Abscisic Acid Signaling and Methyl Jasmonate
Signaling in Arabidopsis Guard Cells

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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](image)

**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 confirmed 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 crystal 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 $\text{H}_2\text{O}_2$ production, NO production, $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations, activation of $I_{\text{Ca}}$ 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 possess 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 O₂⁻-derived free radicals such as superoxide anions (O₂⁻), hydroxyl radicals (HO⁻), peroxyl (RO₂⁻), alkoxyl (RO⁻), as well as O₂⁻ derived non-radical species such as hydrogen peroxide (H₂O₂) (Takahashi and Asada, 1988; Mittler, 2002). ROS, including ¹⁰₂, H₂O₂, O₂⁻ 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 (Alschcer 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 et 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). H₂O₂ causes an increase in guard cell cytosolic free Ca²⁺, 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 \( \text{H}_2\text{O}_2 \) 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 nia1 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 \( \text{K}^+ \) release from the vacuoles and efflux across the plasma membrane (MacRobbie, 1998), and closure can be induced by elevated level of cytoplasmic \( \text{Ca}^{2+} \). ABA-induced increase in cytoplasmic \( \text{Ca}^{2+} \) can be achieved without \( \text{Ca}^{2+} \) influx, by release from cytoplasmic stores, but do not rule out ABA-induced \( \text{Ca}^{2+} \) influx when external \( \text{Ca}^{2+} \) is present. ABA-induced changes in \( [\text{Ca}^{2+}]_{\text{cyt}} \) and \( \text{pH}_{\text{cyt}} \) affect
inward- ($I_{\text{kin}}$) and outward-rectifying ($I_{\text{kout}}$) $K^+$ channels and of $Cl^-$ channels ($I_{\text{Cl}}$) 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 $I_{\text{kin}}$ current in Arabidopsis (Saito et al., 2008) and simultaneously activates $I_{\text{kout}}$ current in Vicia (Evans, 2003). These results indicate that elevation of $[Ca^{2+}]_{\text{cyt}}$ and $pH_{\text{cyt}}$ 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 $I_{\text{in}}$ 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 $K_{\text{out}}$ channel, and disruption of this gene results in impaired stomatal closure in response to darkness and ABA (Hosy et al., 2003). Regarding $I_{\text{in}}$ channels, Arabidopsis guard cells express at least four genes. Of them $K\text{AT}1$ has been
best characterized. Expression dominant-negative KAT1 variants suppressed $K_{\text{in}}$ channel activity and reduced light-induced stomatal opening (Kwak et al., 2001).

1.8 plasma membrane $\text{H}^+\text{-ATPase}$

The proton-pumping ATPase ($\text{H}^+\text{-ATPase}$) 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 $\text{H}^+$ 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 $\text{H}^+$ pump required for stomatal opening is known to be the plasma membrane $\text{H}^+\text{-ATPase}$ (Kinoshita and Shimazaki 1999). BL activates the $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ (Shimazaki et al. 1986, Goh et al. 1996). Inhibition of the $\text{H}^+\text{-ATPase}$ by ABA was due to a decrease in the phosphorylation level of the $\text{H}^+\text{-ATPase}$ (Zhang et al. 2004).

1.9 Purposes of the study

To gain detail information about ABA and MeJA signaling in guard cells, the
present 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, $I_{\text{kin}}$ 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; McNair 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 $I_{\text{kin}}$ 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 ($80 \mu$mol 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$_2$DCF-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 pHcyt of guard cells as described previously (Islam et al. 2010).

2.3.5 Whole cell patch clamp recording of the inward-rectifying K⁺ current (IKin)

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 MgCl₂, 3.35 mM CaCl₂, 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 MgCl₂, 40 mM CaCl₂, 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

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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$_2$O$_2$, 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$_2$O$_2$ 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 $I_{\text{Kin}}$ in guard cells

Inactivation of inward-rectifying K$^+$ ($K^+_{\text{in}}$) 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 $I_{\text{Kin}}$ in isolated guard cell protoplasts (GCPs). Without ABA treatment, no significant difference in amplitude of $I_{\text{Kin}}$ was observed between wild-type and quadruple mutant GCPs (Fig. 3A-C). Treatment of GCPs with 10 μM ABA for 2 hours significantly reduced $I_{\text{Kin}}$ in the wild type. $I_{\text{Kin}}$
**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$_2$DCF fluorescence images (top panel) and vertical scale represents the percentage of H$_2$DCF 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 $k_{\text{kin}}$. However, these receptors are not the only factors upstream of the $k_{\text{kin}}$ 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...
\( I_{\text{kin}} \). 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 (\( \alpha = 0.05 \)).
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, $I_{\text{kin}}$ 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 $I_{\text{kin}}$ in *srk2e* (Fig. 7) but inhibited $I_{\text{kin}}$ 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 $I_{\text{kin}}$ 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 $H^+\text{-ATPase}$ (Fig. 8). Together, these results show that the $H^+\text{-ATPase}$ 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.,
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$_2$DCF fluorescence images (top panel) and vertical scale represents the percentage of H$_2$DCF 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
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 $k_{\text{Kin}}$ in the quadruple mutant (Fig. 3) may be due to NO or ROS, which are known to inhibit $k_{\text{Kin}}$ (Köhler et al., 2003; Garcia-Mata et al., 2003). Alkalization may also be involved in the regulation of $k_{\text{Kin}}$ (Blatt, 1992; Blatt and Armstrong, 1993; Grabov and Blatt, 1997; Wang et al., 2012). In this study, the partial inactivation of $k_{\text{Kin}}$ 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 $K_{\text{in}}$ 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$_2$O$_2$ 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 $k_{\text{Kin}}$ is closely related with kinetics of stomatal opening (Eisenach et al. 2012) and $k_{\text{Kin}}$ 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 \( k_{in} \) by cytosolic alkalization.

In a protein phosphatase 2C mutant \( abi1-1 \), ABA does not induce stomatal closure but NO production (Desikan et al., 2002). On the other hand, ABA-induced \( \text{H}_2\text{O}_2 \) 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 \(K_{\text{in}}\) channel and \(\text{H}^+\)-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 \(K_{\text{in}}\) channel inactivates \(k_{\text{in}}\) (Sato et al., 2009). This agrees with the lack of \(k_{\text{in}}\) regulation in \(srk2e\) mutant (Fig. 7). Other OST1/AAPK substrates, which are involved in \(\text{H}^+\)-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), glyceraldehyde-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 INTRODUCTION

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 connects 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 et 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 $k_{\text{in}}$ 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\(_2\)DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) and DAF-2 DA (CAS 205391-02-2), respectively.

3.3.4 Measurement of cytosolic pH (pH\(_{\text{cyt}}\)) of guard cells

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

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

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 MgCl₂, 3.35 mM CaCl₂, 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 MgCl₂, 40 mM CaCl₂, 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 both 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, \( \alpha = 0.05 \)).

**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$_2$O$_2$, 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$_2$O$_2$ 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, $\alpha = 0.05$).

The ROS production was examined in srk2e mutant guard cells (Fig. 2B). The elevation of H$_2$O$_2$ 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$_2$DCF 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 \( I_{\text{Kin}} \) in guard cells

Suppression of inward-rectifying K\(^+\) (\( K_{\text{in}}^{+} \)) 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 \( I_{\text{Kin}} \) in isolated guard cell protoplasts (GCPs). Without MeJA treatment, significant difference in amplitude of \( I_{\text{Kin}} \) was observed between wild-type and quadruple mutant GCPs (Fig. 3A-F). Treatment of GCPs with 10 µM MeJA for 2 hours significantly reduced \( I_{\text{Kin}} \) in the wild type. But \( I_{\text{Kin}} \) was no reduced in the quadruple mutant. This indicates that PYR1, PYL1, PYL2 and PYL4 ABA receptors are involved in the suppression of \( I_{\text{Kin}} \).
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 signaling 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


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


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.


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


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


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


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


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


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


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


