Abscisic Acid Signaling and Methyl Jasmonate Signaling in Arabidopsis Guard Cells

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Ye Yin

Graduate School of Natural Science and Technology

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OKAYAMA UNIVERSITY, JAPAN

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Ye Yin

Department of Biofunctional Chemistry

Graduate School of Natural Science and Technology

Okayama University, Japan

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Abbreviations used

ABA, abscisic acid

MeJA, methyl jasmonate

PYR, pyrabactin resistance

PYL, pyrabactin resistance-like

RCAR, regulatory component of ABA receptors

OST1, open stomata 1

ROS, reactive oxygen species

NO, nitric oxide

pH_{cyt}, cytosolic pH

K_{in} channels, inward rectifying K^+ channels

H^+-ATPase, plasma membrane protein pump

H_2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate

BCECF-AM, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetomethylester

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Chapter 1

General Introduction

1.1. Plant physiology

The branch of plant science that aims to understand how plant live and function. Its ultimate objective is to explain all life processes of plants by a minimal number of comprehensive principles founded in chemistry, physics, and mathematics. Plant physiology seeks to understand all the aspects and manifestations of plant life. In agreement with the major characteristics of organisms, it is usually divided into three major parts: the physiology of nutrition and metabolism, which deals with the uptake, transformations, and release of materials, and also their movement within and between the cells and organs of the plant; the physiology of growth, development, and reproduction, which is concerned with these aspects of plant function; and environmental physiology, which seeks to understand the manifold responses of plants to the environment. The part of environmental physiology which deals with effects of and adaptations to adverse conditions and which is receiving increasing attention is called stress physiology.

1.2. Stress and stress responses in plants

Stress in plants can be defined as any external factor that negatively influences plant growth, productivity, reproductive capacity or survival. This includes a wide range of factors which can be broadly divided into two main categories: abiotic or environmental stress factors, and biotic or biological stress

factors. Biotic stress can be imposed by other organisms, while abiotic stress can be aroused from an excess or deficit in the physical or chemical environment including drought, waterlogging, high or low solar radiation, extreme temperatures, ozone, salinity, and inadequate mineral nutrient in the soil. A stress triggers plant responses ranging from altered gene expression to changes in plant metabolism and growth. To survive with these challenges, plant defense responses rely on signaling mechanisms of hormones and other plant specific substances (Bostoc, 2005; Zhang et al., 2008)

1.3. Stomata

Stomata are tiny pores on the leaf epidermis of higher plants that regulate the uptake of carbon for photosynthesis and loss of water vapor by transpiration (Hetherington and Woodward 2003). The aperture of the pore is controlled by the state of turgor of the two guard cells. Guard cells have complex sensory and signal transduction machinery that allows them to respond to a variety of factor such as light, CO_2, and water stress. In drought condition guard cells reduce stomatal aperture, thereby protecting the plant from transpirational water loss. Guard cells regulate stomatal pore apertures. Via integration of endogenous hormonal stimuli and environmental signals, and guard cells have been highly developed as a model system to dissect the dynamics and mechanisms of plant cell signaling (Kim et al., 2010). Several key physiological stimuli regulate stomatal aperture, including light, CO_2 concentration, humidity, temperatures, plant hormones like abscisic acid (ABA), methyl jasmonate (MeJA) (Schroeder et al., 2001; Murata et al., 2001; Hetherington and

Woodward 2003; Suhita et al., 2004; Munemasa et al., 2007; Akter et al., 2010; Islam et al., 2010)

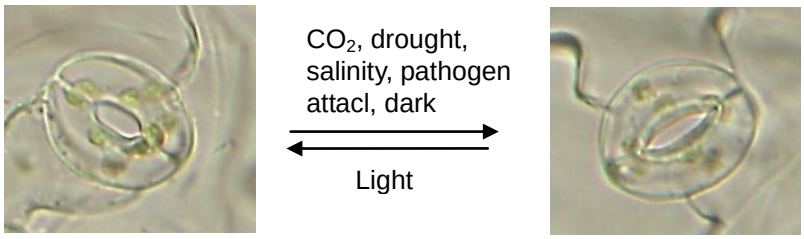

Fig. 1.1. Open and closed of Arabidopsis

1.4. Abscisic acid

Abscisic acid (ABA) is a phytohormone that controls important developmental and abiotic stress responses, including seed dormancy, seed development, growth regulation and stomatal closure. ABA has a central role in protecting plant against water deficiency by regulating the stomatal aperture and minimizing water loss through stomata. ABA accumulation induces stomatal closure and differential gene expression. Furthermore, ABA inhibits light-induced stomatal opening to prevent water loss by transpiration (Garcia-Mata and Lamattina, 2007).

Protein phosphorylation and dephosphorylation are important mediators of ABA signal transduction (Schroeder et al., 2001). ABA-activated protein kinase (AAPK), clucium dependent protein kinases (CPK3, CPK4, CPK6, and CPK11, mitogen-activated protein kinases MPK3, MPK9, and MPK12 all

mediate ABA signaling in guard cells (Li et al., 2000; Mori et al., 2006). Protein phosphatase type 2C enzymes ABI1, ABI2, and HAB1 are all negative regulators of ABA signaling (Merlot et al., 2001; Saez et al., 2004). In contrast, the protein phosphatase type 2A (PP2A) RCN1 functions as a positive regulator of ABA signaling (Kwak et al., 2002). It has been proposed that ABA can be perceived both inside and outside the cell, thus multiple ABA receptors have been proposed to exist (Bray et al., 1997). The existence of cytosolic ABA receptors was recently confirmend by the discovery of the PYR/PYL/RCAR proteins (Ma et al., 2009; Park et al., 2009). After the identification of PYR/PYL/RCAR receptors, the cryetal structure was reported by several groups (Melcher et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Yin et al., 2009)

1.5. Methyl jasmonate

The volatile phytohormone methyl jasmonate (MeJA) is a linolenic acid derivate initially identified from flowers of jasminum grandiflorum, and has proven to be distributed ubiquitously in the plant kingdom (Cheong et al., 2003). MeJA regulates various physiological processes, including pollen maturation, tendril coiling, and responses to wounding and pathogen attack (Liechti and Farmer, 2002; Turner et al., 2002). Similar to ABA, MeJA plays a role in the induction of stomatal closure (Gehring et al., 1997; Suhita et al., 2004; Munemasa et al., 2007; Islam et al., 2010).

MeJA- and ABA-induced stomatal closure comprise of similar events such as cytosolic alkalization, NADPH oxidase dependent H_2O_2 production, NO production, $[Ca^{2+}]_{cyt}$ oscillations, activation of I_{Ca} channels, S-type anion

channels and K^+ outward rectifying channels (K_{out}). However, MeJA as well as ABA induce stomatal closure with their signaling crosstalk. The jasmonate insensitive *Arabidopsis* mutant, *jar1-1* (Suhita et al., 2004) and *coi1* (Munemasa et al., 2007) lack the ability to close stomata in response to MeJA, whereas these mutants prossess responsiveness to ABA. Elevation of ROS, activation of I_{Ca} channels and S-type anion channels induced by MeJA were not observed in *coi1* mutant (Munemasa et al., 2007). Meanwhile, MeJA did not increase ROS level in *jar1-1* guard cells (Suhita et al., 2004). On the other hand, *ost1-2* guard cells are responsive to MeJA in stomatal closure and ROS accumulation, but insensitive to ABA (Suhita et al., 2004). Little is known about the physiological mechanism of stomatal movement by apart from ABA, other phytohormones including MeJA and about these signaling interactions in guard cells.

1.6. Second messengers in guard cell signaling

1.6.1. Reactive oxygen species

Reactive oxygen species (ROS) is a collective term that broadly describes O_2-derived free radicals such as superoxide anions (O_2^-), hydroxyl radicals (HO·), peroxyl (RO_2·), alkoxyl (RO·), as well as O_2^- derived non-radical species such as hydrogen peroxide (H_2O_2) (Takahashi and Asada, 1988; Mittler, 2002). ROS, including $^1O_2$, H_2O_2, O_2^- and HO^- are toxic molecules capable of causing oxidative damage to proteins, DNA and lipids (Apel and Hirt 2004). Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as chloroplast, mitochondria, apoplast and peroxisomes. However, during stress condition the rate of ROS production is drastically

increased. Under physiological steady state conditions these molecules are scavenged by different antioxidative defense components that are often confined to particular compartments (Alscher et al., 1997). The equilibrium between production and scavenging of ROS may be perturbed by a number of adverse environmental factors (Mitller et al., 2004). Antioxidants such as ascorbic acid (AsA), glutathione (GSH) and ROS scavenging enzymes such as superoxide dismutase (GPX) and peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR) are essential for ROS detoxification during normal metabolism, and particularly during stress (Takahashi and Asada, 1998; Apel and Hirt, 2004; Mittler et al., 2004).

There are different sources of ROS like mitochondria, chloroplasts, plasma membrane bound NADPH oxidases, cell wall peroxidases, peroxisomes and glyoxysomes (Pei te al., 2000; Zhang et al., 2001; Asada, 2006). ROS function as second messengers in ABA and MeJA signal transduction cascade in guard cells (Suhita et al., 2004; Munemasa et al., 2007; Saito et al., 2008; Islam et al., 2010). NADPH oxidase-mediated ROS generation is considered as major sources for ABA- and MeJA-induced stomatal closure in Arabidopsis (Kwak et al., 2003; Suhita et al., 2004). H_2O_2 causes an increase in guard cell cytosolic free Ca^{2+}, anion channel activation and leading to stomatal closure (McAinsh et al., 1996; Zhang et al., 2001).

1.6.2. Nitric Oxide

Nitric Oxide (NO) is a versatile signaling molecule regulating basic physiological processes throughout the plant life cycle like seed germination,

growth, development, flowering, fruit maturation, senescence, programmed cell death and stress in different plant species and is suggested to increase plant drought tolerance in stress conditions by reducing transpirational water loss (Garcia-Mata and Lamattina, 2007). NO has been found to act in guard cell ABA signaling and its generation is dependent on H_2O_2 production by NADPH oxidases (Neill et al., 2002; Garcia-Mata and Lamattina, 2002; Desikan et al., 2002). NO accompanied with ROS can regulate diverse physiological functions including ABA- and MeJA-induced stomatal closure (Garcia-Mata and Lamattina 2001; Bright et al., 2006; Munemasa et al., 2007). However, the involvement of NO to signal transduction leading to stomatal closure is still controversial since Lozano-Juste and Leon (2010) have reported that the *Arabidopsis nial nia2 noa1-2* triple mutant, which is deficient endogenous NO production, is hypersensitive to ABA.

1.6.3. Cytosolic pH

Intracellular pH changes have long been proposed to function in signaling and transport control in plant cells, such as plant defense responses and response to hormone activity such as abscisic acid (ABA) and methyl jasmonate (MeJA) (Suhita et al., 2004). Stomatal closure is associated with K^+ release from the vacuoles and efflux across the plasma membrane (MacRobbie, 1998), and closure can be induced by elevated level of cytoplasmic Ca^{2+}. ABA- induced increase in cytoplasmic Ca^{2+} can be achieved without Ca^{2+} influx, by release from cytoplasmic stores, but do not rule out ABA-induced Ca^{2+} influx when external Ca^{2+} is present. ABA-induced changes in $[Ca^{2+}]_{cyt}$ and pH_{cyt} affect

inward- (I_{kin}) and outward-rectifying (I_{kout}) K^+ channels and of Cl^- channels (I_{Cl}) to bias the membrane for KCl efflux and a loss of cell turgor (Blatt, 2000). Blatt and Armstrong (1993) showed that acidification of the cytoplasm by external sodium butyrate inhibited the outward K^+ channel, prevented the ABA-induced alkalization of the cytoplasm, and blocked the ABA-induced activation of the outward K^+ channel, arguing for regulation by cytoplasmic pH, and positioning pH in the signaling chain leading from ABA to this channel. Like ABA, MeJA also suppresses I_{kin} current in Arabidopsis (Saito et al., 2008) and simultaneously activates I_{kout} current in Vicia (Evans, 2003). These results indicate that elevation of $[Ca^{2+}]_{cyt}$ and pH_{cyt} function in ion mobilization.

1.7 Plasma membrane ion channels in guard cells

1.7.1. Potassium channels

K^+ efflux across the plasma membrane of guard cells is stimulated by long-term depolarization of the plasma membrane (Schroeder et al., 1987). It has been shown that cytosolic alkalization induced by ABA inactivates K_{in} channels (Schroeder and Hagiwara, 1989; Grabov and Blatt, 1999), resulting in K^+ efflux from guard cells. K^+ channel, which function in regulation of guard cell turgor pressure, are encoded by Shaker type K^+ channel genes. Shaker type K^+ channels are the archetypal voltage-gated K^+ channels, which consist of six transmembrane domains. Arabidopsis *GOPK*, encodes the guard cell K_{out} channel, and disruption of this gene results in impaired stomatal closure in response to darkness and ABA (Hosy et al., 2003). Regarding K_{in} channels, Arabidopsis guard cells express at least four genes. Of them KAT1 has been

best characterized. Expression dominant-negative KAT1 variants suppressed K_{in} channel activity and reduced light-induced stomatal opening (Kwak et al., 2001).

1.8 plasma membrane H^+-ATPase

The proton-pumping ATPase (H^+-ATPase) of the plant plasma membrane generates the proton motive force across the plasma membrane that is necessary to activate most of the ion and metabolite transport. Stomata open through activation of a H^+ pump in guard cells responding to blue light (BL) (Assmann et al. 1985, Shimazaki et al. 1986). The BL-activated pump creates an inside-negative electrical potential across the plasma membrane and drives K^+ uptake through voltage-gated inward-rectifying K^+ channels (Schroeder et al. 1987). The H^+ pump required for stomatal opening is known to be the plasma membrane H^+-ATPase (Kinoshita and Shimazaki 1999). BL activates the H+-ATPase through phosphorylation of a penultimate threonine in the C-terminus, followed by binding of the 14-3-3 protein to the phosphorylated C-terminus in stomatal guard cells (Kinoshita and Shimazaki 1999, Kinoshita and Shimazaki 2002). ABA-dependent depolarization of the plasma membrane is suggested to be achieved, in part, by the inhibition of the plasma membrane H^+-ATPase (Shimazaki et al. 1986, Goh et al. 1996). Inhibition of the H^+-ATPase by ABA was due to a decrease in the phosphorylation level of the H^+-ATPase (Zhang et al. 2004).

1.9 Purposes of the study

To gain detail information about ABA and MeJA signaling in guard cells, the

presnt researches were carried out with the following objectives: To dissect ABA and MeJA signaling in guard cells using *Arabidopsis pyr1 pyl1 pyl2 pyl4* quadruple ABA receptor knockout mutant and a knockout mutant of the SNF1-related protein kinase, *srk2e.*

Chapter 2

Difference in Abscisic Acid Perception Mechanisms between Closure Induction and Opening Inhibition of Stomata

2.1 ABSTRACT

Abscisic acid (ABA) induces stomatal closure and inhibits light-induced stomatal opening. The mechanisms in these two processes are not necessarily the same. It has been postulated that the ABA receptors involved in opening inhibition are different from those involved in closure induction. Here, we provide evidence that four recently identified ABA receptors (PYR1, PYL1, PYL2 and PYL4) are not sufficient for opening inhibition in *Arabidopsis thaliana*. ABA-induced stomatal closure was impaired in the *pyr1 pyl1 pyl2 pyl4* quadruple ABA receptor mutant. ABA inhibition of the opening of the mutant's stomata remained intact. ABA did not induce either the production of reactive oxygen species (ROS) and nitric oxide (NO), or the alkalization of the cytosol in the quadruple mutant in accordance with the closure phenotype. Whole cell patch clamp analysis of inward-rectifying K^+ current in guard cells showed a partial inhibition by ABA, indicating that the ABA sensitivity of the mutant was not fully impaired. ABA substantially inhibited blue light-induced phosphorylation of H^+-ATPase in guard cells in both the mutant and wild type. On the other hand, in a knockout mutant of the SNF1-related protein kinase, *srk2e*, stomatal opening and closure, ROS and NO production, cytosolic alkalization, I_{Kin} inactivation, and H^+-ATPase phosphorylation were not sensitive to ABA.

2.2 INTRODUCTION

The phytohormone abscisic acid (ABA), which is synthesized in response to abiotic stresses, plays a key role in the drought hardiness of plants. Reducing transpirational water loss through stomatal pores is a major ABA response (Schroeder et al., 2001). ABA promotes the closure of open stomata and inhibits the opening of closed stomata. These effects are not simply the reverse of one another (Allen et al., 1999; Wang et al., 2001; Mishra et al., 2006).

A class of receptors of ABA was identified (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). The sensitivity of stomata to ABA was strongly decreased in quadruple and sextuple mutants of the ABA receptor genes *PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE-LIKE/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR*, *PYR/PYL/RCARs,* (Nishimura et al., 2010; Gonzalez-Guzman et al., 2012). The PYR/PYL/RCAR receptors are involved in the early ABA signaling events, in which a sequence of interactions of the receptors with protein phosphatase 2Cs (PP2Cs) and subfamily 2 SNF1-related protein kinases (SnRK2s) leads to the activation of downstream ABA signaling targets in guard cells (Weiner et al., 2010; Kim et al., 2010; Cutler et al., 2010). Studies of *Commelina communis* and *Vicia faba* suggested that the ABA receptors involved in stomatal opening are not the same as the ABA receptors involved in stomatal closure (Assmann 1994; Allan et al., 1994; Anderson et al., 1994; Schwartz et al., 1994). The roles of PYR/PYL/RCAR in either stomatal opening or closure remained to be elucidated.

Blue light induces stomatal opening through activation of plasma membrane H^+-ATPase in guard cells that generates an inside-negative electrochemical gradient across the plasma membrane and drives K^+ uptake through voltage-dependent inward-rectifying K^+ channels (Assmann et al. 1985, Shimazaki et al. 1986; Blatt, 1987; Schroeder et al., 1987; Thiel et al., 1992). Phosphorylation of the penultimate threonine (Thr) of the plasma membrane H^+-ATPase is a prerequisite for blue light-induced activation of the H^+-ATPase (Kinoshita and Shimazaki, 1999 and 2002). ABA inhibits H^+-ATPase activity through dephosphorylation of the penultimate Thr in the C-terminus of the H^+-ATPase in guard cells, resulting in prevention of the opening (Goh et al., 1996; Zhang et al., 2004; Hayashi et al., 2011). Inward-rectifying K^+ currents (I_{Kin}) of guard cells are negatively regulated by ABA, in addition to through the decline of the H^+ pump-driven membrane potential difference (Schroeder and Hagiwara, 1989; McAinsh et al. 1990; Blatt, 1990; Schwartz et al., 1994; Grabov and Blatt, 1999; Saito et al., 2008). This down-regulation of ion transporters by ABA is essential for the inhibition of stomatal opening.

A series of second messengers has been shown to mediate ABA-induced stomatal closure. Reactive oxygen species (ROS) produced by NADPH oxidases play a crucial role in ABA signaling in guard cells (Pei et al., 2000; Zhang et al., 2001; Kwak et al., 2003; Sirichandra et al, 2009; Jannat et al., 2011). Nitric oxide (NO) is an essential signaling component in ABA-induced stomatal closure (Desikan et al., 2002; Guo et al., 2003; Garcia-Mata and Lamattina, 2007; Neill et al., 2008). Alkalization of cytosolic pH (pH_{cyt}) in guard cells is postulated to mediate ABA-induced stomatal closure in *Arabidopsis*,

Pisum and *Paphiopedilum* (Irving et al., 1992; Grabov and Blatt, 1997; Gehring et al., 1997; Suhita et al., 2004; Gonugunta et al., 2008). These second messengers transduce environmental signals to ion channels and ion transporters that create the driving force for stomatal movements (MacRobbie 1998; Ward et al., 1995; Garcia-Mata et al., 2003).

In this study, we examined the mobilization of second messengers, the inactivation of I_{Kin} and the suppression of H^+-ATPase phosphorylation evoked by ABA in *Arabidopsis thaliana* mutants to clarify the downstream signaling events of ABA signaling in guard cells. The mutants included a quadruple mutant of *PYR/PYL/RCARs*, *pyr1 pyl1 pyl2 pyl4,* and a mutant of a SnRK2 kinase, *srk2e.*

2.3 Materials and Methods

2.3.1 Plant materials

Arabidopsis thaliana wild type (ecotype Columbia-0), *pyr1 pyl1 pyl2 pyl4* quadruple mutant (Park et al., 2009) and *srk2e* mutant (Yoshida et al., 2002; Umezawa et al., 2009) were grown in plastic pots filled with 70% (v/v) vermiculite (Asahi-kogyo, Okayama, Japan) and 30% (v/v) Kureha soil (Kureha Chemical, Tokyo, Japan) in a growth chamber (80 μmol m^{-2} s^{-1} of photon flux with white fluorescent tubes under a 16-h-light/8-h-dark regime). Temperature and relative humidity in the growth chamber were controlled at 22 ± 2 °C and 60 ± 10%, respectively.

2.3.2 Measurement of stomatal aperture

Stomatal closing assay: Stomatal aperture was examined as previously

described (Uraji et al., 2012). In brief, excised rosette leaves from 4- to 6-week-old plants were floated on the opening buffer containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 μmol m^{-2} s^{-1}) to pre-open stomata. Subsequently epidermal fragments were collected on a piece of nylon net (opening: 200 μm) after blending with a Waring blender. Aperture width was microscopically measured after a 2-h incubation under the illumination in the presence of ABA or the solvent control (0.1 % ethanol).

Stomatal opening assay: rosette leaves of 4- to 6-week-old dark-adapted plants (overnight in the dark, 8 hour) were blended with a Waring commercial blender before the light period and suspended in the opening buffer. After a succeeding 2-h incubation in the dark, the widths of stomatal aperture were measured, which serves as the dark control. The blended leaf specimens were subsequently incubated in the light for 2.5 h in the presence of ABA or the solvent control (0.1 % ethanol). Twenty randomly selected stomatal apertures were measured per leaf. Typically, 3 to 5 independent biological repeats were conducted. Reproducibility of the experimental results was independently ensured by one or two other experimenter(s) beside presented data.

2.3.3 Measurement of reactive oxygen species (ROS) and nitric oxide (NO) production

The production of ROS and NO in guard cells was estimated by the methods described by Munemasa et al. (2007) utilizing the fluorescence indicators, H_2DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) and DAF-2 DA (CAS 205391-02-2), respectively.

2.3.4 Measurement of cytosolic pH (pHcyt) of guard cells

A pH-sensitive fluorescent dye, BCECF-AM (CAS 117464-70-7), was used to examine the change in pH_{cyt} of guard cells as described previously (Islam et al. 2010).

2.3.5 Whole cell patch clamp recording of the inward-rectifying K^+ current (I_{Kin})

Patch clamp experiments were carried out essentially as described previously (Munemasa et al., 2007; Saito et al., 2008). Guard cell protoplasts (GCPs) were enzymatically isolated from rosette leaves of 4- to 6-week-old plants. Whole cell currents were measured using a patch-clamp amplifier (model CEZ-2200; Nihon Kohden, Tokyo). Data were acquired and analyzed with pCLAMP 8.2 software (Molecular Devices, Sunnyvale, CA). The pipette solution contained 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$, 3.35 mM $CaCl_2$, 6.7 mM EGTA, and 10 mM HEPES adjusted to pH 7.1 with Tris, and the bath solution contained 30 mM KCl, 2 mM $MgCl_2$, 40 mM $CaCl_2$, and 10 mM MES titrated to pH 5.5 with Tris. Osmolarity of the pipette solution and the bath solution was adjusted with D-sorbitol to 500 and 485 mmol kg^{-1}, respectively. In order to examine the effect of ABA, GCPs were treated with 10 μM ABA (for solvent control, 0.1 % ethanol) for 2 h before giga ohm seal establishment.

2.3.6 Phosphorylation of the penultimate threonine of the plasma membrane H^+-ATPase in guard cells

Blue light-induced phosphorylation of the penultimate Thr in the C-terminus of plasma membrane H^+-ATPase in guard cells was detected immunohistochemically using a specific antibody against the phosphorylated penultimate Thr of H^+-ATPase (anti-pThr) according to the previous report (Hayashi et al., 2011). In brief, epidermis prepared by blending rosette leaves from dark-adapted plants was incubated under background red light for 20 min. ABA or ethanol (solvent control) was added to the incubation medium, followed by a 2.5-min blue light treatment. The specimens were fixed with paraformaldehyde and stuck on a coverslip. After permeabilizing the cells, phosphorylated H^+-ATPases were visualized with anti-pThr and the Alexafluor488-labeled secondary antibody. The fluorescence of Alexafluor488 was imaged with a fluorescent microscope and semi-quantified with image J software.

2.3.7 Statistical Analysis

Significance of differences between data sets was assessed by Student's t-test analysis in all parts of this article unless otherwise stated. Difference at the level of $p < 0.05$ was regarded as significant.

2.3.8 Acknowledgements

The authors are most grateful to Dr. Taishi Umezawa and Dr. Sean Cutler for providing *srk2e* and *pyr1 pyl1 pyl2 pyl4* seeds.

2.4 RESULTS

2.4.1 Differential responses of *pyr1 pyl1 pyl2 pyl4* quadruple mutant in ABA-induced stomatal closure and ABA-inhibited stomatal opening

The effects of exogenous ABA on stomatal movements were examined in the wild type and the *pyr1 pyl1 pyl2 pyl4* quadruple ABA receptor knockout mutant. Stomatal closure was induced when ABA was externally applied to fully open stomata of the wild type. By contrast, ABA-induced stomatal closure was incomplete in the quadruple mutant (Fig. 1A, supplemental Fig. 1), essentially as reported previously (Nishimura et al., 2010). Aperture width of the pre-opened stomata in the light was wider in the quadruple mutant than in the wild type. This indicates that ABA sensitivity was different between stomata of wild type and the mutant with respect to the degree of closure induction.

We next examined the inhibition of light-induced stomatal opening. Exogenously applied ABA to the dark-adapted epidermis strongly inhibited subsequent light-induced stomatal opening in wild type (Fig 1B). ABA inhibited the opening of the mutant's stomata to a similar extent, in contrast to its effects on closure induction (Fig. 1B). The mean aperture of the mutants was wider than that of the wild type in the absence of ABA.

In the quadruple mutant, the ABA responsivity of stomatal movements appeared to be different between opening and closure. The above results suggest that different receptors are involved in ABA-induced closure and ABA-inhibited opening.

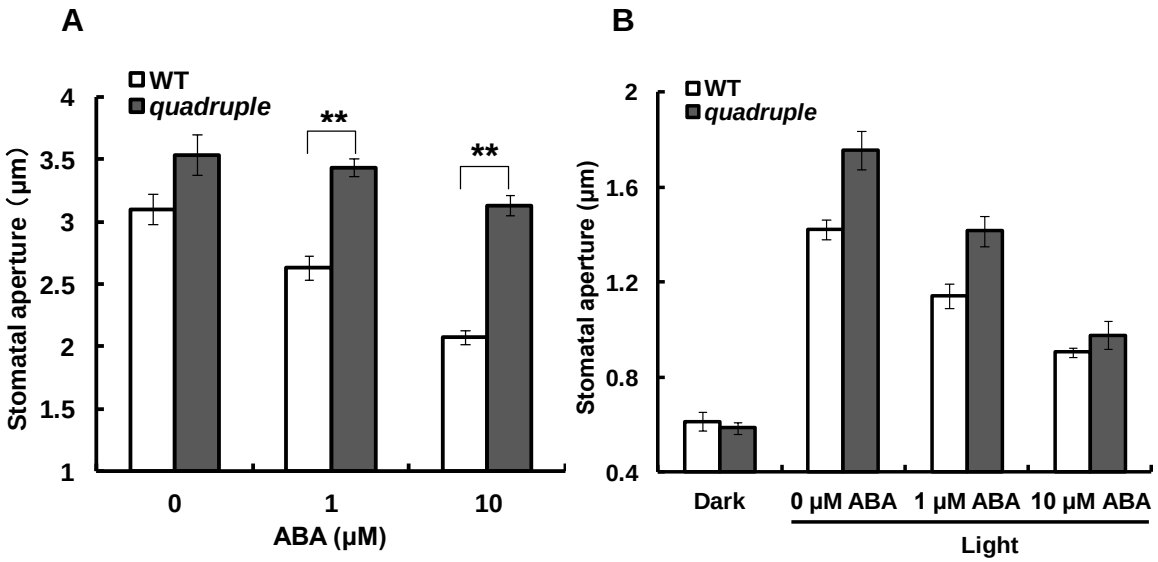

Fig. 2.1. Induction of stomatal closure and inhibition of light-induced stomatal opening by ABA in wild type and *pyr1 pyl1 py2 pyl4* quadruple mutant. A, ABA-induced stomatal closure in wild-type (WT) and the *quadruple* mutant (*quadruple*). Averages from five independent experiments are (n = 5, 100 total stomata) shown. B, ABA inhibition of stomatal opening in wild-type (WT) and *quadruple* plants. Dark represents fully dark-adapted stomata. Control (0 μM ABA) indicates 2.5-h light-treatment after the dark adaption with 0.1% ethanol as the solvent control. 1 and 10 μM ABA represents an ABA addition at same time of the initiation of light-treatment. Averages from three independent experiments are (n = 3, 60 total stomata) shown. ** indicates a significant difference (α = 0.01) by Student's t-test. A data set obtained by Y.Y. is shown out of the results carried out by 3 independent examiners, Y.Y., Y.A. and I.C.M. obtaining essentially same results. Error bars represent standard error.

2.4.2 Impairment of ROS and NO productions, and cytosolic alkalization in the *quadruple* mutant

ABA-induced stomatal closure is accompanied by the productions of ROS and NO and an increase of cytosolic pH in guard cells (Irving et al., 1992; Blatt and Armstrong, 1993; Neill et al., 2002; Kwak et al., 2003). ROS and NO have been shown to act as second messengers (Pei et al., 2000; Neill et al., 2002). Exogenous ABA induced an increase of H_2O_2, as shown by 2',7'-dichlorofluorescin (DCF) fluorescence, in wild-type guard cells (Fig 2A) in accordance with previous results (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003; Munemasa et al., 2007). Unlike the wild type, the quadruple mutant showed no elevation of H_2O_2 level (Fig. 2A). NO production in guard cells, as shown by diaminofluorescein (DAF) fluorescence, was induced by ABA in the wild-type but not in the mutant (Fig. 2B). ABA elicited cytosolic alkalization, as shown by an increase of BCECF fluorescence, in guard cells in the wild type but not in the mutant (Fig. 2C).

2.4.3 ABA inhibition of I_{Kin} in guard cells

Inactivation of inward-rectifying K^+ (K^+_{in}) channels is one of the key events in the repression of stomatal opening (Blatt and Thiel, 1994; Schwartz et al., 1994; Eisenach et al, 2012). Here we employed the whole cell patch clamp technique to examine ABA inactivation of I_{Kin} in isolated guard cell protoplasts (GCPs). Without ABA treatment, no significant difference in amplitude of I_{Kin} was observed between wild-type and quadruple mutant GCPs (Fig. 3A-C). Treatment of GCPs with 10 μM ABA for 2 hours significantly reduced I_{Kin} in the wild type. I_{Kin}

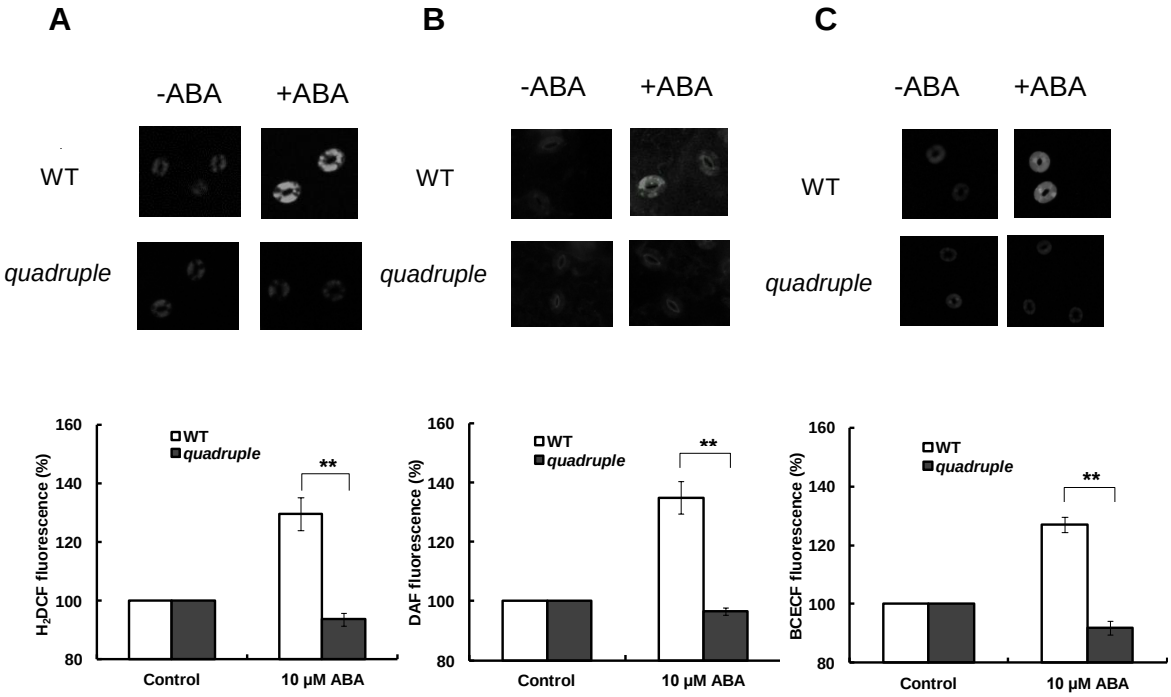

Fig. 2.2. ABA-induced ROS and NO production and cytosolic alkalization in wild-type (WT) and *pyr1 pyl1 pyl2 pyl4* (*quadruple)* mutant guard cells. A, ROS production. The representative gray-scale H_2DCF fluorescence images (top panel) and vertical scale represents the percentage of H_2DCF fluorescence levels (bottom panel). B, NO production. Representative gray-scale DAF fluorescence images (top panel) and DAF fluorescence levels (bottom panel). C, cytosolic alkalization. Representative BCECF images (top panel) and BCECF fluorescence levels (pH, bottom panel). Fluorescent intensities are normalized to the control value taken as 100% for each experiment. The epidermis was pre-loaded with fluorescent dyes before treatments. Ten μM ABA or 0.1% ethanol (solvent control) was added to the suspension of epidermal preparations for 20 min. Bars indicate averages of five independent experiments (n = 5, 100 total guard cells per bar). ** indicates a significant difference (α = 0.01) by Student's t-test. Error bars represent standard error.

was significantly but partially reduced in the quadruple mutant. This indicates that PYR1, PYL1, PYL2 and PYL4 ABA receptors are partially involved in the inactivation of I_{Kin}. However, these receptors are not the only factors upstream of the I_{Kin} regulation.

2.4.4 Inhibition of blue light-induced phosphorylation of plasma membrane H+-ATPase in guard cells by ABA

We examined the inhibition of phosphorylation of H^+-ATPase in the guard cells of the quadruple mutant by immunohistochemical staining (Hayashi et al., 2011). In red light, the fluorescence intensity, an indication of phosphorylation of H^+-ATPase, was low in the guard cells of the quadruple mutant and wild type (Fig. 4A, left panels). Addition of blue light over the background red light increased the phosphorylation level both in wild type and the mutant to a similar extent (Fig. 4A, middle panels). When 10 μM ABA was included, the blue light-induced H^+-ATPase phosphorylation was substantially inhibited in the wild type, as previously reported (Hayashi et al., 2011), as well as in the mutant (Fig. 4A, right panels). Fig. 4B shows the result of semi-quantitative analysis of the fluorescence images. The fluorescence was increased by 5-8 times by blue light treatment. ABA inhibited the phosphorylation and the significant difference was not observed. Together, these results suggest that ABA inhibition of stomatal opening in the mutant was intact.

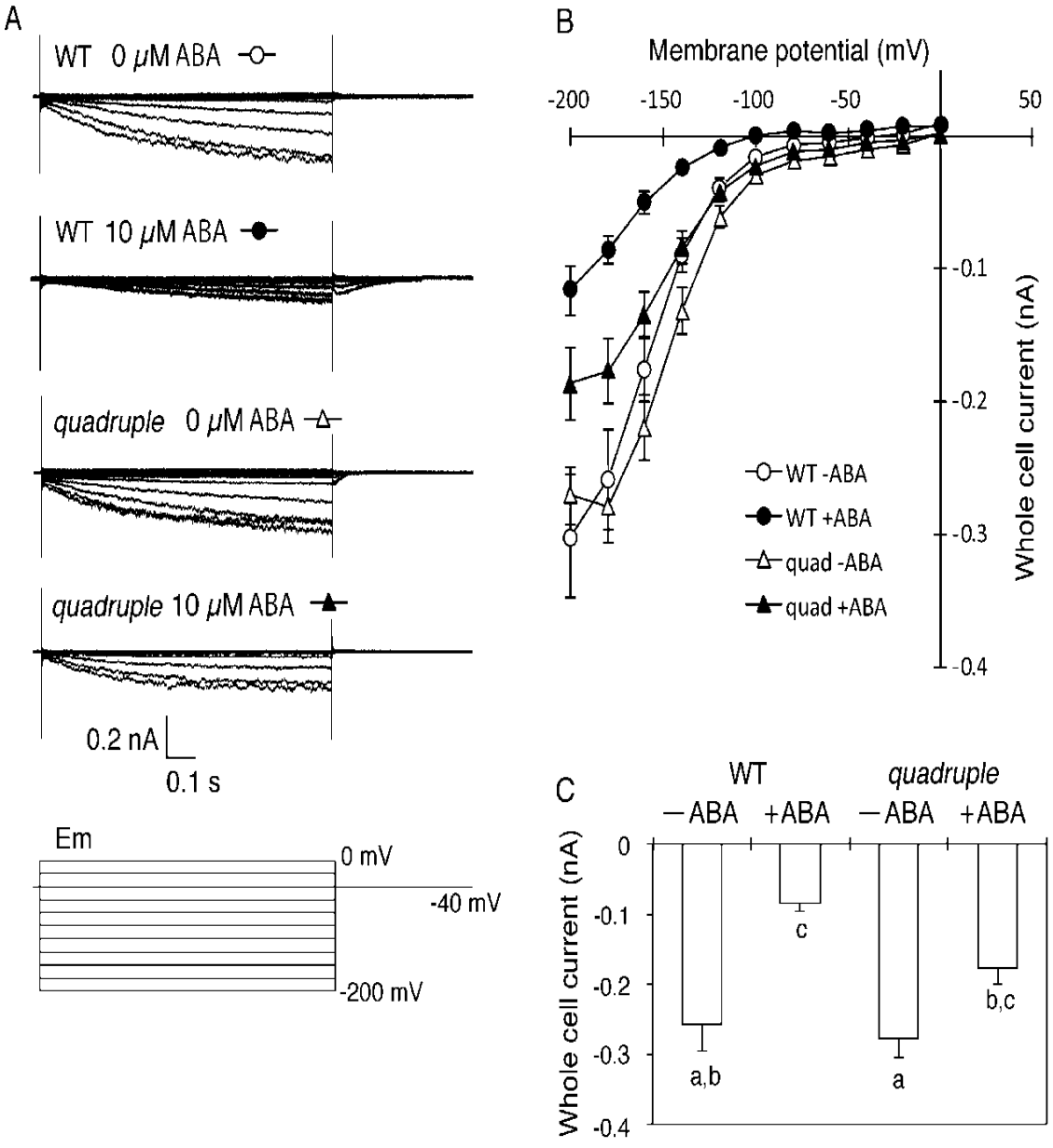

Fig. 2.3. Inactivation of inward-rectifying K^+ channel current by ABA in wild-type and *pyr1 pyl1 pyl2 pyl4* (*quadruple)* guard cell protoplasts. A, Typical raw traces of inward-rectifying K^+ channel current (I_{Kin}) of wild type (WT) and the quadruple mutant with (+) and without (-) 10 μM ABA. The bottom panel indicates the applied step pulse membrane voltage (Em) protocol. B, Current-voltage curve of

I_{Kin}. Open circles, WT without ABA (n =12); closed circles WT with 10 µM ABA (n = 7); open triangles, quadruple mutant without ABA (n = 20); closed triangles, quadruple mutant with 10 µM ABA (n = 14). C, Whole cell current at -180 mV represented from (B). Letters below bars show subgroups indicated by ANOVA with Tukey HSD post-hoc analysis (α = 0.05).

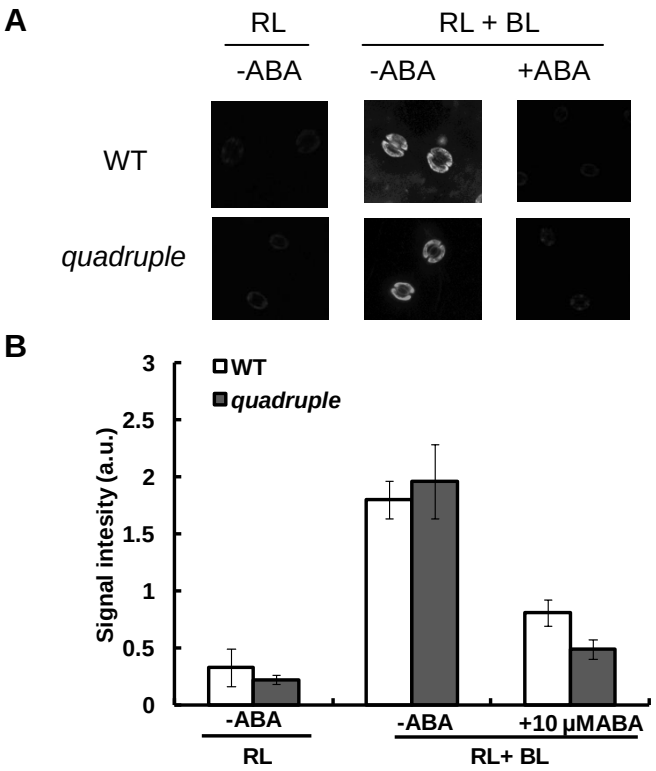

Fig. 2.4. Inhibition of blue light-induced phosphorylation of H^+-ATPase by ABA in wild-type (WT) and *pyr1 pyl1 pyl2 pyl4* (*quadruple)* guard cells. Typical fluorescence images of stomata from the wild type and *quadruple* mutant (A). Quantification of fluorescence images of stomata determined (B). Fluorescence intensity of guard cells visualized with anti-pThr antiserum and Alexa Fluor 488-conjugated secondary antibody was semi-quantified as described in Materials and Methods. Isolated epidermal fragments were illuminated with red light (RL) for 20 min and successively illuminated with RL or blue light with RL (RL + BL) for 2.5 min. Where indicated, 10 µM ABA was added immediately before RL + BL treatment. Bars indicate averages of four independent experiments (n = 4, 120 total guard cells per bar). Error bars represent standard error. a.u. indicates arbitrary unit. We detected no significant difference between

the WT and *quadruple* mutant by Student's t-test (α = 0.05).

2.4.5 ABA responses of guard cells in *srk2e* mutant

A key node in the ABA signaling network includes OST1/SnRK2.6/SRK2E, which is an ABA-activated protein kinase predominantly expressed in *Arabidopsis* guard cells, protein phosphatase 2Cs and the PYR/PYL/RCAR ABA receptors (Yoshida et al., 2006; Hirayama and Shinozaki, 2007; Weiner et al., 2010; Nishimura et al., 2010; Cutler et al., 2010). We examined stomatal phenotypes, second messengers, I_{Kin} inactivation and H^+-ATPase phosphorylation in an *OST1/SnRK2.6/SRK2E* null mutant.

ABA-induced stomatal closure was abolished in the presence of 1 and 10 μM ABA in *srk2e* (Fig. 5A) as well as in the *pyr1 pyl1 pyl2 pyl4* quadruple mutant (Fig. 1A). Light-induced stomatal opening was not inhibited by 1 or 10 μM ABA in *srk2e* (Fig. 5B), which is different from the result of the quadruple mutant (Fig. 1B).

ABA-induced stomatal closure is accompanied by ROS production (Kwak et al., 2003). Ten μM ABA did not induce ROS production in the *srk2e* (Fig. 6) or the *pyr1 pyl1 pyl2 pyl4* (Fig. 2), indicating that SRK2E kinase, like the PYR1, PYL1, PYL2 and PYL4 ABA receptors, is involved in ABA-induced stomatal closure.

Pre-treatment of GCPs with 10 μM ABA for 2 hours did not significantly inhibit I_{Kin} in *srk2e* (Fig. 7) but inhibited I_{Kin} in the *pyr1 pyl1 pyl2 pyl4* (Fig. 3), suggesting that SRK2E, unlike PYR1, PYL1, PYL2 and PYL4, is involved in ABA-inhibition of stomatal opening. The amplitude of I_{Kin} of *srk2e* was smaller

than that of WT in the absence of ABA (-146 ± 28 and -279 ± 27 pA at -180 mV, respectively; n = 20 for Col-0 and n = 5 for *srk2e*, $p < 0.01$) (Fig. 3 and Fig. 7). This may be attributed to the regulation of expression or post-translational modification of K_{in} channels by the impairment of OST1-mediated ABA signaling.

srk2e, unlike the wild type and the quadruple mutant, did not show ABA inactivation of light-induced phosphorylation of H^+-ATPase (Fig. 8). Together, these results show that the H^+-ATPase phosphorylation level is well correlated with stomatal opening inhibition by ABA in all examined plant lines, wild type, the quadruple mutant and *srk2e*.

2.5 DISCUSSION

In this study we examined the differences in ABA signaling components during open inhibition and closure induction in the quadruple *pyr/pyl/rcar* ABA receptor mutant of *Arabidopsis thaliana*. Our results demonstrate that ABA receptors PYR1, PYL1, PYL2 and PYL4, which control ABA-induced stomatal closure, are not strongly involved in the inhibition of stomatal opening by ABA. This suggests that one or more other ABA receptors are involved in the inhibition of stomatal opening. This difference should make it easier to identify the ABA receptors involved in inhibition of stomatal opening by ABA.

2.5.1 ABA receptors involved in opening inhibition have not been identified

In 2009, PYR/PYL/RCAR proteins, which constitute a 14-member family, were identified as ABA receptors in *Arabidopsis* (Ma et al., 2009; Park et al.,

2009). All of them except PYL13 were able to activate ABA-responsive gene expression in protoplast transfection assays (Fujii and Zhu, 2009). PYR1, PYL1, PYL2 and PYL4 appear to be predominately expressed in guard cells and their

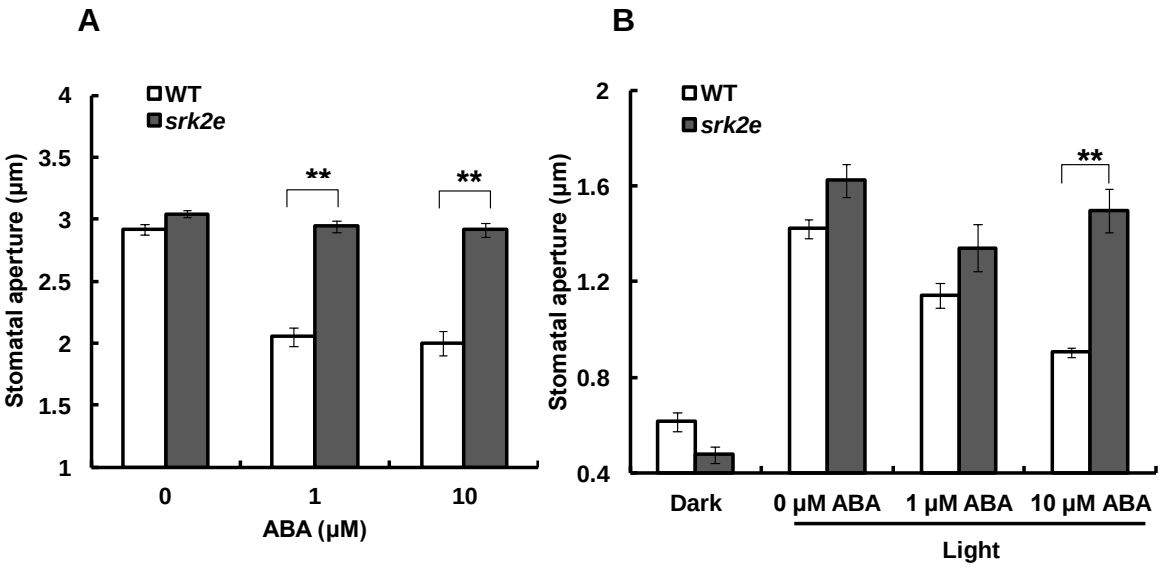

Fig. 2.5. Induction of stomatal closure and inhibition of light-induced stomata opening by ABA in wild type and *srk2e* mutant. A, ABA-induced stomatal closure in wild-type (WT) and *srk2e* plants. Averages from eight independent experiments (n = 8, 160 stomata per bar) are shown. B, ABA-inhibition of stomatal opening in wild-type (WT) and *srk2e* plants. Dark represents fully dark-adapted stomata. Control (0 μM ABA) indicates 2.5-h light-treatment after the dark adaption with 0.1% ethanol as the solvent control. 1 and 10 μM ABA represents an ABA addition at same time of the initiation of light-treatment. Averages from three independent experiments are shown (n = 3, 60 stomata per bar). ** indicates a significant difference (α = 0.01) by Student's t-test. Error bars represent standard error.

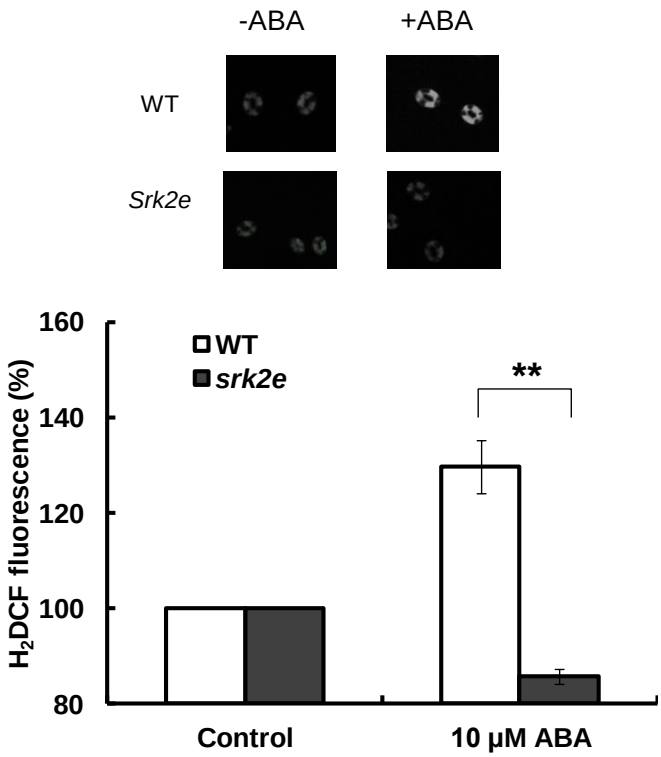

Fig. 2.6. ABA-induced ROS production in wild-type and *srk2e* guard cells. The representative gray-scale H_2DCF fluorescence images (top panel) and vertical scale represents the percentage of H_2DCF fluorescence levels (bottom panel), when fluorescent intensities of ABA-treated cells are normalized to the control value taken as 100% for each experiment. Ten μM ABA or solvent control (0.1% ethanol) was added to the epidermal preparation for 20 min. Wild type (WT) and *srk2e*. Bars indicate averages of five independent experiments (n = 5, 100 total guard cells per bar). ** indicates a significant difference (α = 0.01) by Student's t-test. Error bars represent standard error.

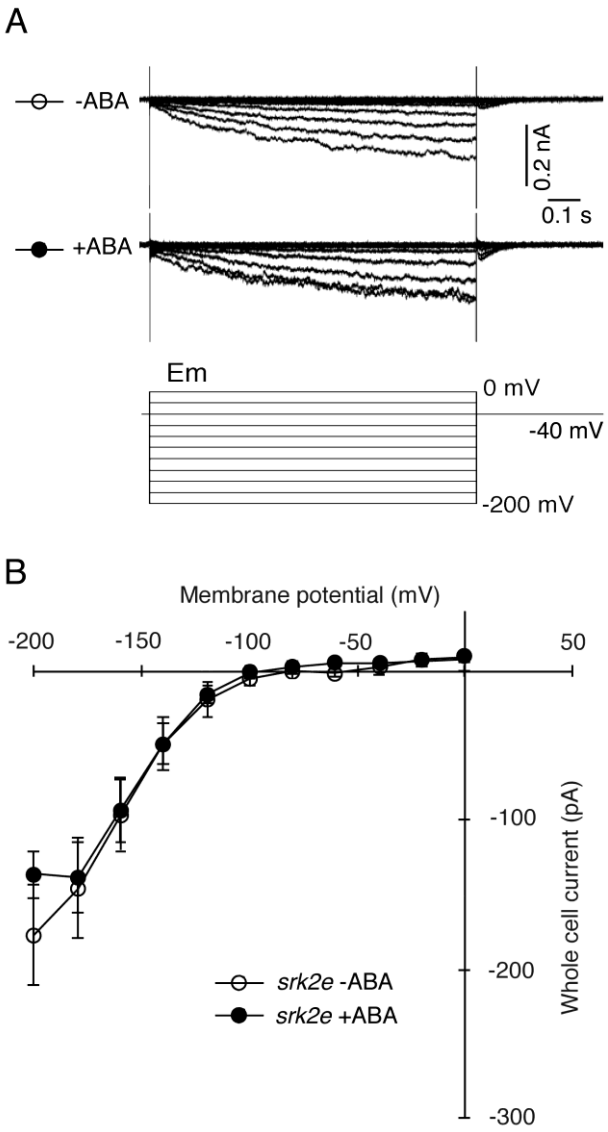

Fig. 2.7. Inactivation of inward-rectifying K^+ channel current (I_{kin}) by ABA in *srk2e* guard cell protoplasts. A, Representative current in the absence (-ABA) and presence (+ABA) of 10 μM ABA. The bottom panel (Em) indicates applied step pulse protocol. B, Current-voltage curve of I_{Kin}. GCPs of *srk2e* were treated with 0.1% ethanol (solvent control, open symbols, n= 5) or 10 μM ABA (closed symbols, n = 6). Error bars indicate standard error.

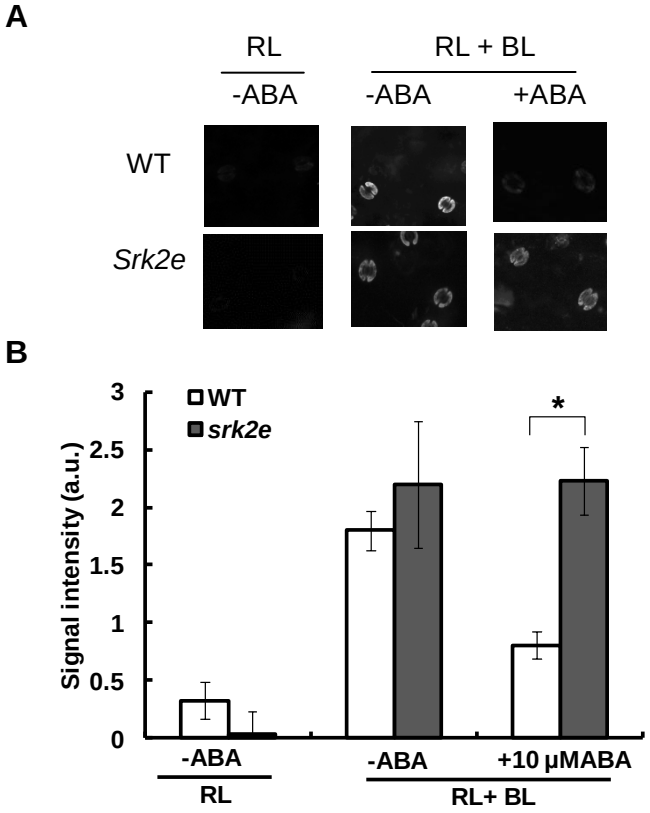

Fig. 2.8. ABA-inhibition of blue light-induced phosphorylation of H^+-ATPase in wild-type and *srk2e* guard cells. Typical fluorescence images of stomata using anti-H^+-ATPase in the epidermis from the wild type and *srk2e* mutant (A). Quantification of fluorescence images of stomata determined (B). Fluorescence intensity of guard cells visualized with anti-pThr antiserum and Alexa Fluor 488-conjugated secondary antibody was semi-quantified (a.u., arbitrary unit). Isolated epidermal fragments were illuminated with red light (RL) for 20 min and subsequently illuminated with RL or blue light with RL (RL + BL) for 2.5 min. Where indicated, 10 µM ABA was added immediately before RL + BL treatment. Bars indicate averages of four independent experiments (n = 4, 120 total guard cells per bar). * indicates a significant difference (α = 0.05) by Student's t-test. Error bars represent standard error.

disruption resulted in insensitivity to ABA in the stomata (Nishimura et al., 2010). The involvement of other ABA receptors in opening inhibition is deduced.

Stomata of a plasma membrane ABA uptake transporter mutant, *abcg40*, opened faster than the wild type in the presence of 1 µM ABA (Kang et al., 2010), indicating that ABA that was transported to the insides of the cells functioned in opening inhibition. This suggests that the unidentified ABA receptor localizes in the cells. Moreover, an excised inside-out patch clamp analysis of Ca^{2+}-permeable channels in *V. faba* GCPs revealed that the ABA perception sites were on the cytosolic side of the patched membrane (Hamilton et al., 2000). Hence, some of the other ten PYR/PYL/RCAR members, which are relatively low in abundance compared to the 4 PYR/PYL/RCARs in guard cells and localized inside of the cell, might be involved in the opening inhibition.

Microinjection experiments have suggested that the extracellular ABA perception sites mainly function in opening inhibition, while intracellular sites are essential for closure induction (Schwartz et al., 1994; Anderson et al., 1994; Assmann, 1994). One can assume that the four Arabidopsis PYR/PYL/RCARs are the intracellular ABA receptors and an unidentified extracellular ABA receptor remains functional in the quadruple mutant.

2.5.2 ABA responses of stomatal movements: opening and closure are regulated differentially

Based on studies in which plants were exogenously treated with NO and H_2O_2, both compounds were reported to be involved in the signaling process of inhibition of light-induced stomatal opening (Yan et al., 2007; Garcia-Mata and

Lamattina, 2007). We found that ABA did not induce the production of ROS or NO, or cytosolic alkalization in the quadruple mutant guard cells (Fig. 2), suggesting that production of ROS and NO, and cytosolic alkalization in guard cells function downstream of PYR1, PYL1 PYL2 and PYL4. ROS and NO production and cytosolic alkalization may only partly function in inhibition of stomatal opening. The partial inactivation of I_{Kin} in the quadruple mutant (Fig. 3) may be due to NO or ROS, which are known to inhibit I_{Kin} (Köhler et al., 2003; Garcia-Mata et al., 2003). Alkalization may also be involved in the regulation of I_{Kin} (Blatt, 1992; Blatt and Armstrong, 1993; Grabov and Blatt, 1997; Wang et al., 2012). In this study, the partial inactivation of I_{Kin} in the mutant could not have been caused by a pH change because the pipette solution was buffered in our patch clamp experiments. However, the possibility that pH is involved in inactivation of K_{in} channels under the four PYR/PYL/RCAR receptors could not be excluded. The finding that H^+-ATPase was inactivated in the quadruple mutant (Fig. 4) indicates that the regulation of H^+-ATPase is not a downstream event of H_2O_2 production, NO production and alkalization.

Non-ratiometric measurements of BCECF fluorescence revealed that cytosolic pH was not increased by ABA in the quadruple mutant (Fig. 2). This suggests that ABA-induced alkalization is impaired in the mutant or that the cytosolic pH in the mutant is constitutively higher than that in the wild type due to derepression of ABA signaling.

Modulation of I_{Kin} is closely related with kinetics of stomatal opening (Eisenach et al. 2012) and I_{Kin} decreases with increasing cytosolic pH (Grabov and Blatt, 1997; Wang et al., 2012). On the other hand, ABA inhibition of

stomatal opening did not appear to be affected in the quadruple mutant (Fig. 1B), in which cytosolic pH varies little (Fig. 2). There results indicate that the inhibition of stomatal opening is mainly caused by the suppression of H^+-ATPase activity and subsidiarily by the inactivation of I_{Kin} by cytosolic alkalization.

In a protein phosphatase 2C muntant *abi1-1*, ABA does not induce stomatal closure but NO production (Desikan et al., 2002). On the other hand, ABA-induced H_2O_2 accumulation was observed in *abi1-1* but not in *abi2-1* (Murata et al., 2001). These complicated facts suggest that PYR/PYL/RCAR in guard cells interact with PP2Cs in such way that PP2Cs function in differentiated signal branches in the ABA signaling. Another possibility is that the complicated phenotype is caused by abnormal specificities of substrate of semi dominant mutants, *abi1-1* and *abi2-1*.

The apertures of open stomata in Fig. 1B were even narrower than those of closed stomata in Fig. 1A. This is attributed to the difference in the methods of sample preparation between closure assay and opening assay. For the closure assay, the whole leaves were incubated in the opening buffer (Materials and Methods) and then blended immediately before the observation. On the other hand, the blended epidermal fragment was obtained first and then incubated in the opening buffer in order to examine opening inhibition.

In the Arabidopsis quadruple mutant, the sensitivity to ABA of the light-induced stomatal opening was impaired (Nishimura et al., 2010), which is inconsistent with the present finding that the sensitivity was intact in the quadruple mutant (Fig. 1B). Stomata exhibit light hysteresis: after light illuminates a leaf, stomatal conductance increases and subsequent dark

treatment cannot fully reduce stomatal conductance to the original dark level (Ng and Jarvis, 1980). Our preliminary experiments show that irradiation for an hour makes stomata partially open even after a 3 hours dark treatment. The incomplete closure is likely a hysteresis of stomata to light. In order to avoid this artifact, we used dark-adapted plants. The difference may also be due to difference of growth environments in the two laboratories.

2.5.3 PYR/PYL/RCAR receptors and OST1

OST1 kinase is reported to play a key role to phosphorylate many substrates in the ABA responses of Arabidopsis stomata (Mustilli et al., 2002; Yoshida et al., 2002; Fujii and Zhu, 2009; Nakashima et al., 2009) and AAPK has a similar function in *V. faba* guard cells (Li et al., 1996; Mori et al., 1997; Li et al., 2000). It was proposed that the activity of OST1 kinase is regulated with the complex of PYR/PYL/RCAR receptors and PP2C (Weiner et al., 2010; Kim et al., 2010; Cutler et al., 2010). We examined ABA inhibition of stomatal opening of a mutant of OST1/SnRK2.6/SRK2E. In this study *srk2e*, which is a T-DNA insertion mutant of the Columbia ecotype (Yoshida et al., 2002) was used instead of *ost1-2*. *ost1-2* is a mutant derived from Lansberg *erecta* (Mustilli et al., 2002). The quadruple mutant was generated from three Columbia background mutants and one Lansberg background mutant (Park et al., 2009).

In contrast to the quadruple mutant, the *srk2e* mutant was insensitive to ABA in inhibition of opening. These results suggest that the functions of PYR1, PYL1, PYL2 and PYL4 in ABA-induced stomatal closure and ABA inhibition of stomatal opening in Arabidopsis are different from those of OST1 as shown in

Figure 9. PYR1, PYL1, PYL2 and PYL4 bind ABA and activate OST1 kinase through inhibition of PP2C (Cutler et al., 2010). One of the functions of the activated OST1 kinase is to induce the production of ROS and NO and alkalization of cytosolic pH (Fig. 6 and 7). These signaling events activate the stomatal closure machinery. At the same time, to inhibit opening, other PYR/PYL/RCAR members or an unidentified extracellular ABA receptor might bind ABA and activate OST1 through an unknown mechanism. The OST1 kinase activated by the unknown mechanism regulates the activities of the K_{in} channel and H^+-ATPase, independently of the PYR1 PYL1 PYL2 PYL4-dependent pathway.

Several substrates of OST1/AAPK have been reported. NADPH oxidase is a substrate (Sirichandra et al., 2009). ROS production was revealed to function downstream of the 4 PYR/PYL/RCAR receptors (Fig. 2). SLAC1 slow anion channel (Negi et al., 2008; Vahisalu et al., 2008) and ALMT12 rapid anion channel (Sasaki et al., 2010; Meyer et al., 2010) are substrates of OST1/AAPK (Li et al., 2000; Geiger et al., 2009; Imes et al., 2013). These anion channels are involved in stomatal closure and most likely function under the PYR/PYL/RCAR-dependent pathway. The inward-rectifying K^+ channel is also a substrate of OST1/AAPK (Mori et al., 2000; Sato et al., 2009). The phosphorylation of the K_{in} channel inactivates I_{Kin} (Sato et al., 2009). This agrees with the lack of I_{Kin} regulation in *srk2e* mutant (Fig. 7). Other OST1/AAPK substrates, which are involved in H^+-ATPase activity regulation, would play roles under the unknown ABA receptor(s) in opening inhibition.

In plants other than Arabidopsis, guard cell SnRK2 kinases may be

regulated by other factors, such as SnRK2-interacting Ca^{2+} sensor protein (Bucholc et al., 2011), glyceroaldehyde-3-phosphate dehydrogenase (Wawer et al., 2010) and phosphatidic acid (Testerink et al., 2004). These factors may be involved in PYR1 PYL1 PYL2 PYL4-independent (or -dependent) ABA signaling in *Arabidopsis* guard cells may interact with the unknown ABA receptors.

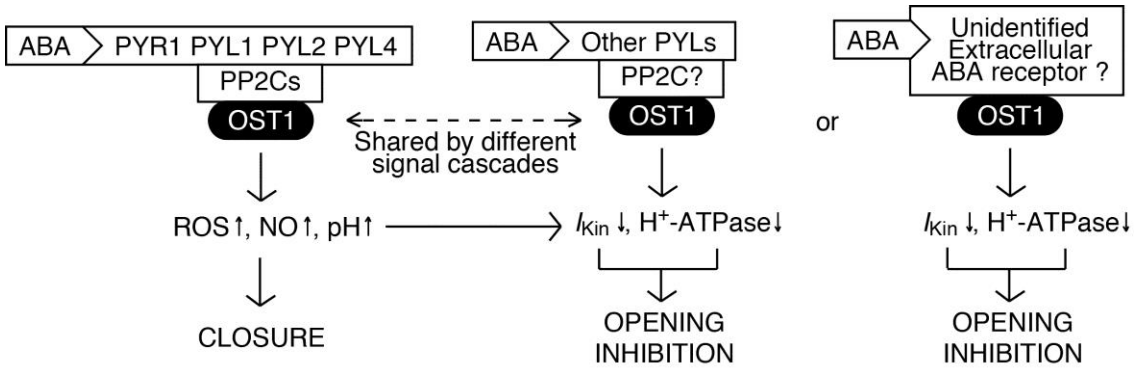

Fig. 2.9. A simple model of the difference in abscisic acid perception mechanisms between closure induction and opening inhibition of stomata in *Arabidopsis* guard cells.

Chapter 3

Methyl Jasmonate Signaling in Arabidopsis Guard Cells

3.1 ABSTRACT

It was suggested that abscisic acid (ABA) is a prerequisite for methyljasmonic acid (MeJA)-induced stomatal closure in the previous study. However, the crosstalk of ABA signaling and MeJA signaling in guard cells remains unclear. In this study, we investigated the involvement of the ABA receptors, PYR1, PYL1, PYL2 and PYL4, and the ABA-activated protein kinase, OST1/SRK2e*,* in MeJA signaling in guard cells. MeJA-induced stomatal closure was not impaired in *pyr1 pyl1 pyl2 pyl4* quadruple mutant, but substantially suppressed in *srk2e* mutant. MeJA-induced reactive oxygen species (ROS) production, nitric oxide (NO) production and cytosolic alkalization were intact in the quadruple mutant. In *srk2e* mutant, MeJA-induced ROS production was completely abolished. Whole cell patch-clamp analysis revealed that inhibition of inward-rectifying K^+ channel by MeJA was impaired in srk2e, unlike wild type and *pyr1 pyl1 pyl2 pyl4*. These results strongly suggest that PYR1 PYL1 PYL2 and PYL4 ABA receptors are not involved in MeJA signaling leading to stomatal closure, however OST1/SRK2E is involved in MeJA signaling in guard cell.

3.2 NTRODUCTION

Stomatal pores, which are formed by a pair of guard cells, respond to various environmental stimuli. Guard cells integrate these environmental signals and transduce the integrated intracellular signal to ion transporters, which drive stomatal movement Many studies have revealed many signaling components in guard cell signaling. However, the crosstalk mechanism that concerts various signals in guard cells has not been understood well.

Methyl jasmonate (MeJA), a linolenic acid derivative, regulates many physiological processes of plant growth and development, and mediates various plant defense responses. Previous studies have demonstrated that MeJA as well as abscisic acid (ABA) induces stomatal closure in several plant species, such as *Arabidopsis thaliana* (Gehring te al., 1997; Liu et al., 2002; Suhita et al., 2004; Munemasa et al., 2007, 2011; Saito et al., 2008; Jannat et al., 2011; Islam et al., 2009).

COI1 is the jasmonic acid (JA)-Ile receptor that is essential for JA-mediated plant immune response (Yan et al., 2009; Melotto et al. 2006) showed that COI1 negatively regulates ABA-induced stomatal closure, through the action of the bacterial toxin, coronatine. On the other hand, the jasmonate insensitive *Arabidopsis* mutant, *jar1-1* (Suhita et al., 2004) and *coi1* (Munemasa et al., 2007) were shown to lack the ability to close stomata in response to MeJA.

A line of evidence has shown that many ABA-insensitive mutants exhibit MeJA insensitivity in stomata, indicating ABA signaling and MeJA signaling in guard cells shares same signaling components (Suhita et al. 2004;Munemasa et al. 2011). It was reported that a very low concentration of ABA is a prerequisite

for MeJA-induced stomatal closure (Hossain et al., 2011). Stomata of the ABA-insensitive mutant, *ost1-2,* is insensitive to ABA but not MeJA (Suhita et al., 2004). The crosstalk of ABA signaling and MeJA signaling in guard cells has not been well elucidated to date.

Recently, the ABA receptor family was identified (Ma et al., 2009; Park et al., 2009). ABA-induced stomatal closure was impaired in the quadruple mutant of ABA receptors, *pyr1 pyl1 pyl2 pyl4* (Nishimuta et al., 2010; Yin et al., in preparation). In this study, we investigated MeJA response in the ABA receptor quadruple mutant and a ost1/snrk2.6/srk2e mutant to dissect the cross talk of ABA and MeJA in guard cells. We examined the mobilization of second messengers, the inactivation of I_{Kin} as well as stomatal closure.

3.3 Materials and Methods

3.3.1 Plant materials

Arabidopsis thaliana wild type (ecotype Columbia-0), *pyr1 pyl1 pyl2 pyl4* quadruple mutant (Park et al., 2009) and *srk2e* mutant (Yoshida et al., 2002; Umezawa et al., 2009) were grown in plastic pots filled with 70% (v/v) vermiculite (Asahi-kogyo, Okayama, Japan) and 30% (v/v) Kureha soil (Kureha Chemical, Tokyo, Japan) in a growth chamber (80 μmol m^{-2} s^{-1} of photon flux with white fluorescent tubes under a 16-h-light/8-h-dark regime). Temperature and relative humidity in the growth chamber were controlled at 22 ± 2 °C and 60 ± 10%, respectively.

3.3.2 Measurement of stomatal aperture

Stomatal closure was examined as previously described (Uraji et al., 2012). In brief, excised rosette leaves from 4- to 6-week-old plants were floated on the opening buffer containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 μmol m^{-2} s^{-1}) to pre-open stomata. Aperture width was microscopically measured after a 2-h incubation under the illumination in the presence of MeJA or the solvent control (0.1 % ethanol).

3.3.3 Measurement of reactive oxygen species (ROS) and nitric oxide (NO) production

The production of ROS and NO in guard cells was estimated by the methods described by Munemasa et al. (2011) utilizing the fluorescence indicators, H_2DCF-DA (2',7'- dichlorodihydrofluorescein diacetate) and DAF-2 DA (CAS 205391-02-2), respectively.

3.3.4 Measurement of cytosolic pH (pHcyt) of guard cells

A pH-sensitive fluorescent dye, BCECF-AM (CAS 117464-70-7), was used to examine the change in pH_{cyt} of guard cells as described previously (Islam et al. 2010).

3.3.5 Whole cell patch clamp recording of the inward-rectifying K^+ current (I_{Kin})

Patch clamp experiments were carried out essentially as described previously (Munemasa et al., 2007; Saito et al., 2008). Guard cell protoplasts

(GCPs) were enzymatically isolated from rosette leaves of 4- to 6-week-old plants. Whole cell currents were measured using a patch-clamp amplifier (model CEZ-2200; Nihon Kohden, Tokyo). Data were acquired and analyzed with pCLAMP 8.2 software (Molecular Devices, Sunnyvale, CA). The pipette solution contained 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$, 3.35 mM $CaCl_2$, 6.7 mM EGTA, and 10 mM HEPES adjusted to pH 7.1 with Tris, and the bath solution contained 30 mM KCl, 2 mM $MgCl_2$, 40 mM $CaCl_2$, and 10 mM MES titrated to pH 5.5 with Tris. Osmolarity of the pipette solution and the bath solution was adjusted with D-sorbitol to 500 and 485 mmol kg^{-1}, respectively. In order to examine the effect of MeJA, GCPs were treated with 10 μM MeJA (for solvent control, 0.1 % ethanol) for 2 h before giga ohm seal establishment.

3.3.6 Statistical Analysis

Significance of differences between data sets was assessed by Student's t-test analysis in all parts of this article unless otherwise stated. Difference at the level of $p < 0.05$ was regarded as significant.

3.4 RESULTS

3.4.1 MeJA-induced stomatal closure impaired in the *srk2e* mutant but not impaired in pyr1 pyl1 pyl2 pyl4 quadruple mutant

The effects of exogenously applied MeJA on stomatal closure were examined in wild type, *pyr1 pyl1 pyl2 pyl4* quadruple mutant and *srk2e* mutant. MeJA at bouth 1 μM and 10 μM significantly induced stomatal closure in WT, as well as in *quadruple* mutant (Fig. 1A). By contrast, MeJA-induced stomatal

closure was impaired in *srk2e* in the presence of 1 and 10 μM MeJA (Fig. 1B).

We observed significant difference in wild type and the quadruple mutant at 0, 1, 10 μM MeJA, but in wild type and *srk2e* mutant at 1, 10 μM MeJA in stomatal aperture. (Student t-test, α = 0.05).

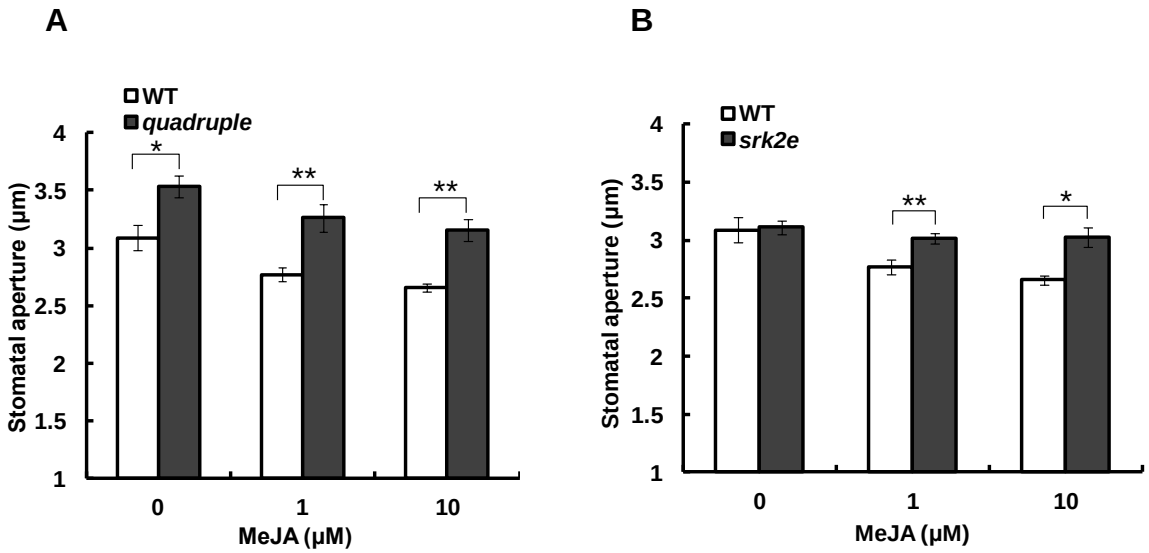

Fig. 3.1. Induction of stomatal closure by MeJA in wild type and *pyr1 pyl1 pyl2 pyl4* quadruple mutant and *srk2e* mutant. A, MeJA-induced stomatal closure in wild-type (WT) and the *quadruple* mutant (*quadruple*). Averages from six independent experiments are (n = 6, 120 total stomata) shown. B, MeJA-induced stomatal closure in wild-type (WT) and the *srk2e* mutant (*srk2e*). Averages from six independent experiments are (n = 6, 120 total stomata) shown. Error bars represent standard error.

3.4.2 Induction of reactive oxygen species and nitric oxide productions, and cytosolic alkalization in the *quadruple* mutant and *srk2e* mutant

The productions of reactive oxygen species (ROS) induced by the application exogenous MeJA in guard cell were examined in wild-type plants and quadruple mutant plants (Fig. 2A). Exogenous MeJA induced an increase of H_2O_2, as shown by 2',7'-dichlorofluorescin (DCF) fluorescence, in wild-type guard cells (Fig 2A, B). This result is consistent with previous studies (Suhita et al., 2004; Mnemasa et al., 2007; Saito et al., 2008; Anowar et al., 2011; Jannat et al., 2011). Like wild type, the quadruple mutant showed an elevation of H_2O_2 level in guard cells (Fig. 2A). A significant difference in DCF fluorescent intensity was not observed between wild type and the quadruple mutant (Student t-test, α = 0.05).

The ROS production was examined in *srk2e* mutant guard cells (Fig. 2B). The elevation of H_2O_2 level was substantially impaired in *srk2e* guard cells (Fig. 2B). This indicates that OST1/SRK2E is involved in MeJA signaling in guard cells and that OST1/SRK2E is shared by ABA signaling and MeJA signaling in guard cells.

Nitric oxide (NO) production in guard cells, as shown by diaminofluorescein (DAF) fluorescence, was induced by MeJA in wild-type guard cells as well as in the quadruple mutant's (Fig. 2C). MeJA induced cytosolic alkalization, as shown by an increase of BCECF fluorescence, in wild-type guard cells, as well as in guard cells in the quadruple mutant (Fig. 2D)

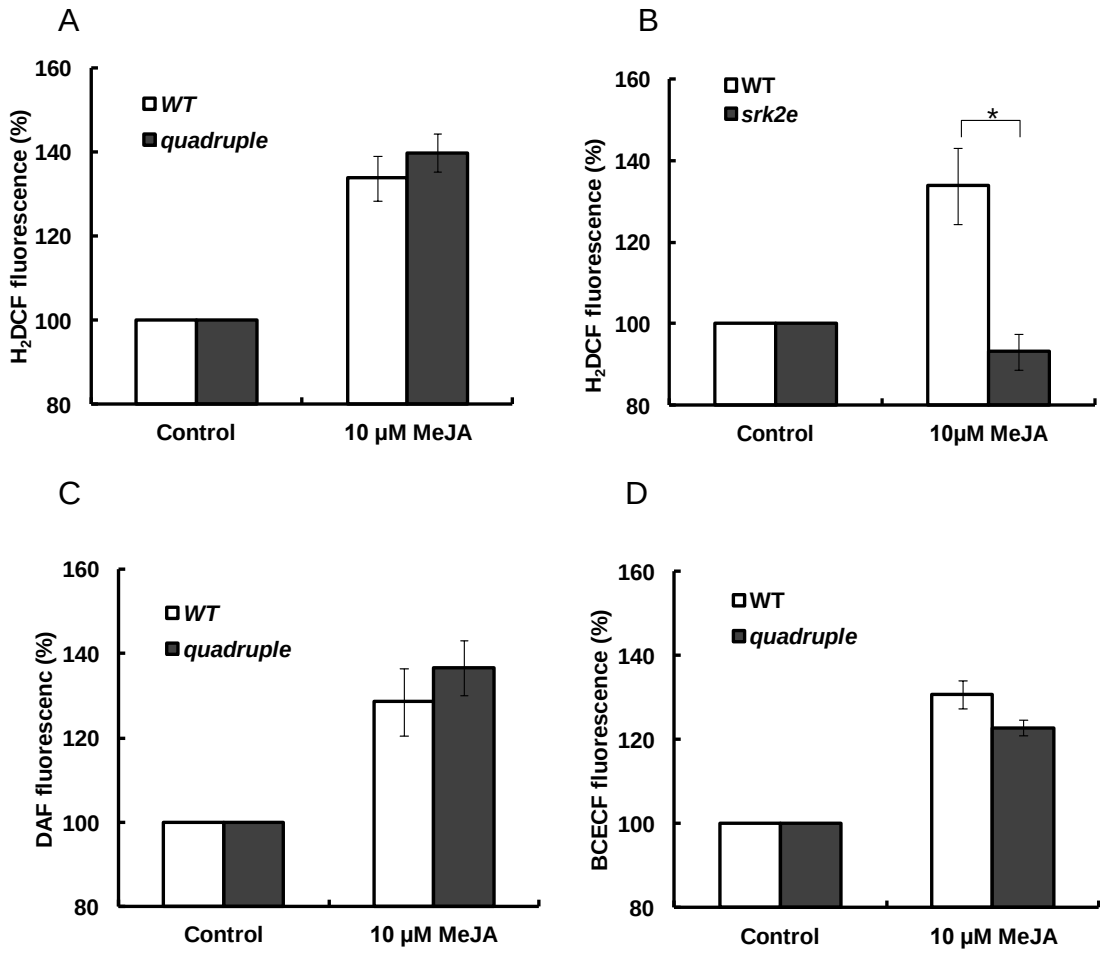

Fig. 3.2. ABA-induced ROS and NO production and cytosolic alkalization in wild-type (WT) and *pyr1 pyl1 pyl2 pyl4* (*quadruple)* mutant and *srk2e* mutant guard cells. A and B, ROS production. The vertical scale represents the percentage of H_2DCF fluorescence levels in wild-type (WT) and *pyr1 pyl1 pyl2 pyl4* (*quadruple)* mutant and *srk2e* mutant. C, NO production. Representative gray-scale DAF fluorescence levels in wild-type (WT) and *pyr1 pyl1 pyl2 pyl4* (*quadruple)* mutant. D, cytosolic alkalization. Representative BCECF fluorescence levels (pH) in wild-type (WT) and *pyr1 pyl1 pyl2 pyl4* (*quadruple)*

mutant. Fluorescent intensities are normalized to the control value taken as 100% for each experiment. The epidermis was pre-loaded with fluorescent dyes before treatments. Ten μM ABA or 0.1% ethanol (solvent control) was added to the suspension of epidermal preparations for 20 min. Bars indicate averages of five independent experiments (n = 5, 100 total guard cells per bar). Error bars represent standard error.

3.4.3 MeJA inhibition of I_{Kin} in guard cells

Suppression of inward-rectifying K^+ (K^+_{in}) channels is one of the key events in the induction of stomatal closure (Saito et al., 2008). Here we employed the whole cell patch clamp technique to examine MeJA inactivation of I_{Kin} in isolated guard cell protoplasts (GCPs). Without MeJA treatment, significant difference in amplitude of I_{Kin} was observed between wild-type and quadruple mutant GCPs (Fig. 3A-F). Treatment of GCPs with 10 μM MeJA for 2 hours significantly reduced I_{Kin} in the wild type. But I_{Kin} was no reduced in the quadruple mutant. This indicates that PYR1, PYL1, PYL2 and PYL4 ABA receptors are involved in the suppression of I_{Kin}.

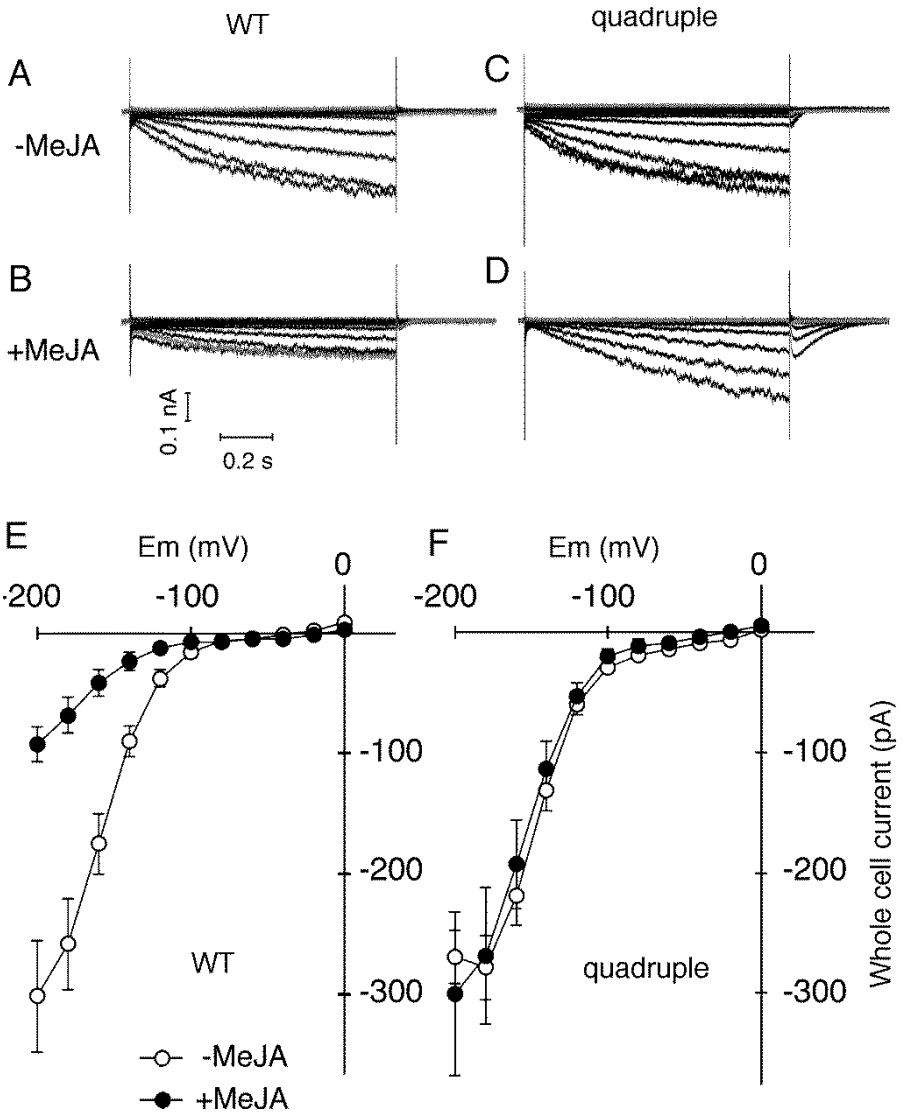

Fig. 3.3. Inactivation of inward-rectifying K^+ channel current (I_{kin}) by MeJA in wild-type (WT) and *pyr1 pyl1 pyl2 pyl4* (*quadruple)* guard cell protoplasts. Representative current in the absence (A, WT, -ABA) and presence (B, WT, +ABA), representative current in the absence (C, *quadruple*, -MeJA) and presence (D, *quadruple*, +MeJA), of 10 µM MeJA. The bottom panel (Em) indicates applied step pulse protocol. E and F, Current-voltage curve of I_{Kin}. GCPs of WT and *quadruple* were treated with 0.1% ethanol (solvent control,

open symbols, n= 5) or 10 μM ABA (closed symbols, n = 6). Error bars indicate standard error.

3.5 DISCUSSION

Hossain et al. (2011) showed that ABA is a prerequisite for MeJA-induced stomatal closure, using ABA deficient mutant. Many genetic components are shared with MeJA signaling and ABA signaling. These facts led us to initially hypothesize that the MeJA signaling is the upstream event of ABA singlaing and mediated by ABA signaling to lead stomatal closure. If so, the ABA receptor quadruple mutant, in which ABA-induced stomatal closure is impaired (Nishimura et al., 2010; Yin et al., in preparation), would be MeJA insensitive. However, stomata of the quadruple mutant were MeJA sensitive (Fig. 1) in an opposite to the hypothesis.

Munemasa (2007) showed that *coi1* stomata were MeJA insensitive, while they were ABA sensitive. This study and previous studies showed that *pyr1 pyl1 pyl2 pyl4* stomata were MeJA sensitive, while they were ABA insensitive. These facts are tempting to speculate that ABA and MeJA were recognized independent receptors and two signals are integrated at an early step in the signaling pathway. After ABA perception, PYR/PYL/RCAR ABA receptors activate OST1/SRK2E kinase by a sequential reaction as follows: (1) inhibition of protein phosphatase 2C by ABA-PYR/PYL/RCAR complexes and (2) disinhibition of OST1/SRK2e kinase via inhibition of protein phosphatase 2C. Stomata of the *OST1/SRK2E* knockdown mutant, *srk2e*, was insensitive to both ABA and MeJA. We infer that MeJA signaling and ABA signaling in guard cells

are integrated at or before OST1/SRK2E kinase activation. The COI1 JA-Ile receptor is a F-box protein that is involved in specific ubiquitination and subsequent protein degradation of JAZ proteins in JA signaling. The mechanism to link COI1 and OST1 remained unknown.

It was suggested that ABA primes or activates MeJA signaling pathway by unresolved mechanism in guard cells (Hossain et al., 2011). It was our surprise that PYR1, PYL1, PYL2 and PYL4 were not involved in the ABA recognition mechanism to render the priming/activation of MeJA signaling. Other ABA receptors are involved in the priming/activation of MeJA signaling. There are 14 members of PYR/PYL/RCAR exist in the Arabidopsis genome. Some of the other 10 members may be the ABA receptor(s) function in the crosstalk of ABA and MeJA in guard cells.

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References

Akter N, Sobahan MA, Hossain MA, Uraji M, Nakamura Y, Mori IC, Murata Y (2010) the involvement of intracellular glutathione in methyl jasmonate signaling in Arabidopsis guard cells. Biosci Biotechnol Biochem **74**: 2504-2506.

Allan AC, Fricker MD, Ward JL, Beale MH, Trewavas AJ (1994) Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. Plant Cell **6**: 1319-1328

Allen GJ, Kwak JM, Chu SP, Llopis J, Tsien RY, Harper JF, Schroeder JI (1999) Cameleon calcium indicator reports cytoplasmic calcium dynamics in *Arabidopsis* guard cells. Plant J **19**: 735-747

Alscher RG, Donahue JL, Gramer CL (1997) Reactive oxygen species and antioxidants: relationships in green cells. Physiol Plant **100**: 224-233

Anderson BE, Ward JM, Schroeder JI (1994) Evidence for an extracellular

reception site for abscisic acid in *Commelina* guard cells. Plant Physiol **104**: 1177-1183

Apel K, Hirt H (2004) Active oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**: 373-399

Asada K (2006) production and scavenging reactive oxygen species in chloroplasts and their functions. Plant Physiology **141**: 391-396

Assmann SM (1994) Ins and outs of guard cell ABA receptors. Plant Cell **6**: 1187-1190

Assmann SM, Simoncini L, Schroeder JI (1985) Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba.* Nature **318**: 285-287

Blatt MR (1987) Electrical Characteristics of Stomatal Guard Cells: The Contribution of ATP-Dependent, "Electrogenic" Transport Revealed by Current-Voltage and Difference-Current-Voltage Analysis. J Membrane Biol **98**: 257-274

Blatt MR (1990) Potassium channel currents in intact stomatal guard cells: rapid enhancement by abscisic acid. Planta **180**: 445-455

Blatt MR (1992) K^+ channels of stomatal guard cells: characteristics of the inward rectifier and its control by pH. J Gen Physiol **99**: 615-644

Blatt MR, Armstrong F (1993) K^+ channels of stomatal guard cells: abscisic acid-evoked control of the outward rectifier mediated by cytoplasmic pH. Planta **191**: 330-341

Blatt MR, Thiel G (1994) K^+ channels of stomatal guard cells: bimodal control of the K^+ inward-rectifier evoked by auxin. Plant J **5**: 55–68

Bostoc RM (2005) Signal cross talk and induced resistance: straddling the line between cost and benefit. Annual Review of phytopathology **43**: 545-580.

Bray EA (1997) Plant responses to water deficit. Trends in Plant Science **2**: 48-54

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ (2006) ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis. The Plant J **45**: 113-122

Bucholc M, Ciesielski A, Goch G, Anielska-Mazur A, Kulik A, Krzywińska E, Dobrowolska G (2011) SNF1-related Protein Kinases 2 Are Negatively Regulated by a Plant-specific Calcium Sensor. J Biol Chem **286**: 3429–3441

Cheong J-J, Choi YD (2003) Methyl jasmonate as a vital substance in plants. Trends in genetics **19**: 409-413

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol **61**: 651-679

Desikan R, Griffiths R, Hancock J, Neill S (2002) A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. PNAS **99**: 16314–16318

Eisenach C, Chen Z-H, Grefen C, Blatt MR (2012) The trafficking protein SYP121 of Arabidopsis connects programmed stomatal closure and K^+ channel activity with vegetative growth. Plant J **69**: 241-251

Evans NH (2003) Modulation of guard cell plasma membrane potassium currents by methyl jasmonate. Plant Physiol **131**: 8-11

Fujii H, Zhu J-K (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. PNAS **106**: 8380–8385.

Gehring CA, Irving HR, Mcconchie R, Parish RW (1997) Jasmonates induce intracellular alkalinization and closure of *Paphiopedilum* guard cells. Ann Bot **80**: 485–489

Garcia-Mata C, Gay R, Sokolovski S, Hills A, Lamattina L, Blatt MR (2003) Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. PNAS **100**: 1116-11121

Garcia-Mata C, Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol **126**: 1196-1204

Garcia-Mata C, Lamattina L (2002) Nitric oxide and abscisic acid cross talk in guard cells. Plant Physiology **128**: 790-792

Garcia-Mata C, Lamattina L (2007) Abscisic acid (ABA) inhibits light-induced stomatal opening through calcium- and nitric oxide-mediated signaling pathways. Nitric Oxide **17**: 143–151

Geiger D, Scherzer S, Mumm P, Stange A, Marten I, Bauer H, Ache P, Matschi S, Liese A, Al-Rasheid KAS, Romeis T, Hedrich R (2009) Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. PNAS **106**: 21425–21430

Goh C-H, Kinoshita T, Oku T, Shimazaki K (1996) Inhibition of blue light-dependent H^+ Pumping by abscisic acid in *Vicia* guard-cell protoplasts. Plant Physiol **111**: 433-440

Gonugunta VK, Srivastava N, Puli MR and Raghavendra AS (2008) Nitric oxide production occurs after cytosolic alkalinization during stomatal closure induced by abscisic acid. Plant Cell Env **31**: 1717–1724

Gonzalez-Guzman M, Pizzio GA, Antoni R, Vera-Sirera F, Merilo E, Bassel GW, Fernández MA, Holdsworth MJ, Perez-Amador MA, Kollist H, Rodriguez PL (2012) *Arabidopsis* PYR/PYL/RCAR receptors play a major role in quantitative regulation of stomatal aperture and transcriptional response to abscisic acid. Plant Cell **24**: 2483–2496

Grabov A, Blatt MR (1997) Parallel control of the inward-rectifier K^+ channel by cytosolic free Ca^{2+} and pH in *Vicia* guard cells. Planta **201**: 84-95

Grabov A, Blatt MR (1999) A steep dependence of inward-rectifying potassium channels on cytosolic free calcium concentration increase evoked by hyperpolarization in guard cells. Plant Physiol **119**: 277-287

Guo FQ, Okamoto M, Crawford NM (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science **302**: 100–103

Hamilton DWA, Hills A, Köhler B, Blatt MR (2000) Ca^{2+} chennels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. PNAS **97**: 4967-4972

Hayashi M, Inoue S-I, Takahashi K, Kinoshita T (2011) Immunohistochemical detection of blue light-induced phosphorylation of the plasma membrane H^+-ATPase in stomatal guard cells. Plant Cell Physiol **52**: 1238–1248

Hetherington AM, Woodward FI (2003) the role of stomatal in sensing and driving environmental change. Nature **424**: 901-908

Hirayama T, Shinozaki K (2007) Perception and transduction of abscisic acid

signals: keys to the function of the versatile plant hormone ABA. Trend Plant Sci **12**: 343-351.

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

Irving HR, Gehring CA, Parish RW (1992) Changes in cytosolic pH and calcium of guard cells precede stomatal movements. PNAS **89**: 1790–1794

Islam MM, Hossain MA, Jannat R, Munemasa S, Nakamura Y, Mori IC, Murata Y (2010) Cytosolic alkalization and cytosolic calcium oscillation in Arabidopsis guard cells response to ABA and MeJA. Plant Cell Physiol **51**: 1721–1730

Jannat R, Uraji M, Morofuji M, Islam MM, Bloom RE, Nakamura Y, McClung CR, Schroeder JI, Mori IC, Murata Y (2011) Roles of intracellular hydrogen peroxide accumulation in abscisic acid signaling in *Arabidopsis* guard cells. J Plant Physiol **168**: 1919-1926

Kang J, Hwang J-U, Lee M, Kim Y-Y, Assmann SM, Martinoia E, Lee Y (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. PNAS **107**: 2355-2360

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

Kinoshita T, Shimazaki K (2002) Biochemical evidence for the requirement of 14-3-3 protein binding in activation of the guard-cell plasma membrane

H^+-ATPase by blue light. Plant Cell Physiol **43**: 1359–1365

Kim TH, Böhmer M, Hu H, Nishimura N, Schroeder JI (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu Rev Plant Biol **61**: 561–591

Köhler B, Hills A, Blatt MR (2003) Control of guard cell ion channels by hydrogen peroxide and abscisic acid indicates their action through alternate signaling pathways. Plant Physiol **131**: 385-388.

Kwak JM, Moon JH, Murata Y, Kuchitsu K, Leonhardt N, DeLong A, Schroeder JI (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, *RCN1*, confers abscisic acid insensitivity in Arabidopsis. The Plant Cell **14**: 2849-1861

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

Liechti R, Farmer EE (2002) The jasmonate pathway. science **296**: 1649-1650

Li J, Assmann SM (1996) An abscisic acid-activated and calcium-independent protein kinase from guard cells of fava bean. Plant Cell **8**: 2359–2368

Li J, Wang X-Q, Watson MB, Assmann SM (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science **287**: 300–303

Lozano-Juste J, Leon J (2010) Nitric oxide modulates sensitivity to ABA. Plant Signaling and Behavior **5**: 314-316

MacRobbie EAC (1998) Signal transduction and ion channels in guard cells.

Phil Trans R Soc Lond B **353**: 1475-1488

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science **324**: 1064-1068

McAinsh MR, Brownlee C, Hetherington AM. (1990) Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. Nature **343**: 186–188

McAinsh MR, Clayton H, Mansfield TA, Hetherington AM (1996) Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. Plant Physiol **111**: 1031-1042

Melcher K, Ng LM, Zhou XE, et al. (2009) A gate-latch-lock mechanism for hormone signaling by abscisic acid receptors. Nature **462**: 602-608

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

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

Mishra G, Zhang W, Deng F, Zhao J, Wang X (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. Science **312**: 264-266

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci **7**: 405-410

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9**: 490-496

Mori IC, Murata Y, Yang Y, Munemasa S, Wang YF, Andreoli S, Tiriac H, Alonso JM, Harper JF, Ecker JR, Kwak JM, Schroeder JI (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. PLoS Biology **4:** e327

Mori IC, Muto S (1997) Abscisic acid activates a 48-kilodalton protein kinase in guard cell protoplasts. Plant Physiol **113**: 833–839

Mori IC, Uozumi N, Muto S (2000) Phosphorylation of the inward-rectifying potassium channel KAT1 by ABR kinase in *Vicia* guard cells. Plant Cell Physiol **41**: 850–856

Munemasa S, Oda K, Watanabe-Sugimoto M, Nakamura Y, Shimoishi Y, Murata Y (2007) The *coronatine-insensitive 1* mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in Arabidopsis guard cells. Specific impairment of ion channel activation and second messenger production. Plant Physiol **143**: 1398-1407

Murata Y, Pei Z-M, Mori IC, Schroeder J (2001) Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase 2C mutants. Plant Cell **13**: 2513–2523

Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J (2002) Arabidopsis

OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell **14**: 3089–3099

Nakashima K, Fujita Y, Kanamori N, Katagiri T, Umezawa T, Kidokoro S, Maruyama K, Yoshida T, Ishiyama K, Kobayashi M, Shinozaki K, Yamaguchi-Shinozaki K (2009) Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. Plant Cell Physiol **50**: 1345–1363

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

Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I (2008) Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot **59**: 165–176

Neill SJ, Desikan R, Clarke A, Hancock JT (2002) Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. Plant Physiol **128**: 13–16

Ng PAP, Jarvis PG (1980) Hysteresis in the response of stomatal conductance in *Pinus sylvestris* L. needles to light: observations and a hypothesis. Plant Cell Environ **3**: 207-216

Nishimura N, Hitomi K, Arvai AS, Rambo RP, Hitomi C, Cutler SR, Schroeder JI, Getzoff ED (2009) Structural mechanism of abscisic acid

binding and signaling by dimeric PYR1. science **326**: 1373-1379

Nishimura N, Sarkeshik A, Nito K, Park S-Y, Wang A, Carvalho PC, Lee S, Caddell DF, Cutler SR, Chory J, Yates JR, Schroeder JI (2010) PYR/PYL/RCAR family members are major *in-vivo* ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. Plant J **61**: 290–299

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu J-K, Schroeder JI, Volkman BF, Cutler SR (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science **324**: 1068-1071

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

Saez A, Apostolova N, Gonzalez-Guzman M, Gonzalez-Garcia MP, Nicolas C, Lorenzo O, Rodriguez PL (2004) Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signaling. plant J **37**: 354-369

Saito N, Munemasa S, Nakamura Y, Shimoishi Y, Mori IC, Murata Y (2008) Roles of RCN1, regulatory a subunit of protein phosphatase 2A, in methyl jasmonate signaling and signal crosstalk between methyl jasmonate and abscisic acid. Plant Cell Physiol **49**: 1396–1401

Santiago J, Rodrigues A, Saez A, Rubio S, Antoni R, Dupeux F, Park S-Y,

Márquez JA, Cutler SR, Rodriguez PL (2009) Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. Plant J **60**: 575-588

Sasaki T, Mori IC, Furuichi T, Munemasa S, Toyooka K, Matsuoka K, Murata Y, Yamamoto Y (2010) Closing plant stomata requires a homolog of an aluminum-activated malate transporter. Plant Cell Physiol **51**: 354-365

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

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

Schroeder JI, Kwak JM, Allen GJ (2001) Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature **410**: 327-330

Schroeder JI, Raschke K, Neher E (1987) Voltage dependence of K^+ channels in guard-cell protoplasts. PNAS **84**: 4108-4112

Schwartz A, Wu W-H, Tucker EB, Assmann SM (1994) Inhibition of inward K^+ channels and stomatal response by abscisic acid: an intracellular locus of phytohormone action. PNAS **91**: 4019-4023

Shimazaki K, Iino M, Zeiger E (1986) Blue light-dependent proton extrusion by guard-cell protoplasts of *Vicia faba.* Nature **319**: 324-326

Sirichandra C, Gu D, Hu H-C, Davanture M, Lee S, Djaoui M, Valot B, Zivy M, Leung J, Merlot S, Kwak JM (2009) Phosphorylation of the Arabidopsis

AtrbohF NADPH oxidase by OST1 protein kinase. FEBS Lett **583**: 2982-2986

Suhita D, Raghavendra AS, Kwak JM, Vavasseur A (2004) Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. Plant Physiol **134**: 1536-1545

Takahashi M, Asada K (1998) Superoxide production in aprotic interior of chloroplast thylakoids. Arch Biochem Biophysics **267**: 714-722

Testerink C, Dekker HL, Lim ZY, Johns MK, Holmes AB, Koster CG, Ktistakis NT, Munnik T (2004) Isolation and identification of phosphatidic acid targets from plants. Plant J **39**: 527-536

Thiel G, MacRobbie EA, Blatt MR (1992) Membrane transport in stomatal guard cells: the importance of voltage control. J Membr Biol **126**: 1-18

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. The plant cell **14**: 153-164

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. PN AS **106**: 17588–17593

Uraji M, Katagiri T, Okuma E, Ye W, Hossain MA, Masuda C, Miura A, Nakamura Y, Mori IC, Shinozaki K, Murata Y (2012) Cooperative function of PLDδ and PLDα1 in abscisic acid-induced stomatal closure in Arabidopsis. Plant Physiol **159**: 450–460

Vahisalu T, Kollist H, Wang YF, Nishimura N, Chan WY, Valerio G,

Lamminmäki A, Brosché M, Moldau H, Desikan R, Schroeder JI, Kangasjärvi J (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature **452**: 487-491

Wang X-Q, Ullah H, Jones AM, Assmann SM (2001) G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. Science **292**: 2070-2072

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

Ward JM, Pei Z-M, Schroeder JI (1995) Roles of ion channels in initiation of signal transduction in higher plants. Plant Cell **7**: 833-844

Wawer I, Bucholc M, Astier J, Anielska-Mazur A, Dahan J, Kulik A, Wysłouch-Cieszynska A, Zaręba-Kozioł M, Krzywinska E, Dadlez M, Dobrowolska G, Wendehenne D (2010) Regulation of Nicotiana tabacum osmotic stress-activated protein kinase and its cellular partner GAPDH by nitric oxide in response to salinity. Biochem J **429**: 73–83

Weiner JJ, Peterson FC, Volkman BF, Cutler SR (2010) Structural and functional insights into core ABA signaling. Curr Opin Plant Biol **13**: 495–502

Yan J, Tsuichihara N, Etoh T, Iwai S (2007) Reactive oxygen species and nitric oxide are involved in ABA inhibition of stomatal opening. Plant Cell Environ **30**: 1320–1325

Yin P, Fan H, Hao Q, Yuan X, Wu D, Pang Y, Yan C, Li W, Wang J, Yan N

(2009) Structural insights into the mechanism of abscisic acid signaling by PYL proteins. Nature Structural and Molecular Biology **16**: 1230-1236

Yoshida R, Hobo T, Ichimura K, Mizoguchi T, Takahashi F, Aronso J, Ecker JR, Shinozaki K (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. Plant Cell Physiol **43**: 1473–1483

Yoshida R, Umezawa T, Mizoguchi T, Takahashi S, Takahashi F, Shinozaki K (2006) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in *Arabidopsis* J Biol Chem **281**: 5310-5318

Zhang X, Wang H, Takemiya A, Song C-P, Kinoshita T, Shimazaki K (2004) Inhibition of blue light-dependent H^+ pumping by abscisic acid through hydrogen peroxide-induced dephosphorylation of the plasma membrane H^+-ATPase in guard cell protoplasts. Plant Physiol **136**: 4150–4158

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song C-P (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in Vicia faba. Plant Physiol **126**: 1438–1448

Zhang Y, Xu W, Li Z, Deng XW, Wu W, Xue W (2008) F-Box protein DOR function as a novel inhibitory factor for abscisic acid-induced stomatal closure under drought stress in Arabidopsis guard cell signaling. plant physiol 148: 2121-2133.