaired in the activation of S-type anion channels (Khanna et al., 2014).

1.4.3 Low humidity-induced stomatal closure

One of the aspects of climate change is the decrease of relative air humidity in the atmosphere due to increased temperature (Vicente-Serrano et al., 2018). It leads to an increase in vapor pressure deficit (VPD), which is a difference between water-saturated vapor pressure at a given temperature and the actual water vapor pressure. Since an increased VPD leads to high transpiration in plants, stomatal closure is initiated to reduce excessive water loss. Despite extensive studies, it is still not known in all detail how guard cells sense changes in VPD (Pantin & Blatt, 2018; Grossiord et al., 2020; Jalakas et al., 2021). It should be noted that a rapid increase in VPD initially causes transient stomatal opening, also called "wrong way response", followed by stomatal closure. During the first minutes after an abrupt increase in VPD, epidermal cells rapidly lose water and turgor; this reduces their pressure on guard cells, leading to transient stomatal opening (Peak & Mott, 2011). High VPD-induced stomatal closure can involve hydroactive and hydropassive components (Peak & Mott, 2011; Buckley, 2016). The hydropassive mechanism suggests that guard cells in high VPD passively lose water and this results in a reduction of their turgor pressure and stomatal closure. Hydroactive regulation implies that high VPD activates signaling pathways in guard cells, which control ion channels (Pantin & Blatt, 2018; Hsu, et al., 2021a; Hsu, et al., 2021b; Jalakas et al., 2021).

A decrease in atmospheric humidity accompanied by enhanced water loss leads to conditions similar to drought stress. Therefore, high VPD-triggered stomatal closure is considered to be connected to ABA signaling (Sussmilch et al., 2017). It was reported that low humidity might induce ABA biosynthesis and increase the concentration of ABA in guard cells (Bauer et al., 2013; Sussmilch et al., 2017). The sextuple ABA receptor mutant *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* demonstrates a delayed stomatal response to high VPD (Merilo et al., 2013, 2018). However, a transgenic guard-cell-specific ABA-insensitive line with the *abi1-1* mutation demonstrates a reduction in stomatal conductance under high VPD, suggesting a minor role of ABA in fast VPD-induced stomatal closure (Yaaran et al., 2019). Initially, a genetic screen employing thermal imaging under low air humidity led to the identification of ABA biosynthesis and signaling mutants (*aba2-13* and *ost1-4*) (Xie et al., 2006). However, other studies showed that ABA deficiency does not affect stomatal response to humidity treatment (Assmann et al., 2000; Merilo et al., 2013, 2018). The ABA biosynthesis mutants *nced3 nced5* and *aba2-11* demonstrate a lack of the transient opening a short time upon an abrupt increase in VPD ("wrong way response") (Merilo et al., 2018; Buckley, 2019; Jalakas et al., 2021). ABA biosynthesis mutants might retain stomatal sensitivity to high VPD due to remaining trace ABA levels and/or due to the hydropassive mechanism. At the same time, the ABA-insensitive *ost1* mutants demonstrate a severely impaired stomatal response to high VPD (Merilo et al., 2018; Jalakas et al., 2021). Another ABA-insensitive mutant *ghr1-3* demonstrates only partially impaired stomatal closure in response to high VPD (Hsu, et al., 2021b). It can be explained by the findings showing that OST1 can be

activated by the B3-family Raf-like MAP Kinase Kinase Kinases (M3Ks), playing a role in stomatal closure induced by high VPD. The B3-family Raf-like M3Ks (M3Kδ1/RAF3, M3Kδ3/EDR1/RAF2, M3Kδ5/RAF6, M3Kδ6/SIS8/RAF5, M3Kδ7/RAF4, and CTR1/RAF1) are expressed in guard cells (Katsuta et al., 2020; Hsu, et al., 2021b). A central role in stomatal VPD response play M3Kδ5 and M3Kδ6, which were shown to activate OST1/SnRK2.6 in Arabidopsis mesophyll protoplasts. The Raf-like kinase *m3kδ1/δ5/δ6/δ7* quadruple mutant demonstrates slower stomatal response to elevated VPD treatment (Hsu, et al., 2021b) (Figure 6).

Although mutants lacking the anion channel SLAC1 maintain delayed stomatal responsiveness to high VPD, the triple *quac1-1 slac1-3 slah3-1* mutant demonstrated almost complete stomatal insensitivity to high VPD, suggesting that activation of both R- and S-type anion channels is required in dry air (Jalakas et al., 2021).

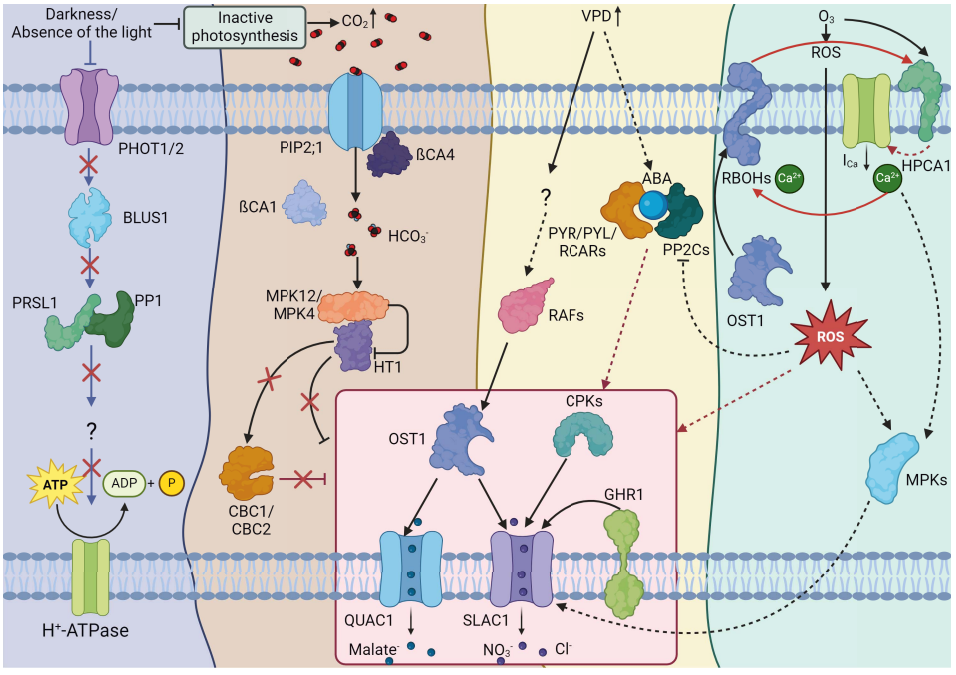

Figure 6. The simplified model of guard cell signaling in response to environmental stimuli. **Darkness**. In the presence of blue light, the photoreceptors PHOT1 and PHOT2 phosphorylate the protein kinase BLUS1, leading to the activation of PP1 and PRSL1. These events upregulate plasma membrane H^+-ATPases, leading to stomatal opening. In darkness, this signaling pathway is not active, promoting stomatal closure. At the same time, photosynthesis is not functioning, resulting in an increase in C_i levels and the activation of high CO_2 signaling. The purpura color indicates the inactive light opening pathway in darkness. **Elevated CO_2** leads to an enhanced influx of CO_2 into the guard cells via the aquaporin PIP2;1. ßCA1 and ßCA4 catalyze CO_2 conversion to bicarbonate (HCO_3^-) that acts as an intercellular signaling molecules. HCO_3^- promotes the formation of the complex between MPK12/MPK4 and HT1, leading to the inhibition of HT1 and

the activation of the downstream signaling components, including OST1, GHR1, and SLAC1, leading to stomatal closure. When C_i is low, HT1 is active and phosphorylates CBC1/CBC2, leading to inhibition/activation of complex signaling components, inducing stomatal opening. The brown color represents high CO_2 signaling in guard cells. **High VPD.** Sensing high VPD by plants is not yet fully understood. Increased VPD induces stomatal closure via OST1 activation, which can be promoted by Raf-like kinases or ABA signaling. A prolonged exposure to elevated VPD might induce ABA synthesis and activate ABA signaling, including PYR/PYL/RCAR, PP2C, OST1, GHR1 and CPKs. The yellow color represents high VPD signaling in guard cells. **O_3.** Ozone induces an increase in apoplastic ROS, which can be sensed by some proteins, potentially including HPCA1. Exogenous ROS amplifies production of apoplastic ROS. Accumulation of ROS within the apoplast triggers a rapid increase in cytosolic Ca^{2+}, which contributes to stomatal closure. OST1 regulates the activity of RBOHF. CPKs and MPKs contribute to stomatal closure mediated by ROS; however, the exact mechanism has not been fully identified. The green color represents signaling triggered by ozone in guard cells. The dotted lines represent possible signaling pathways, while the solid lines show the known signaling events in guard cells. The red arrows show activation or deactivation of OST1, GHR1, SLAC1, and QUAC1. Created with BioRender.com.

1.4.4 Ozone-mediated guard cell regulation

Ozone is a strong oxidant that is harmful to plants and animals and negatively affects crop yields. A prolonged exposure to high ozone concentrations leads to photosynthesis depression and triggers cell death in plants, which can be observed as collapsed leaves (Vainonen & Kangasjärvi, 2015). Ozone enters leaves through stomata and immediately degrades into ROS that activate multiple signaling cascades in the apoplast. Due to this, ozone is used as a tool to study apoplastic ROS signaling in plants. Since ozone uptake depends on stomatal function, ozone was successfully used in genetic screens for Arabidopsis stomatal mutants. For example, SLAC1, GHR1, and dominant HT1 mutations were identified in ozone mutant screens (Vahisalu et al., 2008; Sierla et al., 2018). Activation of apoplastic ROS signaling by ozone leads to fast stomatal closure, which can be observed as a fast reduction of stomatal conductance (Kollist et al., 2007; Vahisalu et al., 2010).

Plant cells are capable of producing ROS in various cellular compartments. ROS include hydroxyl radical ($HO^{\bullet}$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and various forms of organic and inorganic peroxides (Sierla et al., 2016; Waszczak et al., 2018). Since ROS are extremely reactive molecules and can cause direct oxidative damage to DNA, proteins, and lipids, plants have to balance ROS production and accumulation (Castro et al., 2021). A rapid production of large amounts of ROS, termed as oxidative burst, can be triggered in response to plant infections as a part of plant defense mechanisms. Ozone-induced ROS burst mimics ROS accumulation during a pathogen attack (Mittler et al., 2022). ROS are involved in the regulation of various other processes in plants, including stomatal closure. It has been demonstrated that ROS

play a role in regulation of several plant hormone signaling, particularly ABA, JA, SA and ET (Hasan et al., 2021). Plant cells express specialized enzymes that produce ROS involved in signaling in plants. Respiratory Burst Oxidase Homologues (RBOHs) are calcium-dependent NADPH oxidases that control extracellular ROS. Other apoplastic enzymes, including SHAM-sensitive peroxidases, can be also involved in the ROS formation (Sierla et al., 2016; Waszczak et al., 2018). Aquaporins can facilitate ROS transportation across plasma membrane. PIP1,4 and PIP2,1 mediate an influx of apoplastic H_2O_2 to the cytosol during immune signaling and ABA-dependent stomatal closure (Tian et al., 2016; Rodrigues et al., 2017).

Although many ozone-sensitive mutants have been identified, the exact mechanism of ozone or ROS perception still remains obscure. A possible receptor for ROS/ozone is HYDROGEN-PEROXIDE-INDUCED CA^{2+} INCREASES 1 (HPCA1), that is a receptor-like protein kinase involved in ROS sensing (Wu et al., 2020). The *hpca1* mutants demonstrate lower increases in cytosolic Ca^{2+} concentration in response to H_2O_2 as well as impaired H_2O_2-triggered stomatal closure. OST1 and GHR1 are involved in guard cell signaling triggered by ABA and other environmental stimuli and are also required for ROS/ozone-triggered stomatal closure (Mustilli et al., 2002; Kwak et al., 2003; Shang et al., 2016; Sierla et al., 2018). CPKs, in particular CPK3, CPK6, CPK21, and CPK23, are also involved in ozone-induced stomatal closure (Mori et al., 2006; Munemasa, et al., 2011; Merilo et al., 2013; Maierhofer et al., 2014).

In plants, various biotic and abiotic stresses activate MPK signaling cascades. Many MPKs are highly expressed in guard cells (Jammes et al., 2009). The MPK cascades consist of three subsequently acting protein kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and MAP kinase (MAPK) (Davis, 2000; Schaeffer & Weber, 1999). It was shown that exposure to ozone leads to the activation of MPKs and increases their gene expression (Hasan et al., 2021). Ozone-induced activation of MPK3 and MPK6 (Ahlfors et al., 2004) plays a role in plant sensitivity to ozone as MPK6-silenced plants and *mpk3* loss-of-function lines develop more ozone-induced lesions than wild-type plants (Miles et al., 2005). However, in another study, sensitivity to ozone in the *mpk6* mutant was not detected (Leppälä et al., 2022). Cold and salt stress activate the MEKK1-MKK1/2-MPK4/6 signaling cascade, which also interacts with ROS. MEKK1 is activated and stabilized by H_2O_2, and MPK4/6 are upregulated by ROS and biotic stresses (Teige et al., 2004; Vainonen & Kangasjärvi, 2015). In response to pathogen attack, MKK4 is activated by MEKK1 (Suarez-Rodriguez et al., 2007). Moreover, in *Brassica napus,* H_2O_2 activates MPK4 that positively regulates ROS production (Zhang et al., 2015) (Figure 6).

Some phytohormones regulate ROS-dependent signaling and control gene expression in response to ozone-induced oxidative stress (Xia et al., 2015). Thus, it was shown that ET promotes O_3-induced cell death, while JA prevents ozone-triggered lesions (Rao et al., 2002; Tuominen et al., 2004; Xu et al., 2015). An exposure to ozone can increase the total level of SA (Marchica et al., 2022), protecting plants against the development of lesions after ozone treatments (Rao &

Davis, 1999). Ozone might increase ABA concentration through a direct oxidation of ABA precursors (McAdam et al., 2017). Sensing of ozone by guard cells depends on ABA signaling, as evidenced by impaired stomatal closure induced by ozone in the *pyr1 pyl1 pyl2 pyl4* mutant in gas-exchange experiments (Merilo et al., 2013).

2. AIMS

The role of ABA in promoting stomatal closure is well-known. Many publications demonstrated that ABA biosynthesis and signaling are involved in controlling stomatal conductance and stomatal reactions to changing CO_2, darkness, low air humidity, and ozone. However, contributions of other hormones to modulating stomatal conductance are studied significantly less. The aim of the current thesis was to study the roles of plant hormones, such as JA, SA, ET, and SLs, in controlling stomatal functioning of intact plants using a whole rosette gas-exchange system.

The specific aims of the research were as follows:

- estimate impacts of hormones on controlling whole-plant stomatal conductance;
- study a possible connection between SLs and ABA signaling in the regulation of stomatal conductance;
- examine the roles of JA, SA, and ET signaling and biosynthesis in stomatal responses to various stimuli promoting stomatal closure, such as elevated CO_2, darkness, ABA spray, low relative air humidity, and ozone pulse.

3. MATERIALS AND METHODS

The detailed experimental procedure used for preparing articles I–III described in the Materials and Methods sections of the corresponding publications. The main methods and methodology data are described briefly below.

Plants growth

The soil for growing plants contained peat and vermiculite in the ratio of 4:3 (v:v).

Arabidopsis plants for gas exchange measurements were grown in specialized pots covered with glass plates with holes in the middle. A few seeds were put on soil in each pot and after germination one plant was allowed to grow through the hole in the glass plate. The holes in the glass plate were waxed before the experiments to prevent water evaporation from the soil. Arabidopsis plants were grown in the growth chambers (AR-66LX and AR-22L, Percival Scientific, IA, USA) with the 12 h/12 h (or 10 h/14 h for ET mutants) light/darkness photoperiod, 23/18 °C day/night temperature, 150 µmol m^{-2} s^{-1} (or 250 µmol m^{-2} s^{-1} for ET mutants) light intensity and 70% relative air humidity. Arabidopsis plants were watered twice a week.

Tomato plants were grown in the growth chambers with 12 h/12 h light to dark photoperiod, 23/18 °C day to night temperature, 250 µmol m^{-2} s^{-1} light intensity and 70% relative air humidity. Plants were watered twice a week. The 3 to 4 weeks-old plants were used for gas-exchange measurements.

Mutants used in this study

Table 1 contains a brief description of the mutants used in this study. All Arabidopsis mutants are in the Col-0 genetic background. The tomato *jai1-1* mutant is in the Castlemart genetic background.

Table 1. Description of the mutants used in this study:

Genotype	Mutation	Description	Reference
JA biosynthesis and signaling			
dde2-2 (delayed-dehiscence 2)	transposon insertion	defective in ALLENE OXIDE SYNTHASE (AOS) gene, has non-functional JA biosynthesis	(Von Malek et al., 2002)
coi1-16 (coronatine insensitive 1)	ethylmethane sulfonate (EMS)-induced	defective in JA receptor	(Ellis & Turner, 2002)
jai1-1 (jasmonic acid-insensitive-1)	fast-neutron-induced deletion in tomato (*S. lycopersicum*)	does not express the JAI1 protein, which is homologous to the Arabidopsis COI1	(Li et al., 2001, 2004b)
SA biosynthesis and signaling			
sid2-1 (salicylic acid induction-deficient 1)	EMS-induced	defective in isochorismate synthase in the ICS pathway of SA biosynthesis	(Nawrath & Métraux, 1999)
npr1-1 (nonexpressor of pathogenesis-related genes 1)	EMS-induced	defective in SA signaling	(Cao et al., 1997)
ET biosynthesis and signaling			
ein2-1 (ethylene insensitive 2)	EMS-induced	defective in ET signaling	(Guzmán & Ecker, 1990)
etr1-6 (ethylene response 1)	EMS-induced	defective in ET signaling	(Hua & Meyerowitz, 1998)
etr2-3 (ethylene response 2)	T-DNA insertion	defective in ET perception	(Hua & Meyerowitz, 1998)
etr2-3 ein4-4 ers2-3 (ethylene response 2, ethylene insensitive 4, ethylene response sensor 2)	*etr2-3* and *ein4-4* – EMS-induced; *ers2-3* – T-DNA insertion	loss-of-function ET receptor triple mutant	(Hua & Meyerowitz, 1998)
cs16651	T-DNA insertions, amiRNA silencing	ACC-synthase octuple mutant: *acs1-1acs2-1acs4-1acs5-2acs6-1acs7-1acs9-1* with silenced *ACS8* and *ACS11* by amiRNA	(Tsuchisaka et al., 2009)

Genotype	Mutation	Description	Reference
SLs signaling			
max2-4 (more axillary branches 2)	T-DNA insertion, SALK_028336	defective in SL perception	(Bennett et al., 2006)
ABA biosynthesis and signaling			
aba2-11 (ABA deficient 2)	small deletion	disruption in convention of xanthoxin to ABA-aldehyde	(González-Guzmán et al., 2002)
ost1-3 (open stomata 1)	T-DNA insertion, SALK_008068	knockout mutation in ABA-activated protein kinase OST1	(Yoshida et al., 2002)
ghr1-3 (guard cell hydrogen peroxide-resistant 1)	T-DNA insertion, GK_760C07	knockout of the leucine-rich repeat receptor-like protein GHR1 involved in activation of SLAC1	(Hua et al., 2012)
JA and SA double mutants			
coi1-16 sid2-1	EMS-induced	defective in JA signaling and SA biosynthesis	(Xu et al., 2015)
coi1-16 npr1-1	EMS-induced	defective in JA and SA signaling	(Vuorinen et al., 2021)
JA, SA and ET triple mutants			
dde2-2 ein2-1 sid2-2	*dde2-2* – transposon insertion; *ein2-1* and *sid2-1* – EMS-induced	defective in JA biosynthesis, ET signaling, and SA biosynthesis	(Tsuda et al., 2009)
coi1-16 ein2-1 sid2-1	EMS-induced	defective in JA signaling, ET signaling and, SA biosynthesis	(Xu et al., 2015)

Gas-exchange measurements

A custom-made eight-chamber gas-exchange device was used for gas-exchange measurements in Arabidopsis plants (Kollist et al., 2007). Plants were stabilized for at least 1 h before experiments in the device under the following conditions: 150 μmol m^{-2} s^{-1} (or 250 μmol m^{-2} s^{-1} for ET mutants) light intensity, 70% relative air humidity, and ~400 μL L^{-1} CO_2. After pre-incubation of the plants, stimuli of interest were applied: high concentration of CO_2 (~800 μL L^{-1}), darkness, decreased air humidity (about 30% in relative values), 3-min ozone pulse, or spraying with the solutions of ABA, MeJA, and SA. For the mock treatments, the solutions containing only ethanol and Silwet L-77 were sprayed on plants. Changes in stomatal conductance were monitored after treatment applications.

A four-chamber gas-exchange system was used for measurements in tomato plants (Hõrak et al., 2017). Plants were stabilized 1 h before experiments in the sealed chambers with 250 μmol m^{-2} s^{-1} light intensity, 70% relative air humidity and ~400 μL L^{-1} CO_2. After stabilization of plants, high (~800 μL L^{-1}) or low concentrations of CO_2 (~100 μL L^{-1}) were applied and changes in stomatal conductance were monitored.

Stomatal conductance was calculated as described in Kollist et al. 2007.

Stomatal density measurements

The 10^{th} leaves of 8-week-old plants were collected, and the abaxial side of the leaves was covered with a dental resin. The solidified dental resin containing the leaf replica was then covered with nail polish. After solidification, the nail polish was covered with transparent scotch tape and transferred on a microscope glass slide. The samples were analyzed using a microscope. For each line, 10 samples were analyzed. Stomatal density was calculated by dividing the number of stomata by the examined area and expressed in stomata mm^{-2}.

Data analysis

All experiments were repeated at least three times with similar results. To characterize stomatal movements in response to various factors promoting stomatal closure, additional parameters were calculated. Changes in stomatal conductance were calculated as a difference between stomatal conductance values before and at a certain time after a stimulus was applied. The initial rates of changes in stomatal conductance were calculated as linear slopes of the curve, reflecting short-time changes in stomatal conductance after the application of a treatment.

The paired Student's t-test (STATISTICA 7.1) was used to estimate the effect of hormones on stomatal aperture and stomatal conductance. ANOVA (one-way analysis of variance) was used to detect any statistically significant differences between groups in the experiments. HSD test (Tukey's honestly significant difference) was used to compare multiple groups of samples. Dunnett's post hoc test was used to estimate the difference between wild-type plants and mutant lines. All effects were considered significant at $P \leq 0.05$.

4. RESULTS AND DISCUSSION

4.1 Hormonal control of stomatal conductance

4.1.1 The impacts of JA, SA, and ET in the regulation of steady-state stomatal conductance

Steady-state stomatal conductance is influenced by various environmental and inner factors. Hormones are considered to play a significant role in this process. For example, an accumulation of ABA in plants leads to a dramatic reduction in stomatal conductance (Franks & Farquhar, 2001; Pantin et al., 2013). During drought stress, plants synthesize ABA, which initiates the signaling cascade leading to the efflux of water from guard cells and the subsequent stomatal closure, reducing water loss from plants via evaporation. A disruption in ABA biosynthesis or signaling significantly enhances steady-state stomatal conductance even under unstressed conditions (Merilo et al., 2013, 2018; Jalakas et al., 2021). Other plant hormones can be also involved in the control of stomatal apertures regulating the fluxes of water vapor and CO_2. JA and SA are the well-known plant defense hormones, governing the plant defense against mechanical damages and pathogen infections, respectively. Some studies demonstrated an induction of stomatal closure by these hormones (Munemasa et al., 2007; Khokon et al., 2011; Geng et al., 2016). ET also plays a crucial role in plant immunity and is involved in plant-pathogen interaction (Ravanbakhsh et al., 2018; Shekhawat et al., 2023).

In our study, we investigated JA, SA, and ET role in controlling steady-state stomatal conductance under standard conditions. In order to address this question, we compared stomatal conductance in wild-type plants with that of plants carrying mutations in JA, SA, and ET biosynthesis and signaling to reveal possible effects of these hormones on steady-state stomatal conductance. We measured whole-plant stomatal conductance in the tested mutants using a custom-made gas-exchange device under the conditions which either promoted stomatal opening or stomatal closure (Kollist et al., 2007) (Figure 7). The mutants with disrupted JA, SA and ET biosynthesis and signaling have been well-characterized and published before (Table 1).

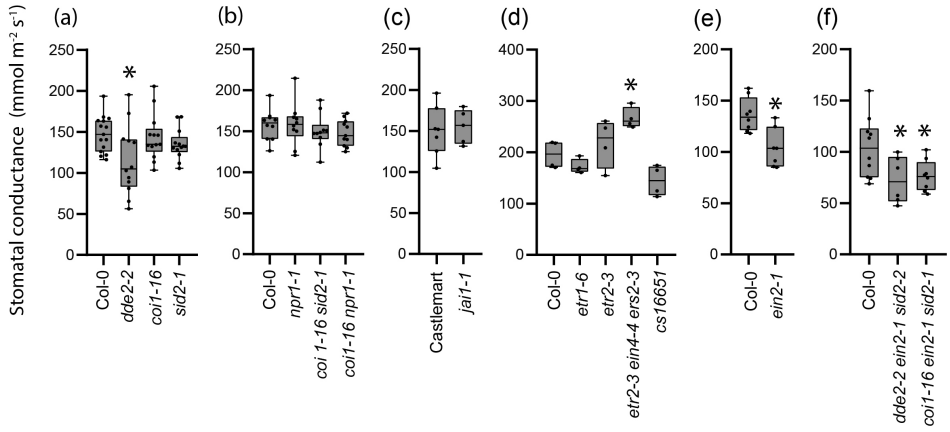

Figure 7. Steady-state stomatal conductance of the JA, SA, and ET biosynthesis and signaling mutants (modified from ref. II). Whole-plant stomatal conductance was monitored in Arabidopsis plants at the age of 3–4 weeks (a, b, d, e, f). Stomatal conductance was also measured in the top leaves of 3–4-weeks-old *Solanum lycopersicum* (tomato) plants (c). Plants were grown under a light intensity of 150 µmol m^{-2} s^{-1} (a, b, e, f) or 250 µmol m^{-2} s^{-1} (c, d). One of three experiments is shown for ET mutants (d). The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots whereas the whiskers are the minimum and maximum values (n = 4–10). Asterisks show significant differences between mutants and the wild type (one-way ANOVA followed by Dunnett's post hoc test; $P < 0.05$).

Stomatal conductance in the JA signaling Arabidopsis *coi1-16* and tomato *jai1-1* mutants was not different from that in the wild-type plants (Figure 7a, c). However, the JA biosynthesis *dde2-2* single mutant demonstrated a 20% reduction in stomatal conductance compared to the wild-type plants (Figure 7a). Interestingly, it was previously reported that JA negatively regulates stomatal development in Arabidopsis cotyledons, potentially controlling steady-state stomatal conductance. In this publication, cotyledons of the *coi1-16* mutant had a higher stomatal index and three times more clustered stomata than wild-type plants (Han et al., 2018). If wild-type seedlings were germinated on MeJA-containing media, the stomatal index in cotyledons was lowered in comparison with plants germinated on the media without adding MeJA (Han et al., 2018). It is not clear how JA could affect stomatal development in 3–4 weeks-old plants that were employed in this study. It is possible that stomatal aperture in *dde2-2* was reduced due to the low level of oxylipins, the precursors of JA in its biosynthetic pathway, that activate signaling pathways independent of COI1 (Savchenko et al., 2014). Further studies are required to reveal potential connections between the *dde2-2* mutation and stomatal regulation.

The levels of stomatal conductance in the *sid2-1* and *npr1-1* single mutants did not differ significantly from that in the wild-type plants (Figure 7a, b), indicating that a disruption in SA biosynthesis or signaling did not affect steady-state stomatal conductance. However, it was previously noticed that SA over-accumulating mutants exhibited decreased stomatal aperture and increased resistance to

drought. Thus, this hormone may have a role in the regulation of stomatal conductance upon activation of its biosynthesis (Miura et al., 2013).

Based on our gas-exchange measurements, stomatal conductance in ET single mutants (*etr1-6* and *etr2-3*) was not statistically different from the control plants. However, we observed that the triple mutant *etr2-3 ein4-4 ers2-3* lacking the ethylene receptors had 35% elevated stomatal conductance compared to the wild-type plants in two of three experiments (Figure 7d). In one of the experiments, stomatal conductance in *etr2-3 ein4-4 ers2-3* was elevated, but not significantly. The *ein2-1* single mutant demonstrates decreased stomatal conductance compared to wild-type plants (Figure 7e). The ethylene biosynthesis octuple ACC synthase mutant *cs16651* mutant had slightly lower stomatal conductance than the wild-type plants, although the difference was not statistically significant. The ET receptors have overlapping functions and a high level of redundancy. When one receptor is inactive, other receptors are potentially able to substitute its function (Theologis, 1998; Cancel & Larsen, 2002; Qu et al., 2007). At the same time, ET signaling is quite sophisticated as the ET receptors have independent roles, which can potentially explain the variations observed in stomatal conductance among various ET receptor mutants (Wilson et al., 2014; Bakshi et al., 2015, 2018). Still, our results indicate that the ET receptors ETR2, EIN4, and ERS2 as well as EIN2 and ET biosynthesis might regulate stomatal conductance, indicating that ET is a positive regulator of stomatal conductance. Some publications have suggested that ET is involved in stomatal opening (Merritt et al., 2001; Tanaka et al., 2005, 2006; Iqbal et al., 2011). Further research should clarify roles of individual ET receptors in controlling whole-plant steady-state stomatal conductance.

In order to investigate possible interactions between JA, SA, and ET in the regulation of stomatal conductance, we tested double and triple mutants impaired in functioning these hormones. The double JA and SA mutants (*coi1-16 sid2-1* and *coi1-16 npr1-1)* demonstrated the wild-type levels of stomatal conductance (Figure 7b). However, stomatal conductance in the triple mutants *dde2-2 ein2-1 sid2-2* and *coi1-16 ein2-1 sid2-1* was lower by around 30% in comparison with wild-type plants (Figure 7f). Thus, adding the *ein2-1* mutation to the defects in JA and SA biosynthesis and signaling resulted in the reduced level of stomatal conductance. Moreover, the *dde2-2 ein2-1 sid2-2* mutant also contains the *dde2-2* mutation, which alone was able to decrease stomatal conductance, while *sid2-1* demonstrated wild-type stomatal conductance. Decreased stomatal conductance in the triple mutants reflects the complex interactions between the hormonal signaling triggered by JA, SA, and ET. The antagonism crosstalk between SA and JA is well documented, showing that one hormone blocks the production and transcription of another (Doares et al., 1995; Spoel et al., 2003; Van der Does et al., 2013; Caarls et al., 2015). However, some studies suggested that the relation between JA and SA is not only antagonistic. Researchers demonstrated that the defense system activated by both JA and SA is involved in protection against pathogens (Yamada et al., 2012; Tamaoki et al., 2013). It was also reported that SA receptors can promote JA signaling (Liu et al., 2016). It is possible that an

outcome from the interaction between JA and SA could depend on the relative concentrations of these two hormones (Mur et al., 2006). Moreover, JA and ET interact in the regulation of development, growth, and defense response (Lorenzo et al., 2003; Adams & Turner, 2010; Song et al., 2014; J. Kim et al., 2015). Experiments using epidermal peels showed that ET signaling inhibits JA signaling in the guard cells (Munemasa et al., 2019). Additional studies are required to reveal which aspects of stomatal regulation are affected in the triple mutants: stomatal aperture or stomatal development.

Taking together, our results indicate that the disruptions in JA, SA and ET biosynthesis and signaling did not dramatically affect steady-state stomatal conductance. However, we noticed some effects related to specific mutations in JA biosynthesis and ET signaling, which might influence steady-state stomatal conductance. Our data also indicate a potential interaction between JA, SA, and ET in regulating stomatal conductance.

4.1.2 MAX2 controls stomatal conductance independently of ABA biosynthesis and signaling

SLs are plant hormones that regulate plant-environment interactions, particularly the plant defense against pathogenic bacteria. It has been proposed that the signaling triggered by SLs regulates stomatal functioning by inducing stomatal closure to protect plants against pathogens, although the exact mechanism is still not well understood. Mutants with impaired SL signaling have more open stomata, leading to enhanced ozone sensitivity and susceptibility to pathogens (Piisilä et al., 2015).

We decided to test the hypothesis that SL and ABA activate the same signaling pathways in guard cells. To address this question, the *max2-4* mutant, lacking the F-box protein MAX2 involved in SL signaling, was crossed with the ABA-insensitive *ghr1-3* and *ost1-3* mutants and the ABA biosynthesis mutant *aba2-11* (González-Guzmán et al., 2002; Yoshida et al., 2002; Sierla et al., 2018). Whole-rosette stomatal conductance was measured in the single and double mutants at the age of 3–4 weeks by using a custom-made gas-exchange system (Kollist et al., 2007). As reported previously by Piisilä et al. (2015), the *max2-4* mutant demonstrated twice higher stomatal conductance than wild-type plants (Figure 8a). Similarly, ABA biosynthesis and signaling mutants had significantly enhanced stomatal conductance. The double mutants (*aba2-11 max2-4*, *ghr1-3 max2-4,* and *ost1-3 max2-4*) demonstrated higher stomatal conductance than the corresponding single mutants (Figure 8a), demonstrating the additive effect between the *max2-4* mutation and impaired ABA biosynthesis and signaling. These results allowed us to conclude that MAX2-dependent SL regulation of stomatal conductance was independent from ABA biosynthesis and signaling.

The control of stomatal conductance by ABA has been well-documented and confirmed in many ABA biosynthesis and signaling mutants. Thus, in the *aba2* mutants, the disrupted convention of xanthoxin to ABA-aldehyde resulted in

~20–25% of the wild-type ABA concentration (González-Guzmán et al., 2002) and dramatically enhanced stomatal conductance (Figure 8a) (Merilo et al., 2013, 2018). The *ost1-3* and *ghr1-3* mutants lacking SLAC1 activation by ABA show constantly enhanced stomatal conductance. In addition, ABA deficient and ABA receptor mutants frequently have increased stomatal density which also contributes to elevated stomatal conductance (Tanaka et al., 2013; Jalakas et al., 2018; Merilo et al., 2018). At the same time, the mechanism leading to enhanced stomatal conductance in *max2* is less understood, however, our results directly indicate that it does not depend on ABA level or signaling. To investigate whether the lack of MAX2 would affect stomatal development, we measured stomatal density in the *max2-4* mutant. We found that stomatal density in *max2-4* (approximately 78 stomata mm^{-2}) was not statistically different from stomatal density in the Col-0 plants (approximately 86 stomata mm^{-2}) (Figure 8b).

To sum up, MAX2-dependent SL signaling in the regulation of stomatal conductance is independent of ABA biosynthesis and signaling. At the same time, it does not influence stomatal density. Since *max2-4* demonstrates increased stomatal aperture (Piisilä et al., 2015) and unaffected stomatal density (Figure 8b), further studies should reveal how SLs regulate turgor pressure in guard cells.

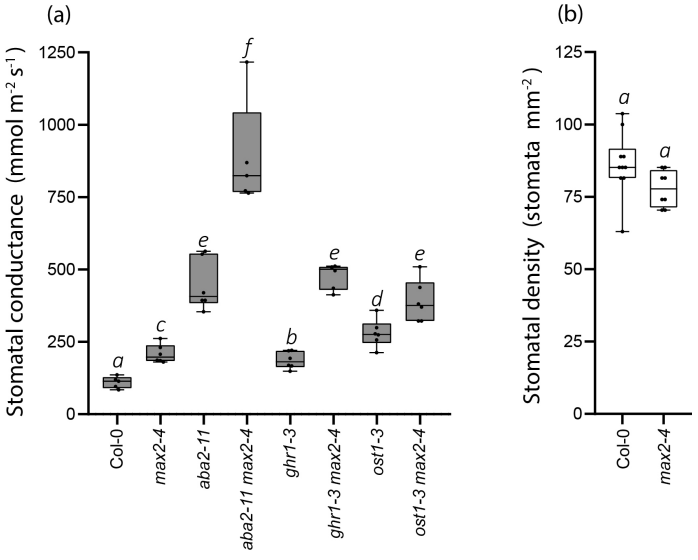

Figure 8. MAX2 regulates stomatal conductance independently of ABA and does not influence stomatal density (modified from ref I). Whole-rosette stomatal conductance of single and double mutants was measured at the age of 3–4 weeks (a). Stomatal density of *max2-4* and the wild-type plants was measured when the plants started to flower at the age of 8 weeks (b). The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots whereas the whiskers are the minimum and maximum values (n = 4–10). Different letters indicate statistically significant differences between groups (one-way ANOVA followed by Tukey HSD (a) and Dunnett's post hoc test (b); $P < 0.05$).

4.1.3 Stomata are significantly more sensitive to ABA than to MeJA and SA

Numerous studies demonstrated that JA and SA can induce stomatal closure in epidermal peels (Mori et al., 2001; Munemasa et al., 2007; Khokon et al., 2011; Geng et al., 2016). Our results showed that plants with reduced levels of SA and JA or impaired JA or SA signaling had only minor alterations in steady-state stomatal conductance, while impairments in ABA biosynthesis or signaling significantly enhanced stomatal conductance (Figures 7 and 8). We decided to address sensitivity of stomata to externally applied ABA, MeJA, and SA by spraying these hormones on intact Arabidopsis plants and monitoring stomatal conductance during 23 h with gas-exchange devices.

Spraying with 5 μM ABA induced fast and prolonged stomatal closure, which was still detectable 23 h after the treatment (II, Supplemental Fig. 9a). MeJA or SA were less efficient regarding triggering stomatal closure even in significantly higher concentrations (Figure 9b, c). Interestingly, stomatal conductance was slightly, but noticeably increased in response to the mock treatments, which could be explained by the brief wetting of leaves or the temporary increase in humidity in the chambers (Panchal et al., 2016; Yokoyama et al., 2019). Spraying MeJA in concentrations from 50 to 200 μM inhibited this type of stomatal opening induced by wetting/increased humidity and resulted in a weak stomatal closure. In 80 min, stomatal conductance was reduced by 5–10% of its initial level in the plants sprayed with MeJA (Figure 9b). Plants treated with SA in the concentration range from 200 to 1000 μM demonstrated the same profile of time-resolved stomatal conductance as the plants sprayed with the mock (Figure 9c).

Potentially, the differences between the hormonal treatments could be explained by inefficient uptake of the sprayed MeJA and SA by plant cells. To confirm that spraying plants with ABA, MeJA and SA led to a penetration of these hormones into the leaves, we measured transcript levels of hormone-responsive marker genes. The spraying with MeJA and SA led to the accumulation of the *JAZ1* and *WRKY38* transcripts, respectively (Figure 9d, e). Additionally, spraying with 5 μM ABA resulted in over a 100-fold increase in ABA-responsive HAI1 transcript ($P<0.01$ by paired t-test) (II, Supplemental Fig. 9f). The uptake of MeJA and SA by guard cells was confirmed by the increased expression levels of *JAZ1* and *WRKY38* in guard cell-enriched epidermal fractions (Figure 9f, g). These results suggest that sprayed MeJA and SA penetrated leaves and were taken up by guard cells, being efficient in the induction of biological effects, although efficient stomatal closure was not observed. According to the previous research, the estimated concentration of JA and SA in guard-cell-enriched epidermal peels were at the levels of approximately 9 pg mg^{-1} fresh weight (or 40 pmol g^{-1}) and 115 pg mg^{-1} fresh weight (or 0.83 nmol g^{-1}), respectively (David et al., 2020). Similar concentration ranges for JA and SA were detected in whole Arabidopsis leaves (Forcat et al., 2008; Pan et al., 2010). In our study, we used the concentrations of MeJA and SA solutions which were significantly higher than the levels

of these hormones in guard cells and were sufficient for studying their biological effects on guard cells.

As a complementary method of studying the effects of JA and SA on stomatal conductance, we measured stomatal apertures in epidermal peels after 3 h in the mock and hormonal solutions (Figure 9h). After incubation of the epidermal peels in 5 μM ABA, stomatal apertures were reduced 2.7-fold compared with the mock treatments. The solutions containing 400 and 1000 μM MeJA induced stomatal closure by 15% and 26%, respectively, compared with the mock treatments. Incubation of epidermal peels in the SA solutions induced detectable stomatal closure only when the SA with a concentration of 1000 μM was applied.

Our results indicate that JA and SA are significantly less efficient in triggering stomatal closure than ABA, which is a key hormone in the regulation of stomatal movements. Conflicting findings regarding the effectiveness of MeJA in inducing stomatal closure have been published. Savchenko et al. (2014) demonstrated that MeJA is less efficient in inducing stomatal closure compared to ABA and ODPA, the JA precursor (Savchenko et al., 2014). In another study, no MeJA-induced stomatal closure was observed (Montillet et al., 2013). However, other studies demonstrated efficient MeJA-triggered stomatal closure (Munemasa et al., 2007; Hossain et al., 2011). Similarly, the involvement of SA in promoting stomatal closure has been established in many studies (Mori et al., 2001; Khokon et al., 2011). Interestingly, a recent report indicated that an accumulation of SA under constant light was associated with stomatal opening and preventing pathogen-induced stomatal closure (Lajeunesse et al., 2023). Stomatal closure induced by MeJA and SA was mostly demonstrated in experiments using epidermal peels, while in this study we used intact plants and measured whole-plant stomatal conductance. It is also possible that growth conditions in various labs can influence guard cell regulation and stomatal sensitivity to hormones. Our results also indicate that MeJA and SA efficiently trigger stomatal closure in response to high hormone concentrations and longer incubation times, as can be achieved by soaking epidermal peels in hormonal solutions. Our results demonstrate that MeJA and SA are significantly less efficient in promoting stomatal closure than ABA.

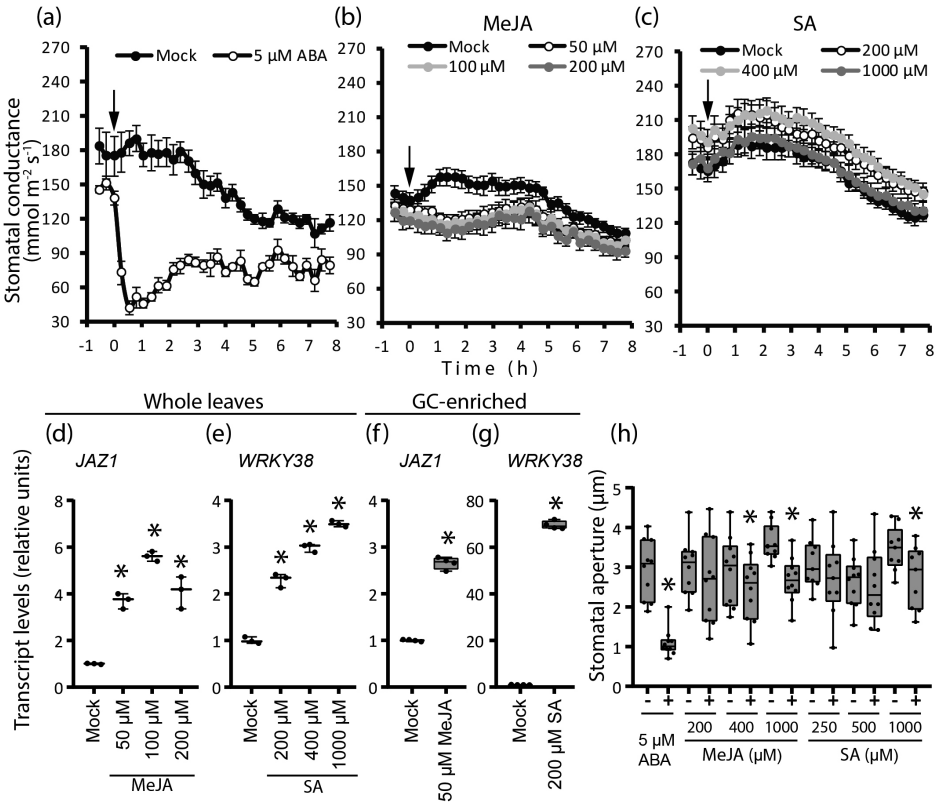

Figure 9. Effects of ABA, MeJA, and SA in promoting stomatal closure in Arabidopsis thaliana (copied from ref II). Arabidopsis plants (Col-0) at the age of 3–4 weeks were sprayed with ABA (a), MeJA (b), or SA (c), in various concentrations. Time courses are shown for stomatal conductance (average ± SE, n = 4 for ABA, n = 16–24 for MeJA and SA). Relative expression of *JAZ1* and *WRKY38* after MeJA (d) and SA (e) treatments, respectively, in plants treated as in (b) and (c) (n = 3). The same transcripts were quantified in guard-cell-enriched epidermal fractions (GC-enriched) collected from MeJA- and SA-treated plants (n = 4) (f and g). Asterisks show significant differences between mock and hormonal treatments (one-way ANOVA, followed by Dunnett's post hoc test; $P < 0.05$). (h) ABA demonstrates a significantly higher potency to induce stomatal closure in stomatal assays than MeJA and SA. Epidermal peels were collected from plants at the age of 4–5 weeks and incubated in stomatal opening buffer supplemented with mock (ethanol), ABA, MeJA, or SA. The width of stomatal apertures was measured after exposure for 3 h (n = 9–10 individual plants). The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots, whereas the whiskers are the minimum and maximum values. Asterisks show statistically significant differences between the mock (−) and the hormonal treatment (+) by repeated-measures ANOVA ($P < 0.05$).

4.2 Roles of hormones for stomatal regulation in response to environmental factors

As demonstrated here and in numerous studies before, ABA signaling is involved in stomatal regulation by environmental stimuli such as elevated CO_2, darkness and low air humidity (Chater et al., 2015; Gonzalez-Guzmann 2012; Merilo et al., 2013, 2018). Potential roles for other hormones, including JA, SA, and ET, controlling stomatal responses to environmental stimuli has not been comprehensively addressed. In order to resolve this question, we tested stomatal responses to stimuli promoting stomatal closure in the mutants defective in JA, SA and ET biosynthesis or signaling. The 3–4 weeks-old plants were incubated in a gas-exchange device and changes in stomatal conductance were monitored upon application of elevated CO_2, darkness, low humidity, ozone, or ABA spray.

4.2.1 CO_2- and light-induced stomatal movements

Light-dependent stomatal reactions are crucial for optimizing photosynthesis and minimizing unnecessary water loss from leaves. Stomata are able to recognize both light conditions and C_i, adjusting stomatal apertures for CO_2 uptake (Engineer et al., 2016; Matthews et al., 2020). Changing concentrations of atmospheric CO_2 influence C_i and also induce stomatal closure or opening. The previous studies performed in our lab and by other researchers showed that ABA signaling can modulate high CO_2- and darkness-induced stomatal closure. Thus, the mutants lacking ABA receptors demonstrated impaired stomatal closure in response to elevated CO_2 and darkness (Merilo et al., 2013; Chater et al., 2015). The protein kinase OST1 and receptor-like protein pseudokinase GHR1, being crucial for ABA signaling in guard cells, are also required for stomatal closure triggered by elevated CO_2 and darkness (Merilo et al., 2013; Sierla et al., 2018). To study the impacts of JA, SA, and ET on stomatal regulation by CO_2 and illumination conditions, we monitored whole-plant stomatal conductance in the mutants defective in biosynthesis and signaling of these hormones upon application of changing CO_2 or an artificial darkness period followed by plant recovery under the initial conditions.

The wild-type Arabidopsis plants closed their stomata in response to the transition from ambient (approximately 420 µl L^{-1}) CO_2 to elevated (approximately 800 µl L^{-1}) CO_2 (Figure 10) or from light to darkness (Figure 11). Both elevated CO_2 and darkness treatments led to a decrease in stomatal conductance in wild-type plants by approximately 40% and 50% of the initial level, respectively (Figure 10 and 11). The subsequent application of ambient CO_2 or re-illumination led to stomatal re-opening and to an increase in stomatal conductance to almost the initial values after approximately 30 min. The same treatments induced similar changes in stomatal movements in all the studied JA and SA biosynthesis and signaling mutants (Figure 10 and 11). Thus, pronounced stomatal closure in response to elevated CO_2 and darkness was observed in the *dde2-2*, *coi1-16, npr1-1*,

and *sid2-1* mutants. The changes in stomatal conductance, triggered by the application of these treatments, were highly similar to those observed in the wild-type plants (Figure 10 and 11). The subsequent application of the initial conditions (the ambient concentration of CO_2 or re-illumination) led to stomatal re-opening in the JA and SA mutants, resembling that in the wild-type plants. Surprisingly, the *sid2-1* mutant demonstrated slightly slower stomatal re-opening during the recovery period under both ambient CO_2 and re-illumination (the initial slopes of stomatal conductance recovery, $P = 0.003$ and $P = 0.028$, respectively, according to Dunnett's post hoc test) (II, Fig. 2i and Supplemental Fig. 4g). It can be related with a role of SA in sustaining stomatal opening under constant light and preventing pathogen- and ABA-triggered stomatal closure (Lajeunesse et al., 2023). Our data show that SA might enhance the recovery of stomata from elevated CO_2 or darkness. Further studies are needed to elucidate SA-dependent signaling pathways in these processes.

To further address the possible involvement of JA signaling on stomatal sensitivity to CO_2, we studied stomatal reactions to changing CO_2 in the tomato *jai1-1* mutant defective in the JA receptor COI1 homolog. The magnitudes of the changes in stomatal conductance upon application of elevated (750 µl L^{-1}) or reduced (100 µl L^{-1}) CO_2 levels were highly similar in the *jai1-1* mutant and the wild-type plants (Figure 10c).

Our results demonstrate that stomatal CO_2 sensitivity does not depend on JA in both Arabidopsis and tomato, contradicting the previous research published by Geng et al. (2016). These authors demonstrated an accumulation of jasmonates in guard cells under elevated CO_2 and detected an absence of high CO_2-triggered stomatal closure in the JA-insensitive mutants, such as *coi1, jar1* and *jin1/myc2* (Geng et al., 2016). This discrepancy could result from different experimental approaches employed by Geng et al. and in this study. Thus, guard cells in epidermal peels used by Geng et al. do not receive mesophyll-driven signals, which can be involved in CO_2-induced stomatal regulation. The exact nature of these signals is still under debate; however, some substances have been found to connect stomatal reactions to CO_2 with mesophyll performance. Apoplastic malate promotes activation of the malate-sensitive R-type anion channel QUAC1, contributing to the mechanism of high CO_2-triggered stomatal closure (Lee et al., 2008; Meyer et al., 2010). Additionally, the concentrations of sucrose and glucose in the apoplastic space depend on photosynthetic activity in mesophyll and can regulate stomatal apertures for optimal CO_2 inflow (Santelia & Lawson, 2016; Flütsch, et al., 2020b). Additional studies should explore possible connections between JA signaling, stomatal regulation by CO_2, and mesophyll-driven signals. The discrepancy in CO_2 sensitivity of JA signaling mutants in our study and in Geng et al. (2016) highlights the importance of using intact plants and gas-exchange measurements to monitor stomatal movements.

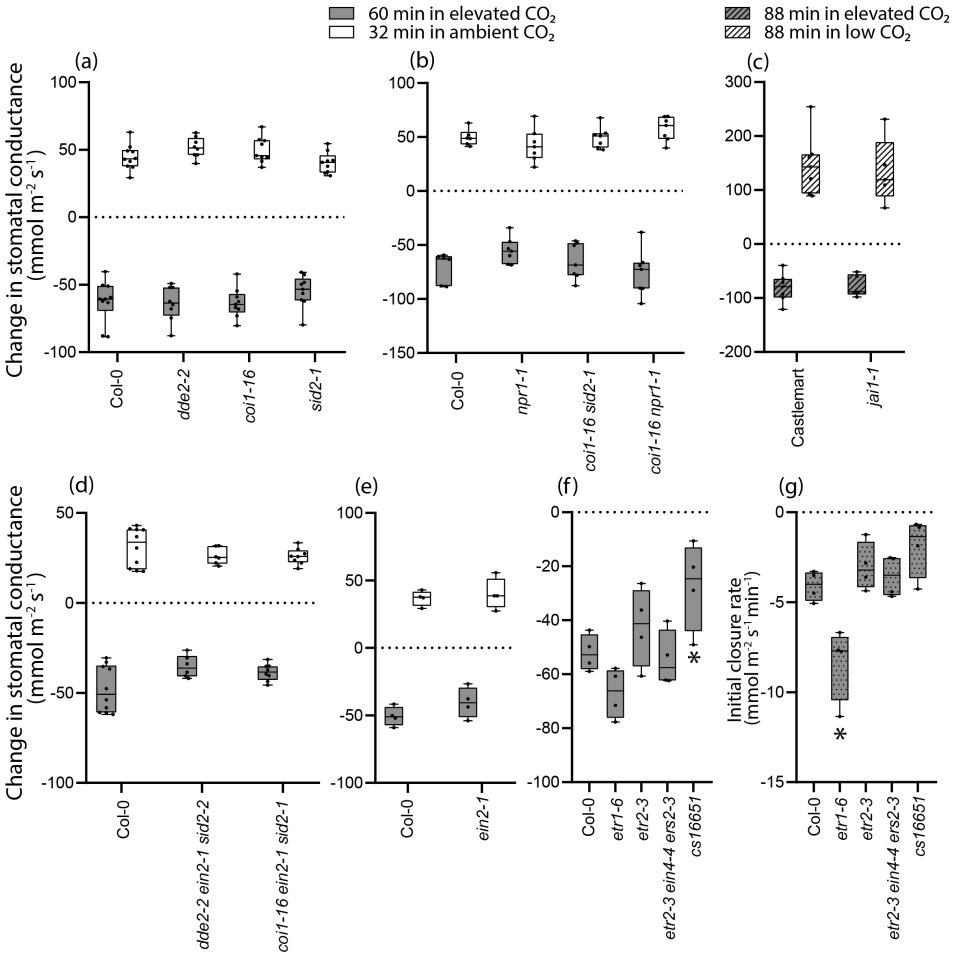

Figure 10. CO_2-triggered changes of stomatal conductance in Arabidopsis JA, SA, and ET biosynthesis and signaling mutants and in the tomato *jai1-1* mutant (based on ref II). The corresponding wild-type (Col-0 and Castlemart) plants are also shown. Changes in stomatal conductance were calculated as a difference between stomatal conductance values before and at the indicated timepoint upon the elevation of CO_2 from 420 to 800 µl L^{-1} (values at 60 min minus values at 0 min) or the recovery in ambient CO_2 by the reduction of CO_2 concentration from 800 to 420 µl L^{-1} (values at 92 min minus values at 60 min) (a, b, d, e, f). Tomato plants were incubated in ambient (approximately 400 µl L^{-1}) CO_2 and were treated either with elevated CO_2 (approximately 750 µl L^{-1}) or low CO_2 (approximately 100 µl L^{-1}). The changes in stomatal conductance in 88 min after the altered CO2 level are shown (values at 88 min minus values at 0 min) (c). Initial rates of stomatal closure were calculated as linear slopes of the stomatal conductance curve within 8 min after the application of high CO_2 (g). The values are average ± SE. The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots, whereas the whiskers are the minimum and maximum values (n = 4–10). Asterisks show significant differences between mutant lines and Col-0 (one-way ANOVA, followed by Dunnett's post hoc test; $P < 0.05$).

To explore the involvement of ET in stomatal reactions to CO_2 and darkness, we employed a mutant deficient in ET biosynthesis as well as mutants lacking ET receptors and EIN2, acting downstream to the ET receptors. The ACC-synthase octuple mutant *cs16651* with impaired ET biosynthesis showed weakened stomatal closure triggered by elevated CO_2 and darkness (Figure 10f and 11f). Interestingly, the mutants lacking various ET receptors demonstrated different results. The single mutant *etr2-3* and triple mutant *etr2-3 ein4-4 ers2-3* demonstrated intact stomatal responses to elevated CO_2 and darkness. In the same experiments, the *etr1-6* mutant had a faster stomatal response to both elevated CO_2 and darkness compared to wild-type plants although the magnitude of stomatal closure was not affected (Figure 10f, g and 11f, g). On the other hand, the *ein2-1* single mutant exhibited wild-type stomatal response to changes from ambient to elevated and from elevated to ambient concentrations of CO_2 (Figure 10e). Similarly, stomatal responses to illumination conditions remained unaffected in the *ein2-1* mutant (Figure 11e).

Since the octuple mutant *cs16651* demonstrated defective stomatal closure in response to elevated CO_2, one can conclude that ET is required for stomatal regulation under changed CO_2 and darkness. ET, as a gaseous molecule, rapidly diffuses and might provide fast controlling stomatal movements triggered by these stimuli. The ET receptors are the negative regulators in ET signaling and the loss-of-function mutants *etr1-6*, *etr2-3* and *etr2*-3 *ein4-4 ers2-3* have constantly activated ET signaling. However, only the *etr1-6* mutant displayed enhanced stomatal closure in response to elevated CO_2 and darkness (Figure 10g and 11g). It should be noted that the exact role of ET receptors in the regulation of stomatal conductance has not been fully studied. The ET receptors can induce different ET-dependent signaling pathways in stomatal movements (Wilson et al., 2014; Bakshi et al., 2015, 2018). The results also can be explained by the activation of noncanonical signal transduction pathways involving ET receptors play without the well-known CTR1/EIN2 signaling cascade (Binder, 2020). In particular, ETR1 can activate cytokinin signaling components, including Arabidopsis thaliana histidine phosphotransfer proteins and Arabidopsis thaliana response regulators, modulating a plant response to ET (Scharein et al., 2008; Zdarska et al., 2019; Binder, 2020).

To explore possible interactions between hormones in stomatal regulation by CO_2 and illumination conditions, we studied stomatal responsiveness to changing CO_2 and darkness/re-illumination in double and triple mutants with defects in JA, SA, and ET biosynthesis and signaling. Despite combining several mutations, stomatal responses to CO_2 in these mutants (*coi1-16 sid2-1*, *coi1-16 npr1-1*, *dde2-2 ein2-1 sid2-2, coi1-16 ein2-1 sid2-1*) were the same as in the wild-type plants (Figure 10b, d). Interestingly, combining *sid2-1* with the *coi1-16* seemingly recovered stomatal re-opening in ambient CO_2 after elevated CO_2 and upon re-illumination as evident from the initial slopes for the recovery of stomatal conductance (II, Fig. 2j and Supplemental Fig. 4d). JA and SA show antagonistic relations in many aspects of hormonal regulations (Spoel et al., 2003; Van der Does et al., 2013; Caarls et al., 2015) and it is possible that the observed delay in

stomatal re-opening in the *sid2-1* mutant resulted from an imbalance in JA- and SA-triggered signaling pathways. Our results with the triple mutants show that the simultaneous disruption of JA and SA biosynthesis and signaling, even combined with the lack of EIN2, does not influence stomatal reactions to CO_2 (Figure 10d and 11d). In contrast to the *coi1-16 ein2-1 sid2-1* mutant, the lack of oxylipins and SA combined with blocked ET signaling in the *dde2-2 ein2-1 sid2-2* mutant resulted in the weakly impaired stomatal response to darkness and re-illumination (Figure 11d). The *dde2-2* mutation affects stomatal movement triggered by illumination only in the *dde2-2 ein2-1 sid2-2* mutant, suggesting that signaling induced by oxylipins interacts with ET and SA signaling in stomatal reactions to light.

Studies of ABA-insensitive mutants demonstrated that stomatal reactions to CO_2 and darkness are influenced by ABA signaling. Further research indicated that high CO_2 triggered stomatal closure (Figure 6) involves both ABA-independent components, such as the HT1/MPK12/MPK4 signaling node (Hõrak et al., 2016; Jakobson et al., 2016; Tõldsepp et al., 2018; Takahashi et al., 2022), and ABA-dependent components, including ABI1, ABI2, OST1, and GHR1 (Merilo et al., 2013; Sierla et al., 2018). The ABA sensor ABAleon (Waadt et al., 2014) did not detect an accumulation of ABA in guard cells under high CO_2, indicating that although ABA signaling modulates stomatal sensitivity to CO_2, but it does not mediate high CO_2-triggered stomatal closure (Hsu et al., 2018; Zhang et al., 2020). Stomatal closure in darkness can involve inactivation of light opening and high CO_2-triggered signaling due to inactive photosynthesis and increasing C_i (Figure 6). A recent study revealed that ABA metabolism and signaling are not necessary for darkness-induced stomatal closure, but instead regulate the speed (Pridgeon & Hetherington, 2021). Our studies show that JA and SA have only a minor influence or no effect on stomatal movements in changing CO_2 concentration and illumination conditions. SA concentration in plants slightly influences the stomatal opening rate, although the magnitude of stomatal opening remains unaffected. We also found that ET level and specific ET receptors regulate stomatal responsiveness to elevated CO_2. Most likely, ABA, JA, SA, and ET do not mediate high CO_2-triggered stomatal closure, however, they might modulate stomatal sensitivity to changing CO_2 and light, regulating stomatal CO_2 inflow in accordance with a plant status and environmental conditions.

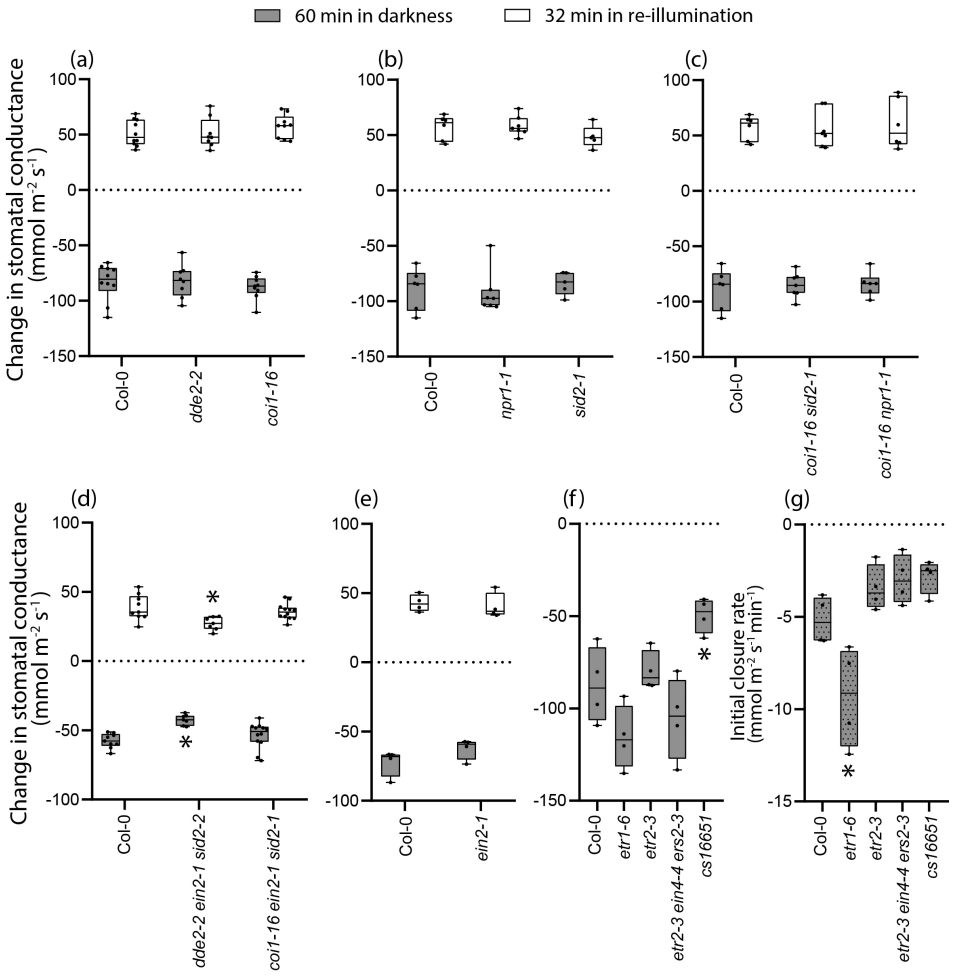

Figure 11. Characterization of stomatal movements induced by darkness in Arabidopsis mutants with disturbed JA, SA, and ET biosynthesis and signaling (based on ref II). The changes in stomatal conductance in the mutants and corresponding wild-type Col-0 plants are shown in (a–f) (average ± SE). They were calculated as differences in stomatal conductance between the initial values and the values at the indicated timepoints (values at 60 min minus values at 0 min for darkness-induced stomatal closure and values at 92 min minus values at 60 min for stomatal re-opening). Initial rates of stomatal closure were calculated as linear slopes of the stomatal conductance curves within 8 min after the darkness treatment starts (g). The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots, whereas the whiskers are the minimum and maximum values (n = 4–10). Asterisks show significant differences between the mutant lines and Col-0 (one-way ANOVA, followed by Dunnett's post hoc test; $P < 0.05$).

4.2.2 High VPD-triggered stomatal closure

Increased temperature and/or decreased relative air humidity lead to an increase in atmospheric VPD, enhancing transpiration. To prevent excessive water loss, plants close stomata upon high evaporative demand. For a long time, it has been considered that plants subjected to high VPD experience drought symptoms, and stomatal closure in dry air is closely related to ABA signaling in guard cells. To confirm it, stomatal responsiveness to reduced air humidity was extensively studied in mutants with disrupted ABA biosynthesis and signaling. It has been demonstrated that OST1 plays a central role in high VPD-triggered stomatal closure, while ABA-deficient mutants still closed their stomata in dry air (Merilo et al., 2013, 2018). Moreover, a rapid accumulation of ABA induced by elevated VPD has not been demonstrated (Merilo et al., 2018; Jalakas et al., 2021). These findings make the role of ABA in stomatal closure under elevated VPD still unresolved. In this study, we decided to verify whether JA, SA and ET regulate high VPD-triggered stomatal closure and tested stomatal responses to dry air in the mutants with defects in biosynthesis and signaling of these hormones.

Plants at the age of 3–4 weeks were subjected to a rapid decrease in air humidity from 70% to 35%, corresponding to an increase in VPD from 1.0 to 2.2 kPa. Typically, the wild-type plants demonstrated a rapid stomatal opening within a few minutes, known as "wrong way response" and caused by the fast dehydration of pavement cells (Merilo et al., 2013; Jalakas et al., 2021). The initial stomatal opening was followed by fast stomatal closure observed as a rapid decrease in stomatal conductance within 15 min after the decrease of air humidity (II, Supplemental Fig. 5). Then, stomatal conductance partially recovered. Due to these complex changes in stomatal conductance upon a rise in VPD, we compared this parameter in the plants 15 min and 60 min after the treatment started. The wild-type Arabidopsis plants demonstrated a decrease in stomatal conductance by approximately 60% after 15 min and by approximately 55% after 60 min of exposure to dry air (Figure 12).

All the studied JA, SA, and ET mutants displayed changes in stomatal conductance upon the abruptly increased VPD, which were similar to those in the wild-type plants. The JA biosynthesis (*dde2-2*) and signaling (*coi1-16*) single mutants and the *npr1-1* mutant with impaired SA signaling demonstrated pronounced stomatal closure in response to elevated VPD (Figure 12a, b) with the same magnitude as the wild-type plants. Interestingly, the SA biosynthesis mutant *sid2-1* demonstrated slightly impaired stomatal closure in dry air both 15 and 60 min after the treatment started (Figure 12b). The changes in stomatal conductance in response to elevated VPD in the *ein2-1* and *etr2-3* single mutants and the *etr2-3 ein4-4 ers2-3* triple mutant were not statistically different from those in the wild-type plants (Figure 12d, e). Similar to the results obtained with elevated CO_2 and darkness, the *cs16651* mutant displayed slightly impaired stomatal closure 15 min in dry air, compared with the wild-type plants (Figure 12e). On the other hand, the mutant lacking ETR1 (*etr1-6*) demonstrated an enhanced reduction of stomatal conductance 15 min after the increase in VPD. Apparently, ET influences

only fast stomatal closure in dry air as both *cs16651* and *etr1-6* mutants demonstrated the wild-type level of stomatal conductance 60 min of the treatment. The *ein2-1* mutant displayed intact stomatal responsiveness to dry air.

The simultaneous disruption of JA and SA does not affect stomatal closure triggered by high VPD. Thus, stomata in the double mutants combining the impairment in JA signaling with defects in SA biosynthesis or signaling (*coi1-16 sid2-1* and *coi1-16 npr1-1*) demonstrated the wild-type responses to low humidity. The addition of the *ein2-1* mutation to the disruptions in JA and SA signaling or biosynthesis in the *dde2-2 ein2-1 sid2-2* and *coi1-16 ein2-1 sid2-1* mutants did not affect stomatal closure triggered by high VPD (Figure 12e, f).

Although the mechanism of stomatal closure has not been characterized in all details, it has been convincingly demonstrated that OST1 plays a central role in the stomatal response to high evaporative demand (Xie et al., 2006; Merilo et al., 2018). OST1 can be activated by Raf-kinases independently of ABA signaling (Katsuta et al., 2020; Soma et al., 2020), and it has been recently shown that the B3 Raf-kinase m3kδ5 and m3kδ1/δ5/δ6/δ7 (raf3/6/5/4) quadruple mutants display delayed stomatal closure upon an increase in VPD (Hsu, et al., 2021b). The long-term adaptation to low air humidity can depend on the activation of ABA biosynthesis in plants (Yaaran et al., 2019). Our results demonstrate only minor effects of JA, SA, and ET on stomatal closure induced by high VPD. Plants with low SA concentrations due to the disruption of the ICS pathway in SA biosynthesis have slightly impaired stomatal responsiveness to high VPD (Figure 12b), suggesting a minor role for SA in promoting stomatal closure in dry air. ET also might modulate fast stomatal closure triggered by high VPD. Previously, it was reported that ET production is enhanced by elevated humidity (low VPD) in tomato plants and might promote stomatal opening under these conditions (Arve & Torre, 2015; Calvo-Polanco et al., 2017). Thus, tomato plants grown at a moderate level of air humidity and treated with ethephon, an ET precursor, showed increased leaf conductance and more open stomata than the control plants (Arve & Torre, 2015). Our results indicate that ET also promotes stomatal closure in dry air in Arabidopsis. The influence of SA and ET on stomatal closure should be studied further in relation to Raf-kinase-dependent activation of OST1.

JA and SA can regulate stomatal opening under high (>95%) air humidity close to the dew point (Panchal et al., 2016; Panchal & Melotto, 2017). It was suggested that these conditions result in stomatal opening due to the activation of JA signaling and the simultaneous downregulation of SA-responsive genes in guard cells (Panchal et al., 2016; Panchal & Melotto, 2017). It was also reported that high air humidity dampens both SA synthesis and signaling processes, leading to a strong inhibition of SA-related signaling pathways (Yao et al., 2022). In contrast to these findings, we observed that plants sprayed with MeJA did not demonstrate stomatal opening triggered by high humidity/wetting (Figure 9b). It should be noted that stomatal opening triggered by high humidity has not been thoroughly examined, and further studies are required to elaborate roles for JA and SA in the stomatal response to this stimulus.

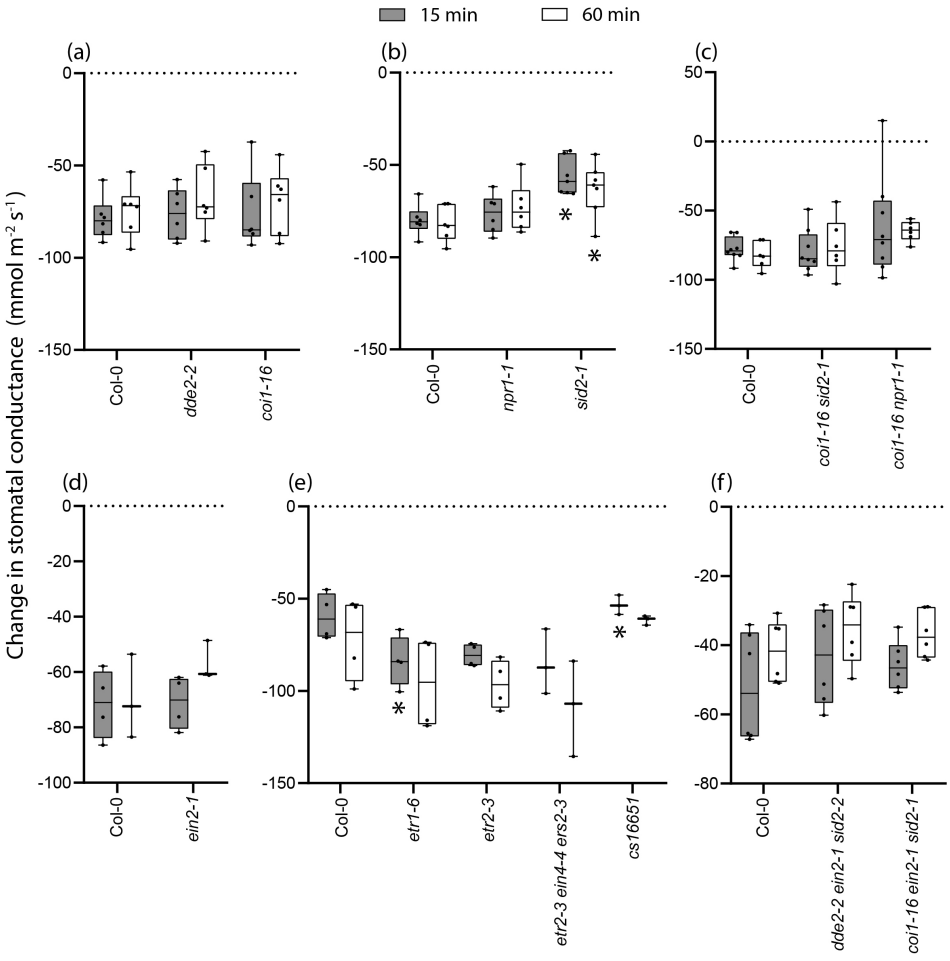

Figure 12. Changes in stomatal conductance of JA, SA, and ET biosynthesis and signaling mutants in response to elevated VPD (based on ref II). The changes in stomatal conductance in the mutants and corresponding wild-type Col-0 plants are shown in (a–f) (average ± SE). They were calculated as differences in stomatal conductance between the initial values and the values at the indicated timepoints (values at 15 min minus values at 0 min and values at 60 min minus values at 0 min) after changing air humidity from ~70% to ~30%. The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots, whereas the whiskers are the minimum and maximum values (n = 3–10). Asterisks show significant differences between mutant lines and Col-0 (one-way ANOVA, followed by Dunnett's post hoc test; $P < 0.05$). Differences for each timepoint were analyzed separately.

4.2.3 Stomatal closure triggered by short-term ozone pulses

Ozone is a gaseous photochemical oxidant that enters plants and triggers an accumulation of various ROS in the apoplast, leading to the activation of ROS-dependent signaling, oxidative stress, cell death, and visible leaf lesions (Vainonen & Kangasjärvi, 2015; Waszczak et al., 2018; Mittler et al., 2022). Ozone-derived ROS mimic oxidative stress triggered by pathogen attack (Vaahtera et al., 2014), suggesting that ozone can be used as a tool to study plant immunity. Phytohormones play an essential role in plant sensitivity to ozone-induced oxidative stress and development of ozone-triggered damage on leaves. Thus, defense hormones JA and SA regulate plant reactions to ozone exposure/ROS (Myers et al., 2023). SA level has been shown to correlate with the formation of lesions in response to ozone exposure (Rao & Davis, 1999). ET promotes O_3-induced cell death, while JA prevents ozone-triggered lesions (Rao et al., 2002; Tuominen et al., 2004). To address possible roles for JA, SA, and ET in stomatal responsiveness to ozone-triggered oxidative stress, we investigated stomatal conductance in the mutants with disrupted JA, SA, and ET biosynthesis and signaling after a short-term exposure to ozone.

Plants at the age of 3–4 weeks were subjected to a 3-min 470 nl L^{-1} ozone pulse, and changes in whole-plant stomatal conductance were monitored. The treatments induced fast stomatal closure in the wild-type plants, which was followed by gradual re-opening and the recovery of stomatal conductance to levels close to the initial values (II, Fig. 5). The maximal stomatal closure corresponding to a decrease in stomatal conductance to 30–40% of the initial level, was detected in all the studied lines 12 min after the ozone treatment started (Figure 13).

The JA biosynthesis (*dde2-2*) and signaling (*coi1-16*) mutants exhibited the wild-type magnitude of stomatal closure. Interestingly, the *coi1-16* mutant showed a faster recovery of stomatal conductance after the ozone-induced stomata closure compared with the Col-0 wild-type plants (the initial opening rate, P=0.001 by Dunnett's post hoc test) (II, Fig. 5h). The *dde2-2* displayed an intact recovery of stomatal conductance (Figure 13a). The *sid2-1* and *npr1-1* mutants with disrupted SA biosynthesis and signaling, respectively, demonstrated unaffected stomatal responses to ozone pulses (Figure 13b). The mutant with a decreased level of ET (*cs16651*) also exhibited wild-type stomatal responsiveness to ozone (Figure 13e). However, the lack of ETR1 in the *etr1-6* mutant enhanced the magnitude of ozone-triggered stomatal closure and accelerated stomatal re-opening after the ozone treatments (Figure 13e). Other studied ET receptors did not control stomatal closure in response to ozone as the *etr2-3 ein4-4 ers2-3* triple mutant displayed the same changes in stomatal conductance after the ozone treatments as the wild-type plants. The lack of EIN2 also did not influence stomatal reactions to ozone (Figure 13d).

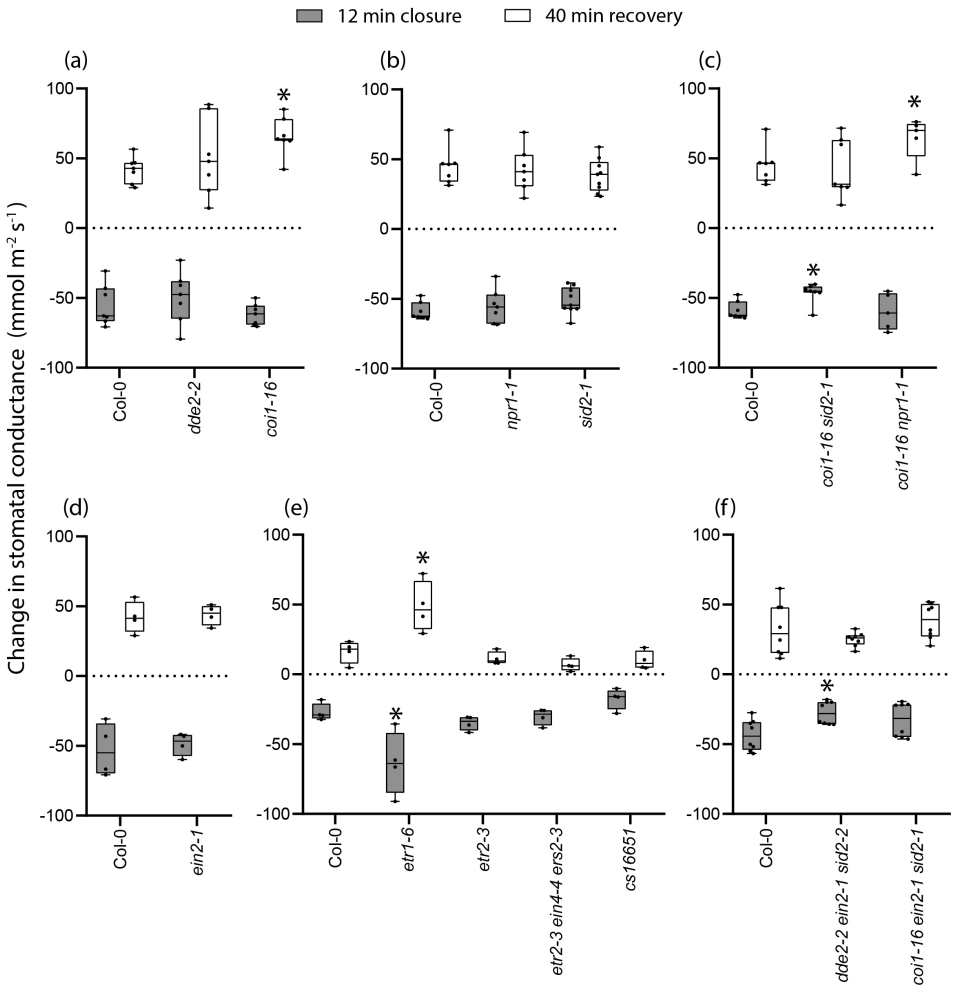

Figure 13. Ozone-induced stomatal closure and recovery of stomatal conductance after ozone pulses in Arabidopsis mutants with disrupted JA, SA, and ET biosynthesis and signaling (based on ref II). The changes in stomatal conductance in the mutants and corresponding wild-type Col-0 plants are shown in (a–f) (average ± SE). They were calculated as differences in stomatal conductance between the initial values and the values at the indicated timepoints. Ozone-induced stomatal closure was characterized by a decrease of stomatal conductance 12 min (values at 12 min minus values at 0 min) after 3-min ozone pulse (approximately 470 nl L^{-1}). Stomatal re-opening after ozone treatments was recorded as an increase in stomatal conductance during 40 min after the maximal ozone-induced decrease of stomatal conductance (values at 52 min minus values at 12 min). The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots, whereas the whiskers are the minimum and maximum values (n = 4–10). Asterisks show significant differences between mutant lines and Col-0 (one-way ANOVA, followed by Dunnett's post hoc test; $P < 0.05$).

The mutants combining defects in JA, SA, and ET biosynthesis and signaling showed some minor changes in time-resolved stomatal conductance after ozone treatments. The *coi1-16 sid2-1* mutant demonstrated slightly impaired stomatal closure. The *coi1-16 npr1-1* exhibited unaffected stomatal closure and marginally accelerated stomatal re-opening after ozone treatment (Figure 13c). The *dde2-2 ein2-1 sid2-2* triple mutant showed moderately impaired ozone-induced stomatal closure, while stomatal conductance in the *coi1-16 ein2-1 sid2-1* mutant changed in the same manner as in the wild-type plants after the ozone exposure (Figure 13f). These results might reflect complex interactions between the hormones in stomatal closure triggered by ozone/ROS.

We did not observe a strong dependency of stomatal responsiveness to apoplastic ROS/ozone on JA, SA, and ET biosynthesis and signaling. However, an involvement of ROS in signaling induced by these hormones has been demonstrated. RBOHF and RBOHD are expressed in guard cells and involved in ABA-induced stomatal closure. The *rbohD/F* double mutants demonstrated partially impaired stomatal responsiveness to ABA and a disruption in ROS elevation triggered by ABA in guard cells. At the same time, application of H_2O_2 induced stomatal closure in the *rbohD/F* double mutants and wild-type plants (Kwak et al., 2003). ROS were increased in guard cells treated with SA, and SA-induced stomatal closure was abolished in the presence of ROS inhibitors (Khokon et al., 2011). Similarly, MeJA induced H_2O_2 production in wild-type guard cell protoplasts, while exogenous application of inhibitors of NAD(P)H oxidases resulted in suppression of MeJA-induced stomatal closure (Suhita et al., 2004). The ET receptor ETR1 was involved in guard cell ROS signaling and stomatal closure. The loss-of-function mutant *etr1-7* demonstrated impaired stomatal closure in response to H_2O_2 (Desikan et al., 2005).

We found that some mutations and their combinations might affect kinetics of the changes in stomatal conductance, triggered by ozone pulses. These effects were minor, but they can reflect the regulation of stomatal sensitivity to ROS by JA, SA, and ET.

4.2.4 ABA-induced stomatal closure

Hormones frequently interact in the regulation of physiological processes (Acharya & Assmann, 2009; Müller, 2021; Myers et al., 2023). Since ABA is one of the key hormones in control of stomatal performance, we intended to test whether JA, SA and ET might influence ABA-mediated stomatal closure. To address this question, we sprayed JA, SA, and ET mutants at the age of 3–4 weeks with a 5 μM ABA solution and monitored changes in stomatal conductance.

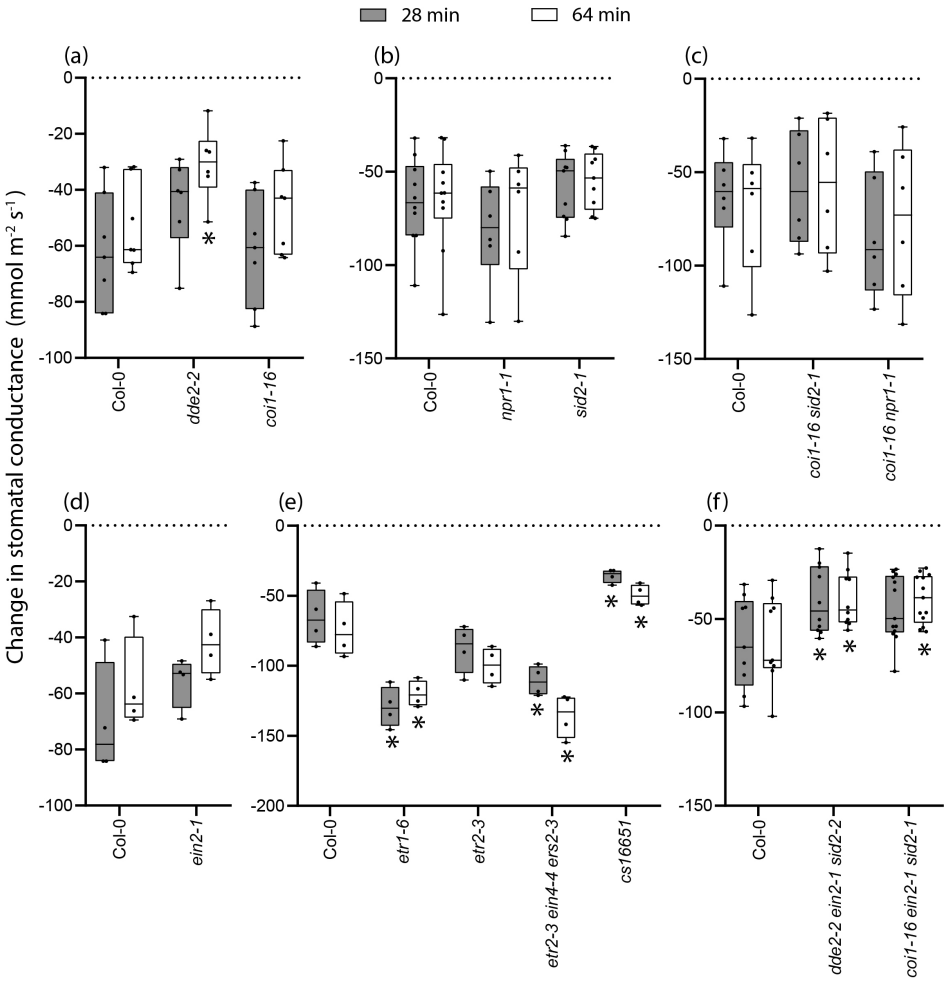

Figure 14. ABA-induced changes in stomatal conductance of Arabidopsis plants impaired in JA, SA, and ET biosynthesis and signaling (based on ref II). The changes in stomatal conductance in the mutants and corresponding wild-type Col-0 plants (a–f) are shown (average ± SE). They were calculated as differences in stomatal conductance between the initial values and the values at the indicated timepoints (values at 28 min minus values at 0 min and values at 64 min minus values at 0 min) after spraying with 5 μM ABA solution. The boxes extend from the 25th to the 75th percentiles, with the horizontal lines plotted at the median values. The individual data points are shown as dots, whereas the whiskers are the minimum and maximum values (n = 4–10). Asterisks show significant differences between mutant lines and Col-0 (one-way ANOVA, followed by Dunnett's post hoc test; $P < 0.05$).

As expected, spraying wild-type Arabidopsis plants with 5 μM ABA resulted in fast and pronounced stomatal closure, which was observed as a decrease in stomatal conductance to approximately 50–60% of the initial level within 28 minutes after treatment (Figure 14). The mutants with impairment of hormonal regulation by JA, SA, and ET also demonstrated a reduction of stomatal conductance after the spraying, indicating ABA-triggered stomatal closure; however, the kinetics of these responses was affected in some of these lines.

Both *dde2-2* and *coi1-16* mutants demonstrated a fast stomatal response to the ABA treatments, which was the same as in the wild-type plants (Figure 13a). However, the JA biosynthesis mutant *dde2-2* demonstrated an accelerated recovery of stomatal conductance that appeared significantly higher than that in the wild-type plants 60 min after ABA spraying. The difference between *dde2-2* and *coi1-16* regarding ABA-triggered changes in stomatal conductance can be related to the reduced level of 12-oxo-phytodienoic acid, a JA precursor, in the *dde2-2* mutant. In epidermal peels, this substance was able to induce stomatal closure by itself independently of COI1 and most efficiently in combination with ABA (Savchenko et al., 2014). Our results indicate that 12-oxo-phytodienoic acid might regulate the duration of ABA-triggered stomatal closure, enhancing the stomatal reaction to ABA.

The *sid2-1* mutant retained an unaffected stomatal response to ABA in the gas exchange experiments (Figure 14b). At the same time, the *npr1-1* mutant demonstrated faster stomatal closure initiated by the ABA treatment in comparison with the wild-type plants (the initial slope of stomatal closure within 12 min, P=0.033 by Dunnett's post hoc test) (II, Fig. 4f), indicating that NPR1-dependent SA signaling modulates ABA-triggered stomatal closure. The *sid2-1* mutant contains a residual level of SA (about 10%) (Nawrath & Métraux, 1999; Genger et al., 2008). The antagonistic relations between SA- and ABA-mediated signaling have been described in the development of SAR (Yasuda et al., 2008; Moeder et al., 2010). Our results show that SA signaling might negatively influence stomatal closure initiated by ABA.

The survey of stomatal responses to ABA in the ET mutants demonstrated that impairments in ET signaling can significantly enhance ABA-triggered stomatal closure. Thus, the lack of the ET receptors in the *etr1-6* and *etr2-3 ein4-4 ers2-3* triple mutants enhanced the reduction of stomatal conductance after the ABA treatments (Figure 14e). Although the *etr2-3* mutant showed a slightly more pronounced stomatal closure than the wild-type plants after ABA spraying, the difference was not statistically significant. The reduction of ET production in the octuple *cs16651* mutant led to animpaired stomatal response to ABA treatment. The *ein2-1* single mutant demonstrated a faintly impaired stomatal response to ABA treatment, although the difference was not significant (Figure 14d).

The double mutants with disruptions in JA and SA biosynthesis or signaling (*coi1-16 sid2-1* and *coi1-16 npr1-1*) had wild-type stomatal closure in response to ABA treatment (Figure 14c). The *coi1-16 npr1-1* mutant retained intact ABA-triggered stomatal closure in contrast to the single *npr1-1* mutant, as shown by the initial slopes for reduction in stomatal conductance after ABA spray

(II, Fig. 4f and Supplemental Fig. 7j). It indicates that an interaction between JA and SA might influence stomatal responsiveness to ABA. The triple mutants with the simultaneous disturbance of JA and SA biosynthesis and signaling combined with blocked EIN2-dependent ET signaling (*dde2-2 ein2-1 sid2-2* and *coi1-16 ein2-1 sid2-1*) had impaired response to the ABA treatment (Figure 14f) in contrast to the double *coi1-16 sid2-1* and *coi1-16 npr1-1* mutants (Figure 14c). It additionally supports the notion that ET enhances stomatal closure triggered by ABA through EIN2 activation.

Our results indicate that JA, SA, and ET biosynthesis and signaling are able to modulate ABA-induced stomatal closure, influencing the rate, magnitude, or duration of the reduction in stomatal conductance that resulted from ABA spraying of intact plants. The crosstalk of JA, SA, and ET with ABA in stomatal regulation has been noticed before. It was shown that stomatal closure induced by MeJA and SA depends on both ABA levels in plants and ABA signaling. Thus, impaired stomatal responses to MeJA and SA have been observed in mutants with disturbed ABA biosynthesis (Zeng & He, 2010; Hossain et al., 2011). Studies of ABA insensitive mutants revealed that MeJA-induced stomatal closure involves ABI1, ABI2, and OST1, but does not require the ABA receptors PYR1, PYL1, PYL2, and PYL4 (Munemasa et al., 2007; Hossain et al., 2011; Yin et al., 2016). On the other hand, SA was shown to trigger the phosphorylation of SLAC1 in guard cells through calcium-dependent protein kinases, but not via OST1 (Prodhan et al., 2018). Positive and negative interactions between ABA and ET were reported before (Müller, 2021). Further investigations are needed to understand the connections between ABA-induced stomatal closure kinetics and other hormones, such as JA, SA, and ET.

The role of ABA signaling in the modulation of stomatal responses to environmental factors, such as elevated CO_2, darkness period, elevated VPD, and ozone, has been convincingly demonstrated. Thus, dominant mutations in ABI1 and ABI2, as well as the lack of ABA receptors, OST1 and GHR1, significantly reduce or abolish high CO_2- and darkness-triggered stomatal closure (Merilo et al., 2013; Chater et al., 2015; Hsu et al., 2018; Sierla et al., 2018). OST1 activation by Raf-kinases plays an important role in stomatal closure in elevated VPD/low air humidity, although the impact of ABA signaling on this type of stomatal closure still should be evaluated (Merilo et al., 2013, 2018; Hsu, et al., 2021b). Many ABA-insensitive mutants, including those lacking ABA receptors, OST1, GHR1, demonstrate an absence of ozone-triggered stomatal closure in addition to the inability to close stomata in response to ABA (Merilo et al., 2013, 2018). In contrast to these results, we did not find complete stomatal insensitivity to elevated CO_2, darkness, elevated VPD, and ozone in the studied mutant with impaired JA, SA, and ET biosynthesis and signaling. However, these hormones can still influence stomatal reactions to environmental cues by modulating the speed and magnitude of the stomatal movements. Seemingly, JA signaling might restrict stomatal opening as the *coi1-16* mutant showed accelerated recovery of stomatal conductance after ozone treatment and spraying with MeJA suppressed stomatal opening triggered by high humidity (Figures 9b). At the same time, the SA-insensitive

npr1-1 mutant demonstrated accelerated ABA-induced stomatal closure. The ET receptors (mostly ETR1) and ET level influence stomatal responsiveness to environmental cues and ABA, suggesting a role for ET in promoting stomatal closure. Although the mechanism underlying the modulation of stomatal responsiveness to environmental stimuli by JA, SA, and ET is not clear, the influence of these hormones on stomatal opening and closing deserves further attention. JA, SA, and ET signaling and/or biosynthesis might be directly activated by environmental cues, interacting with signaling pathways in guard cells and promoting or inhibiting stomatal movements (Casteel et al., 2012; Geng et al., 2016; Z. Li & Ahammed, 2023). It is also possible that JA, SA, and ET control the expression of stomatal regulators in guard cells, affecting rates and magnitudes of stomatal reactions to environmental cues and ABA. Understanding how JA, SA, and ET regulate rates of stomatal movements can be valuable for accelerating stomatal reactions, resulting in improved water-use efficiency and enhanced photosynthetic performance in crops under ever-changing environmental conditions.

5. CONCLUSIONS

This thesis can be summarized as follows:

- We demonstrated that JA, SA, and ET biosynthesis and signaling have only minor roles in controlling steady-state whole-plant stomatal conductance. Only disruptions in JA biosynthesis and ET signaling weakly influence the levels of stomatal conductance under standard growth conditions. At the same time, JA signaling and SA biosynthesis and signaling do not regulate steady-state stomatal conductance.
- Our study of double mutants combining the lack of MAX2 with disrupted ABA biosynthesis and signaling showed that SL signaling controls stomatal conductance independently of ABA. The elevated level of stomatal conductance in the *max2-4* mutant is caused by more open stomata, but not affected by stomatal density.
- Abilities of JA and SA in promoting stomatal closure were compared with that of ABA. By spraying MeJA and SA on intact plants, we showed that, while ABA induced a fast and prolonged stomatal closure, JA and SA caused minor effects on stomatal conductance in the same experiments. It highlights that ABA is the main regulator of stomatal conductance, while JA and SA are significantly less efficient in inducing stomatal closure.
- Roles for JA, SA, and ET in stomatal regulation by environmental cues and ABA were studied. All the studied mutants with affected biosynthesis and/or signaling of these hormones demonstrated stomatal responsiveness to changing CO_2, illumination conditions, low air humidity, ozone, and ABA treatments. However, JA, SA, and ET are able to influence stomatal reactions to environmental cues and ABA by modulating the speed and magnitude of the stomatal movements.
- The level of OPDA, a JA precursor, might regulate stomatal conductance independently of COI1. The low level of this substance in the *dde2-2* mutant can potentially result in reduced stomatal conductance and weakened ABA-triggered stomatal closure.
- The reduced SA level was associated with slower stomatal re-opening after elevated CO_2 and darkness as well as with diminished high VPD-induced stomatal closure. The lack of NPR1 accelerated ABA-triggered stomatal closure, indicating that SA signaling negatively influences ABA in stomatal regulation.
- ET controls stomatal conductance and accelerates stomatal closure in response to elevated CO_2, darkness, high VPD, ozone, and ABA. Analysis of mutants lacking various ET receptors shows that these proteins are able to trigger various signaling pathways, which can be independent of EIN2. Among

analyzed ET receptors, the lack of ETR1 demonstrates the highest impact on the regulation of stomatal movements.

- Simultaneous disruptions of JA, SA and ET biosynthesis and signaling in double and triple mutants caused only weak effects on stomatal responsiveness to environmental cues and ABA, which still might reflect interactions between these hormones in stomatal regulation.

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SUMMARY IN ESTONIAN

Taimehormoonide mõju õhulõhede juhtivusele

Märkimisväärne osa maa pinnast on kaetud taimedega. Taimed on võimelised, päikese energia abil, assimileerima fotosünteesi käigus süsihappegaasi (CO_2) ja vett orgaanilisteks, energiarikasteks ühenditeks ning eraldavad samal ajal hapnikku, mis on vajalik respiratoorsete organismide toimimiseks. Ei ole liialdus väita, et taimed on elu aluseks planeedil maa, nad moodustavad lõviosa inimkonna toidulauast ja on tooraineks mitmetele teistele tööstusharudele, nagu ehitus-, meditsiini- ja tekstiilitööstus. Seetõttu on taimede füsioloogia uurimine oluline küsimus.

CO_2 siseneb ja O_2 väljub taime lehest läbi lehepinnal paiknevate õhulõhede. Õhulõhe moodustub paarina paiknevatest sulgrakkudest, mis muudavad oma mahtu ja kuju, ning läbi selle nende vahel olevat pilu ehk õhulõhe poori. Õhulõhede avanemine ja sulgumine sõltuvad vee ja ioonide transpordist läbi sulgraku membraanide. Õhulõhe avanedes sisenevad ioonid ja vesi sulgrakkudesse ja läbi selle suureneb nende maht ja turgorrõhk. Vastupidi, õhulõhed sulguvad siis, kui vesi ja ioonid väljuvad sulgrakkudest. Sulgrakkude membraanides olevad ioonkanalid reguleerivad nende mahtu ja turgorrõhku. Keskkonnamuutused mõjutavad sulgrakkudes signaalradu, mis omakorda põhjustab õhulõhede avanemist või sulgemist. Näiteks valgus, madal CO_2 kontsentratsioon ja kõrge õhuniiskus soodustavad õhulõhede avanemist, samas kui pimedus, kõrgenenud CO_2, madal õhuniiskus ja õhusaasteained, nagu osoon, põhjustavad õhulõhede sulgumist.

Hoolimata ulatuslikust teadusstööst õhulõhede avanemise ja sulgemise uurimisel, on endiselt palju lünki seoses sellega, kuidas õhulõhed reguleerivad taimede gaasivahetust erinevates keskkonna tingimustes. Edasised uuringud, eesmärgiga selgitada välja molekulaarsed komponendid õhulõhede regulatsioonis, on vajalikud sest nende alusel on võimalik aretada taim mis suudavad kohaneda ebasoodsate keskkonnatingimustega. Taimehormoon abstsiishape (ABA) mängib olulist rolli taime põuavastuse kujundamisel ja õhulõhede avatuse reguleerimisel. Mõned uuringud väidavad ka teiste taimehormoonide osalust õhulõhede funktsiooni reguleerimisel. Käesolevas doktoritöös uuriti taimehormoonide, nagu strigolaktoonid (SL-d), jasmoonhape (JA), salitsüülhape (SA) ja etüleen (ET), rolli õhulõhede regulatsioonis.

Esmalt uurisime JA, SA ja ET mõju taime õhulõhede juhtivusele. Mõõtsime õhulõhede juhtivust taimeliinides, kus oli häiritud JA, SA ja ET biosüntees ning signaalülekanne. Meie tulemused näitasid, et JA biosünteesi häirimine põhjustas õhulõhede juhtivuse kerge languse võrreldes metsiktüübiga. Siiski JA signaaliülekande, SA biosünteesi ning signaaliülekande geenide mutatsioonid ei omanud olulist mõju õhulõhede juhtivusele. Teisalt ET seotud valkude – ETR2, EIN4 ja ERS2 puudumine viis õhulõhede juhtivuse kerge suurenemiseni, samas mutatsioonid ET biosünteesi geenides vähendasid õhulõhede juhtivust. Õhulõhede juhtivus *dde2-2 ein2-1 sid2-2* ja *coi1-16 ein2-1 sid2-1* kolmikmutantides oli

umbes 30% madalam võrreldes metsiktüübiga, mis viitab komplekssetele interaktsioonidele, mis on tingitud JA, SA ja ET poolt vahendatud hormonaalsest signaaliülekandest. Kokkuvõttes võib saadud tulemuste alusel väita, et JA, SA ja ET biosünteesi ning signaaliülekande geenide mutatsioonid omavad vähest mõju taime õhulõhede juhtivusele.

Teiseks uurisime, kas SL-id ja ABA jagavad sama signaalrada õhulõhede juhtivuse reguleerimisel. *max2-4* mutandil oli metsiktüüpi taimedega võrreldes oluliselt suurem õhulõhede juhtivus, sarnaselt ABA biosünteesi ja signaaliülekande geenide mutatsioonidega. Uurisime topeltmutantide õhulõhede juhtivust, kus oli häiritud MAX2 ja üks ABA signaaliülekande või biosünteesi komponentidest. Meie andmed näitasid, et kaksikmutandid (*aba2-11 max2-4*, *ghr1-3 max2-4* ja *ost1-3 max2-4*) omasid oluliselt kõrgemat õhulõhede juhtivust võrreldes vastavate üksikmutantidega, mis viitab *max2-4* mutatsiooni ja häiritud ABA biosünteesi ja signaaliülekande aditiivsele mõjule. *max2-4* mutandil oli normaalne õhulõhede tihedus, kuid suurenenud õhupilu ava. Järgnevalt tuleks selgitada, kuidas SL-id reguleerivad turgorrõhku sulgrakkudes.

Kolmandaks hindasime metüüljasmonaadi (MeJA) ja SA efektiivsust taimede õhulõhede juhtivuses muutuste esilekutsumisel. Piserdasime MeJA ja SA lahustega metsiktüüpi taimi, et uurida nende taimehormoonide mõju õhulõhede juhtivusele. Töötlus MeJA-ga põhjustas õhulõhede juhtivuse kerge languse võrreldes kontrolltaimedega. Samas, pärast SA töötlust, erinevusi ei täheldatud. Töötlus ABAga põhjustas kiire õhulõhede juhtivuse languse. Meie eksperimendid viitavad sellele, et JA ja SA-l on õhulõhede sulgumisele väike mõju, samas kui ABA on peamine hormoon, mis on seotud õhulõhede reguleerimisega. On võimalik, et JA ja SA-st põhjustatud õhulõhede sulgumise korral on vaja kõrgemaid hormoonikontsentratsioone ja pikemat inkubatsiooni aega. Lisaks võivad kasvutingimused mõjutada õhulõhede tundlikkust hormoonide suhtes.

Järgmiseks uurisime JA, SA ja ET signaaliülekande ja biosünteesi mutantide õhulõhede vastuseid erinevatele keskkonnastiimulitele nagu suurenenud CO_2, pimedus, madala suhtelise õhuniiskuse või osooni impulsi rakendamine. JA biosünteesi ja signaaliülekande defektidega mutantsed taimeliinid reageerisid, peaaegu kõikidele mainitud stiimulitele, sarnaselt metsiktüübile. Siiski *dde2-2* üksikmutant õhulõhed reageerisid ABA töötlusele veidi häiritult ning *coi1-16* mutandil oli kiirem taastumine pärast osooniimpulssi võrreldes metsiktüübiga. SA biosünteesi ja signaaliülekande mutantide õhulõhede tundlikkus keskkonnastiimulitele ei erinenud oluliselt metsiktüübist, välja arvatud *sid2-1* mutant, mille puhul täheldati väikesi muutusi valguse taasrakendamisel, CO_2 vähendamise ja madala õhuniiskuse reaktsioonides. ET mutandid näitasid keskkonnatingimustele erinevaid reageerimismustreid. ET biosünteesi defektidega mutandid näitasid häiritud õhulõhede vastust, samas kui ET signaaliülekande katkestused viisid tugevama õhulõhede sulgumiseni vastusena testitud stiimulitele. Leidsime, et ETR1-l oli kõige suurem mõju õhulõhede juhtivuse reguleerimisele viie uuritud ET retseptori hulgas seoses suurenenud CO_2, pimeduse, madala õhuniiskuse, osooniimpulsi ja ABA töötlusega. Huvitavalt näitas *etr2-3 ein4-4 ers2-3* kolmikmutant tugevamat õhulõhede sulgumist ABA pihustamisele, samal ajal kui *etr2-3*

üksikmutant reageeris sarnaselt metsiktüübile. Need tulemused viitavad sellele, et mitme ET retseptori mutatsioonidel võib olla suurem mõju õhulõhede reaktsioonidele.

JA, SA ja ET biosünteesi ja signaaliülekande kombineeritud mutatsioonidega kaksik- ja kolmikmutandid näitasid sarnaseid õhulõhede reaktsioone vastavatele üksikmutantidele. Siiski näitasid kaksik- ja kolmikmutandid konkreetsetes katsetes erinevaid õhulõhede fenotüüpe võrreldes vastavate üksikmutantidega, eriti reageerides osoonile ja ABA'ga pihustamisele. See viitab hormonaalsete signaalradade vahelisele koostoimele.

Kokkuvõttes viitavad antud töös läbi viidud eksperimendid sellele, et JA, SA ja ET avaldavad õhulõhede reaktsioonidele suhteliselt väikest mõju. ABA omab aga olulist rolli taimehormoonina õhulõhede reguleerimisprotsessis. Siiski võib JA, SA ja ET ikkagi mõjutada õhulõhede reaktsioone keskkonnatingimustele, muutes õhulõhede reaktsiooni kiirust ja ulatust. Vigase MAX2-ga taimedes oli õhulõhede juhtivus madalam. SL-de mõju õhulõhede juhtivusele töötab sõltumatult ABA-st ja toimib samaaegselt ABA signaalrajaga.

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PUBLICATIONS

CURRICULUM VITAE

Name:	Olena Zamora
Date of birth:	14th of June 1991
Citizenship:	Ukraine
Contact:	Nooruse 1-301, Tartu 50411, Estonia olena.zamora@ut.ee

Education

2014– …	Doctoral studies in Science and Technology, University of Tartu
2012–2014	Master's studies in Biotechnology, The National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute"
2008–2012	Bachelor's studies in Biotechnology, The National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute"
2004–2008	Rubizhne Lyceum, specialization Chemistry and Biology

Professional employment

September 2021 – … Specialist in plant biology, University of Tartu

November 2018 – March 2021
Researcher, Faculty of Biological and Environmental Sciences, University of Helsinki, Finland

July 2014 –November 2014
Standardization and Quality Engineer, Quality Control Department, ACINO, Kyiv, Ukraine

October 2012 –June 2014
Researcher, Department of General and Soil Microbiology, Danylo Zabolotny Institute of Microbiology and Virology of the NASU, Kyiv, Ukraine

Scientific publications

Kalliola, M., Jakobson, L., Davidsson, P., Pennanen, V., Waszczak, C., Yarmolinsky, D., **Zamora, O.**, Palva, E. T., Kariola, T., Kollist, H., & Brosché, M. (2020). Differential role of MAX2 and strigolactones in pathogen, ozone, and stomatal responses. *Plant Direct*, *4*(2), e00206. https://doi.org/10.1002/pld3.206

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ELULOOKIRJELDUS

Nimi: Olena Zamora
Sünniaeg: 14 Juuni 1991
Kodakondsus: Ukraina
Kontakt: Nooruse 1-301, Tartu 50411, Eesti
olena.zamora@ut.ee

Haridus

2014– …	Tehnika ja tehnoloogia doktoriõpe, Tartu Ülikool
2012–2014	Biotehnoloogia magistriõpingud, Ukraina Riiklik Tehnikaülikool "Igor Sikorsky nimeline Kiievi Polütehniline Instituut"
2008–2012	Bakalaureuseõpingud biotehnoloogia erialal, Ukraina Riiklik Tehnikaülikool "Igor Sikorsky nimeline Kiievi Polütehniline Instituut"
2004–2008	Rubižne Lütseum, keemia ja bioloogia eriala

Erialane teenistuskäik

September 2021 – … Taimebioloogia spetsialist, Tartu Ülikool

November 2018 – märts 2021
Bioloogia- ja keskkonnateaduste teaduskonna teadur, Helsingi Ülikool, Soome

Juuli 2014 – november 2014
Standardi- ja kvaliteediinsener, kvaliteedikontrolli osakond, ACINO, Kiiev, Ukraina

Oktoober 2012 – juuni 2014
Teadur, Üld- ja mullamikrobioloogia osakond, Ukraina Rahvusliku Teaduste Akadeemia Danylo Zabolotny nimeline Mikrobioloogia ja Viroloogia instituut, Kiiev, Ukraina.

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