Regulation of salicylic acid, yeast elicitor, and chitosan signaling in Arabidopsis guard cells

2013, 9

Mohammad Issak

Graduate School of

Natural Science and Technology

(Doctor’s Course)

OKAYAMA UNIVERSITY

Regulation of salicylic acid, yeast elicitor, and chitosan signaling in Arabidopsis guard cells

A thesis
Presented to Graduate School of Natural Science and Technology
Okayama University

In partial fulfillment of the requirement for the degree of
Doctor of Philosophy

Submitted by
Mohammad Issak

Department of Biofunctional Chemistry
Graduate School of Natural Science and Technology
Okayama University, Japan
2013, 9

List of contents

Abbreviations used

ABA	abscisic acid
$[Ca^{2+}]_{cyt}$	cytosolic free calcium concentration
CFP	cyan fluorescent protein
Col-0	*Arabidopsis* ecotype Columbia-0
CHT	chitosan
DAF-2DA	4,5-diaminofluorescein-2 diacetate
FLU	fluridon
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
MeJA	methyl jasmonate
PP	protein phosphatase
PYL	pyrabactin resistance-like
PYR	pyrabactin resistance
ROS	reactive oxygen species
SA	salicylic acid
YC	Yellow Cameleon
YEL	Yeast elicitor
YFP	yellow fluorescent protein

List of Figures

CHAPTER 1

General Introduction

1.1 Plant responses to stress

Any unfavorable condition or substance that negatively affects plants metabolism, growth, development, productive capacity or survival can be considered as stress. Stress includes a wide range of factors which can be divided into two main categories: abiotic or environmental stress factors, and biotic or biological stress factors. Among the environmental conditions that may be considered as stress factors are drought, waterlogging, high or low solar radiation, extreme temperatures, ozone, salinity, and inadequate mineral nutrient in the soil. A stress response is initiated when plant recognizes stress at the cellular level. Stress triggers plant responses ranging from altered gene expression to changes in plant metabolism and growth. Stress recognition activates signal transduction pathways that transmit information within the individual cells and throughout the plant. To survive with these challenges, plant defense responses rely on signaling mechanisms of hormones and other plant specific substances (Zhang *et al*., 2008; Bostoc, 2005).

1.2 Stomata

Stomata are important organs in leaf surfaces of higher plants to regulate carbon dioxide (CO_2) uptake for photosynthesis and transpiration water loss (Schroeder et al. 2001; Hetherington et al., 2003). To adapt various environmental changes including light intensity, humidity, and carbon dioxide (CO_2)

concentration, plants tightly regulate own stomatal apertures. The term “stoma” denotes a mouth in greek and like a mouth, each stoma has a lip surrounding the pore, which is formed by a pair of highly specialized cells called “guard cells” (Fig. 1.1) Stomatal movements strongly dependent on guard cell volumes. Stomata open as guard cell volumes increase. In contrast, stomata close as guard cell volume decrease. Until now, so maney studies have been established to elucidate guard cell signaling and a large number of factors working in this signaling have been found. However, detail mechanism of guard cell signaling is not yet fully understood.

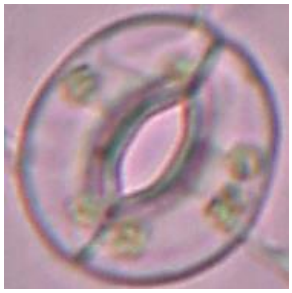

Figure 1.1 Arabidopsis stoma

1.3 Guard cell signaling

Stomatal pores are surrounded by a pair of specialized parenchyma cells known as guard cells. Most importantly stomata control the exchange of water vapor and carbon dioxide (CO_2) between the leaf interior and the atmosphere, thus controlling the water and carbon cycles in the world. In drought condition guard cells reduce stomatal aperture, thereby protecting the plant from transpiration water loss. Because they regulate stomatal pore apertures via integration of endogenous

hormonal stimuli and environmental signals, guard cells have been highly developed as a model system to dissect the dynamics and mechanisms of plant-cell signaling (Kim *et al*., 2010). A number of environmental factors including light, CO_2 concentration, humidity, temperature and plant hormones like abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA), elicitors induce stomatal opening and closing (Schroeder *et al*., 2001*a*, *b*; Hetherington and Woodward, 2003; Suhita *et al*., 2004; Young et at., 2006; Munemasa *et al*., 2007, 2011; Islam *et al*., 2010*a*, *b*; Khokon *et al*., 2010 a,b; 2011a).

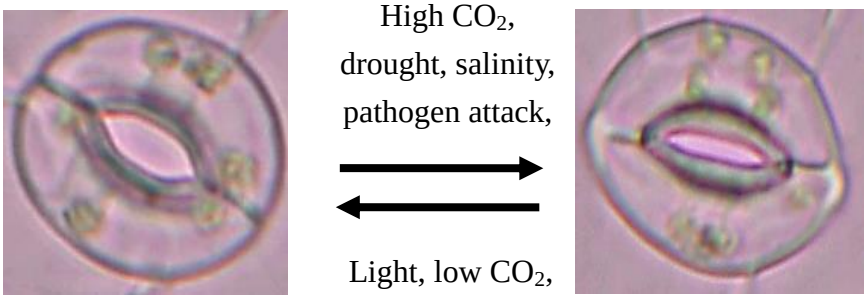

Figure 1.2 A model showing closing and opening of stoma under various conditions.

1.4 Abscisic acid

Abscisic acid (ABA) is a plant hormone that controls important developmental and abiotic stress responses, including seed dormancy, seed development, growth regulation and stomatal closure. ABA was identified in plants in 1960s (Ohkuma *et al*., 1963). ABA has a central role in protecting plant against

water deficiency by regulating the stomatal aperture and minimizing water loss through stomata. In response to water deficiency, enhanced biosynthesis of ABA occurs, foliar ABA moves to apoplast and accumulates preferentially in guard cells where its concentration is increased up to 30-fold (Outlaw, 2003). 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; Schroeder *et al*., 2001*b*).

Protein phosphorylation and dephosphorylation are important mediators of ABA signal transduction (Schroeder *et al*., 2001*a*, *b*). ABA-activated protein kinase (AAPK), calcium 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; Zhu *et al*., 2007; Jammes *et al*., 2009; Gudesblat *et al*., 2007). Protein phosphatase type 2C enzymes ABI1, ABI2, and HAB1 are all negative regulators of ABA signaling (Leung *et al*., 1994; Merlot *et al*., 2001; Rodriguez *et al*., 1998; 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 (Nishimura *et al*., 2009; Santiago *et al*., 2009; Yin *et al*., 2009). Endogenous ABA is required for MeJA signaling in Arabidopsis (Hossain et al., 2011).

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*., 2003, 2004, Munemasa *et al*., 2007; Islam *et al*., 2010*a*).

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* mutants, *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 Salicylic acid

Salicylic acid (SA) (C6H4(OH)COOH) is a monohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid and is widely used in organic synthesis and functions as a phytohormone. SA plays role in plant growth and development, photosynthesis, transpiration, ion uptake and transport. SA also induces specific changes in leaf anatomy and chloroplast structure. SA is involved in endogenous signaling, mediating in plant defense against pathogens (Hayat *et al*. 2007). It plays a role in the resistance to pathogens by inducing the production of pathogenesis-related proteins and involved in the systemic acquired resistance (SAR) in which a pathogenic attack on one part of the plant induces resistance in other parts. The signal can also move to nearby plants by salicylic acid being converted to the volatile ester, methyl salicylate. SA induces stomatal closure (Manthe et al., 1992; Lee and Raskin, 1998; Mori et al., 2001; Khokon et al., 2011; Grant and Lamb, 2006). SA-induced stomatal closure is accompanied by peroxidase-mediated extracellular ROS production and intracellular ROS accumulation (Mori *et al*. 2001; Khokon *et al*. 2011). The detailed mechanism of SA signaling is not yet well understood.

1.7 Elicitors

Elicitors are chemicals or biofactors that can induce physiological changes of the target living organism. Elicitors are generally defined as molecules that stimulate any defense responses of plants. It may include abiotic elicitors such as

metal ions and inorganic compounds, and biotic elicitors from fungi, bacteria, viruses or herbivores, plant cell wall components, as well as chemicals that are released at the attack site by plants upon pathogen or herbivore attack. It is well known that treatment of plants with elicitors, or attack by incompatible pathogens, causes an array of defense reactions, including the accumulation of a range of plant defensive secondary metabolites such as phytoalexins in intact plants or in cell cultures. However, with regard to signal transduction from elicitor perception to defense reactions in plants, the term elicitor as used here only refers to fragments from fungal or plant cell wall components, bacterial, virus or herbivore constituents, or synthetic analogs with elicitor activity (Zhao *et al*. 2005).

1.7.1 Yeast elicitor

Yeast elicitor is a fungal elicitor which stimulates the defense responses of plants (Zhao *et al*. 2005). Crude extract collected from baker's yeast (*Saccharomyces cerevisiae*) was used in this study. Yeast elicitor induces stomatal closure in Arabidopsis requiring extracellular Ca^{2+} (Klüsener *et al*. 2002). Fungal elicitor like *Fusarium oxysporum* cell wall preparation has been reported to induce oxidative burst during defense response in Arabidopsis and conferred resistance to wide range of pathogens (Bindschedler *et al*. 2006). Yeast elicitor has been reported to induce ROS production, $[Ca^{2+}]_{cyt}$ oscillation, and activation of S-type anion channels in guard cells leading to stomatal closure (Klüsener *et al*. 2002; Khokon *et al*. 2010*b*; Wu *et al*. 2011; Salam et al., 2013). However, the detailed signaling of yeast elicitor has not yet been completely elucidated.

1.7.2 Chitosan

Chitosan (β-1,4-linked glucosamine) is a deacylated derivative of chitin that is a component of the cell walls of many fungi (Bartnicki-Garcia, 1970). It can be produced by the activity of the enzyme chitinase, one of the pathogenesis-related proteins that are activated during infection and are toxic to invading pathogens (Mauch *et al*. 1988). Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It has a number of commercial and possible biomedical uses. Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi. Chitosan increases photosynthesis, promotes and enhances plant growth, stimulates nutrient uptake, increases germination and sprouting, and boost plant vigors. Chitosan has been reported to induce stomatal closure in several plant species (Lee *et al*. 1999; Klüsener *et al*. 2002; Srivastava *et al*. 2009; Khokon *et al*. 2010*a*; Salam et al., 2012). Chitosan induces ROS accumulation, cytosolic alkalization, $[Ca^{2+}]_{cyt}$ oscillation, and activation of S-type anion channels (Lee *et al*. 1999; Klüsener *et al*. 2002; Srivastava *et al*. 2009; Khokon *et al*. 2010*a*; Koers *et al*. 2011; Salam et al., 2012). However, the detailed signaling of chitosan has not yet been fully understood.

1.8 Second messengers in guard cell signaling

Second messengers such as reactive oxygen species (ROS), cytosolic alkalization, and Ca^{2+} are crucial mediators in guard cell signaling in response to different factors regulating the stomatal aperture.

1.8.1 Reactive oxygen species

Reactive oxygen species (ROS) are important signals mediating stomatal movements and other physiological processes in plants (Desikan *et al*., 2004; Foyer and Noctor, 2005). ROS comprise of singlet oxygen ($^1O_2$), super oxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot -}$). Plants accumulate ROS continuously as a side product from metabolism, which is strictly controlled. During stress situations the formation of ROS increases and regulates important downstream signaling events in plant defence reactions. ROS control cell growth and cycle, program cell death, stress responses, and hormonal signaling (Allan and Fluhr, 1997; Foreman *et al.*, 2003; Pei *et al*., 2000; Torres *et al*., 2002). When the production of ROS exceeds the degrading capacity, it can lead to oxidative stress and possible injury (Zhang *et al*., 2001).

There are different sources of ROS like mitochondria, chloroplasts, plasma membrane bound NADPH oxidases, cell wall peroxidases, peroxisomes and glyoxysomes (Asada, 2006; del Rio *et al*., 2006; Allan and Fluhr, 1997; Pei *et al*., 2000; Zhang *et al*., 2001). ROS function as a second messenger in ABA and MeJA signal transduction cascade in guard cells (Pei *et al*., 2000; Murata *et al*., 2001; Suhita *et al*., 2004; Bright *et al*., 2006; Munemasa *et al*., 2007; Saito *et al*., 2008; Islam *et al*., 2009; Islam *et al*., 2010*a*). NADPH oxidases 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 stomatal closure (McAinsh *et al*., 1996; Zhang *et al*., 2001).

1.8.2 Cytosolic alkalization (pH_{cyt})

Increment of guard cells cytosolic alkalization (pH_{cyt}) is a common phenomenon in stomatal closure (Irving *et al*., 1992; Gehring *et al*., 1997; Suhita *et al*., 2004; Gonugunta *et al*., 2008). Changes in (pH_{cyt}) in guard cells are closely involved in the modulation of ion mobilization leading to stomatal movement (Islam *et al*., 2010). ABA inhibits plasma membrain H+-ATPase activity and activates vacular H+-ATPase (V-ATPase) activity, resulting in cytosolic alkalization (Goh et al., 1996; Barklay et al., 1999; Zhang et al., 2004) Cytosolic alkalization also activates outward K^+ currents and inactivates inward K^+ currents to promote net efflux of K^+ in guard cells (Blatt and Armstrong, 1993).

1.8.3 Calcium

The calcium ion (Ca^{2+}) plays a role in the regulation of cellular function as a major intracellular messenger. Changes in cytosolic Ca^{2+} concentration take place in response to a wide variety of abiotic and biotic signals (Sanders *et al*., 1999; Sanders *et al*., 2002). Ca^{2+} is stored both in extra- and intracellular stores. Inside the cell, the vacuole is the most prominent Ca^{2+} storage place, but its concentration is higher than in the cytosol also in the endoplasmic reticulum, mitochondria and chloroplasts. The unequal Ca^{2+} distribution in the cell makes rapid Ca^{2+} concentration changes and fluxes possible (Kudla *et al*., 2010). Different effectors can induce an elevation in cytosolic Ca^{2+} in guard cells. Cytosolic Ca^{2+} elevation can be achieved by Ca^{2+} influx from the apoplast into the cytosol via hyperpolarization activated Ca^{2+} channels in the plasma membrane of

guard cells and by its release from intracellular stores, primarily from the vacuole. Cyclic adenosine 5'-diphosphoribose (cADPR) and cyclic guanosine 3',5'-monophosphate (cGMP) mediate intracellular Ca^{2+} release during stomatal closure (Garcia-Mata *et al*., 2003). Both ABA and MeJA activate Ca^{2+}-permeable cation channels and elicit $[Ca^{2+}]_{cyt}$ oscillation of various frequencies and amplitudes (Allen *et al*., 2002; Kwak *et al*., 2003, Munemasa *et al*., 2007, 2011, Islam *et al*., 2010*a*, *b*).

1.9 Arabidopsis mutants

Arabidopsis is a model plants having a large collection of mutants with defect in different defense and stress related signaling pathways and use of these mutants makes it possibility to determine which pathways are controlling an observed response (Glazebrook, 1997). Genetical, pharmacological, cell bioliological and electrophysiological studies have elucidated the multiple regulatory protein components, enzymes and second messengers involved in ABA, MeJA, and SA signaling and biosynthesis (Schroeder *et al*., 2001; Munimasa *et al*., 2007; Khokon *et al*., 2011). In my experiment, several Arabidopsis mutants *aba2-2*, aos, nahG, *sid2*, acd6, *siz1-2* were used to clarify the involvement of phytohormons ABA, MeJA or SA in SA, YEL, and CHT, signaling.

1.9.1 *aba2-2* mutants

ABA-deficient mutant of *Arabidopsis thaliana* has been isolated in order to clarify the physiological role of ABA in seed dormancy and the adaptive response to dehydration (Nambara *et al*., 1998). Genetic analysis showed this

mutation is a new allele of locus *aba2,* and therefore has been designated *aba2-2.* *ABA2* encodes a cytosolic short-chain dehydrogenase/reductase involved in the conversion of xanthoxin to ABA-aldehyde during ABA biosynthesis. The levels of endogenous ABA in fresh and dehydrated tissues of the *aba2-2* mutant were highly reduced compared to those of wild-type plants (Nambara *et al*., 1998). As a consequence, *aba2-2* plants wilt and produce seeds with reduced dormancy. In these experiments, I used *aba2-2* mutant to elucidate the involvement of endogenous ABA in SA, YEL, and CHT, signaling signaling. *Arabidopsis* genome initiative numbers for *ABA2* gene is AT1G52340.

1.9.2 *aos* mutants

An Arabidopsis knock-out mutant defective in the jasmonic acid (JA) biosynthetic gene CYP74A (*allene oxide synthase, AOS*) has been isolated to better understand the biosynthetic mechanism of JA (Park *et al*., 2002). AOS is critical for the biosynthesis of all biologically active jasmonates and this enzyme catalyses dehydration of the hydroxypeeroxide to an unstable allene oxide in the JA biosynthetic pathway. In Arabidopsis AOS is encoded by a single gene (Luddert and Weiler, 1998).Endogenous JA levels, which increase 100-fold 1 h after wounding in the wild-type plants, do not increase after wounding in the *aos* mutants (Park *et al*., 2002). The *aos* mutant shows severe mail sterility due to defects in anther and pollen development. The mail-sterility phenotype was completely rescued by exogenous application of MeJA and by complementation with constitutive expression of the *AOS* gene (Park *et al*., 2002). In these experiments, I used *aos* mutant to investigate the involvement of endogenous

MeJA in SA, YEL, and CHT signaling. Arabidopsis genome initiative numbers for *AOS* gene is AT5G 42650.

1.9.3 *nahG* transgenic plants

Transgenic Arabidopsis plants that express the bacterial nahG gene encoding salicylate hydroxylase can metabolize SA (Lawton et al., 1995). The bacterial nahG gene encodes salicylate hydroxylase, an enzyme produced by *Pseudomonas putida* that metabolizes SA to catechol (You et al., 1991). Salicylate hydroxylase (E.C. 1.14.13.1), encoded by the nahG gene of Pseudomonas putida, is a flavoprotein that catalyzes the decarboxylative hydroxylation of salicylate, converting it to catechol (11 Yamamoto et al., 1965). Salicylate hydroxylase leades to large decreases in endogenous SA levels in nahG transgenic Arabidopsis plants (Friedrich et al., 1995). Leaves of nahG transgenic lines show around 25% SA levels compared with those of wild-type plants (Abreu et al., 2009). Arabidopsis nahG plants are defective in non-host resistance to pathogen. A DNA fragment containing the nahG coding sequence from *P. putida* PpG7 was subcloned from pSR20 into an expression vector in which transcription is controlled by the enhanced 35S cauliflower mosaic virus promoter and terminated by the tmnl 3' terminator.

1.9.4 *acd6* mutants

A dominant gain-of-function Arabidopsis mutant, accelerated cell death (acd6), reveals novel regulation and function of the SA signaling pathway in controlling cell death, defenses, and cell growth (Rate et al., 1999). *ACD6* encodes

a novel protein with putative ankyrin and transmembrain region (Lu et al., 2003). Plants overexpressing ACD6 shows modestly increased SA levels, increased resistance to *P. syringae*. These plants show small spontaneous patches of cell death and high level of resistance to *P. syringae*. acd6 plants have high levels of defenses that require SA as a sole activator as well as defenses that require SA as a coactivator. acd6 acts partially through a *NON EXPRESSOR OF PR1* (NPR1) gene-independent pathway that activates defenses and confers resistance to P syringae (Rate et al., 1999).

1.9.5 *siz1* mutants

AtSIZ1 is a plant small ubiquitin-like modifier (SUMO) E3 ligase and is a focal controller of Pi starvation-dependent responses. AtSIZ1 has SUMO E3 ligase activity in vitro, and immunoblot analysis revealed that the protein sumoylation profile is impaired in siz1 plants. It has been reported that the siz1 mutation, which is impaired in the SIZ-type small ubiquitin-like modifier (SUMO) E3 ligase in Arabidopsis (Miura et al., 2005), conferred ABA hypersensitivity (Miura et al., 2009; Miura and Hasegawa, 2009) and enhanced the accumulation of SA and SA-inducible gene transcripts (Lee et al., 2007). Sumoylation functions in plant development, stress responses and flowering (Miura et al., 2007a; Miura and Hasegawa, 2010), and SIZ1 SUMO E3 ligase plays an important role in the regulation of these functions (Miura et al., 2005, 2007b, 2009, 2010, 2011a,b; Yoo et al., 2006; Catala et al., 2007; Lee et al., 2007; Jin et al., 2008). SIZ1 negatively affects stomatal apertures through the SA-induced ROS accumulation, independent of ABA hypersensitivity.

1.9.6 *sid2* mutants

SA induction-deficient (*sid2*) mutant is more susceptible to *P. syringae* and Peronospora parasitica and show a reduced SAR response. Contrary to our extensive knowledge concerning SA-mediated signal transduction, little is known about the control of SA biosynthesis. The biosynthesis of SA is strongly induced upon pathogen infection. This pathogen-induced SA biosynthesis is controlled by *SID2*, which encodes isochorismate synthase 1 (ICS1; Wildermuth et al., 2001). Arabidopsis *sid2* mutants are defective in pathogen-induced SA accumulation and are severely compromised in disease resistance to biotrophic pathogens (Nawrath and Metraux, 1999; Wildermuth et al., 2001). Thus, the regulation of *SID2* expression is fundamental to plant immunity.

1.10 Purpose of the study

The aim of this study is to obtain novel information about the regulation of SA, YEL, and CHT signaling in the model plant *Arabidopsis thaliana*.

Specific aims were as follows:

1. To investigate involvement of endogenous ABA or JA in SA, YEL, and CHT signal transduction.
2. To investigate involvement of endogenous SA in YEL- and CHT-induced stomatal closure.

Chapter 2

Neither endogenous abscisic acid nor endogenous jasmonate is involved in salicylic acid-, yeast elicitor-, or chitosan-induced stomatal closure in *Arabidopsis thaliana*

2.1 Abstract

Salicylic acid (SA), yeast elicitor (YEL), and chitosan (CHT) induced stomatal closure in Arabidopsis wild-type and *aba2-2* plants, induced stomatal closure in fluridon-treated wild-type plants, and induced stomatal closure in *aos* mutants. These results suggest that neither endogenous abscisic acid nor endogenous jasmonic acid is involved in SA-, YEL-, or CHT-induced stomatal closure.

2.2 Introduction

SA is a phenolic plant hormone that induces stomatal closure in several plant species and plays a crucial role in plant defense (Lee *et al*., 1998; Mori *et al*., 2001; Khokon *et al*., 2011a). YEL induces stomatal closure in *Arabidopsis thaliana* (Klüsener *et al*., 2002; Khokon *et al*., 2010b; Salam *et al*., 2012), and a fungal elicitor, CHT (β-1,4-linked glucosamine), a deacetylated derivative of chitin, induces stomatal closure in several plant species (Lee *et al*., 1999; Srivastava *et al*., 2009; Khokon *et al*., 2010a).

We have found that methyl jasmonate (MeJA)-induced stomatal closure required endogenous abscisic acid (ABA), and that allyl isothiocyanate (AITC)-

induced stomatal closure required endogenous jasmonates (JAs) in Arabidopsis (Hossain *et al*., 2011; Khokon *et al*., 2011b), but it remains to be clarified whether ABA or JA is involved in SA-, YEL-, or CHT-induced stomatal closure.

Short-chain alcohol dehydrogenase, ABA2, is critical to the biosynthesis of ABA. In *aba2* mutants, endogenous ABA levels are lower than in wild-type plants (Nambara *et al*., 1998). Fluridon (FLU: 1-methyl-3-phenyl-5-[3-trifluromethyl (phenyl)]-4-(1*H*)-pyridinone) is an inhibitor of ABA biosynthesis (Gamble and Mullet, 1986), and it decreases endogenous ABA levels (Moore and Smith, 1984). Allene oxide synthase (AOS) is essential to the biosynthesis of Jas (Park *et al*., 2002). In *aos* mutants, the levels of JAs are lower than in wild-type plants (Park *et al*., 2002).

In order to determine whether endogenous ABA or JAs is involved in SA-, YEL-, or CHT-induced stomatal closure, we examined SA-, YEL-, and CHT-induced stomatal closure in *aba2-2* and *aos* mutants and the effects of FLU on SA-, YEL-, and CHT-induced stomatal closure in wild-type plants and *aos* mutants.

2.3 Materials and Methods

2.3.1 Plant materials and growth conditions

Arabidopsis thaliana L. ecotype Columbia-0 (Col-0) was used in this study. Col-0 plants and transgenic Col-0 plants expressing Ca^{2+} reporter Yellow Cameleon 3.6 (YC3.6) were grown in pots containing a mixture of 70% (v/v) vermiculite (Asahi-kogyo, Okayama, Japan) and 30% (v/v) kureha soil (Kuhera chemical, Tokyo, Japan) in a growth chamber (80 µmol m^{-2} s^{-1} under a 16-h-light/8-h-dark regime) as described previously (Islam *et al.*, 2010*a*). The

temperature and relative humidity in the growth chamber were 22±2ºC and 60±10%, respectively. Twice or three times a week, 0.1% Hyponex solution (Hyponex, Osaka, Japan) was provided to plants.

2.3.2 Measurement of stomatal aperture

Stomatal aperture measurements were performed as described previously (Murata *et al*., 2001). Excised rosette leaves were floated on medium 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 induce stomatal opening. Then the SA, YEL, and CHT solution was added to the medium to give the desired concentration. After 2-h incubation on supplemented medium, stomatal apertures were measured. Leaves were shredded in a blender for 30 s and remaining epidermal tissues were collected using nylon mesh (pore size: 30 μm). Stomatal apertures in the epidermal tissues were observed under a microscope. Twenty stomatal apertures were measured for each sample.

2.3.3 Chemicals

SA and $CaCl_2$ was purchased from Wako Pure Chemical Industries (Osaka, Japan), KCl, 2-(N-morpholino)ethanesulfonic acid (MES), and tris(hydroxymethyl)aminomethane (Tris) from Nacalia Tesque (Kyoto, Japan), FLU from Sigma-Aldrich (St. Louis, MO, USA). Chitosan was purchased from Sigma-Aldrich (St. Louis, MO, USA), prepared as previously described (Walker-Simmons *et al*., 1984), and then dissolved in stomatal opening solution. Yeast elicitor was prepared from commercial bakers as previously described (Schumacher *et al*., 1987).

The elicitor precipitate was lyophilized and stored at -20°C until use.

2.3.4 Statistical analysis

The significance of differences between mean values and stomatal apertures were assessed by one-way analysis of variance (ANOVA) with Tukey's test. Differences were considered significant for *p* values of < 0.05.

2.3.5 Accession numbers

The Arabidopsis Genome Initiative numbers for the genes discussed in this thesis as follows: *ABA2-2*, AT1G52340, and *AOS*, AT5G42650.

2.4 Results

2.4.1 SA-, YEL-, and CHT-induced stomatal closure in the wild-type and *aba2-2* mutants

SA at 200 μM, 500 μM, and 1000 μM significantly induced stomatal closure in the wild-type plants (Fig. 1A), in accord with previous results (Khokon *et al*.,2011a). SA at 200 μM, 500 μM, and 1000 μM also significantly induced stomatal closure in the *aba2-2* mutants (Fig. 1B). There were no differences in SA-induced stomatal closure as between the wild-type plants and the *aba2-2* mutants.

YEL at 10 μg/mL and at 50 μg/mL induced stomatal closure in the wild-type plants (Fig. 1C), in accord with previous results (Khokon *et al*., 2010b). YEL at 10 μg/mL and at 50 μg/mL elicited stomatal closure in the *aba2-2* mutants (Fig. 1D). There were no significant differences in YEL-induced stomatal closure as

between the wild-type plants and the *aba2-2* mutants.

CHT at 10 µg/mL and at 50 µg/mL induced stomatal closure in the wild-type plants (Fig. 1E), in accord with previous results (Khokon *et al*., 2010a).[5] CHT at 10 µg/mL and at 50 µg/mL induced stomatal closure in the *aba2-2* mutants (Fig. 1F). There were no significant differences in CHT-induced stomatal closure as between the wild-type plants and the *aba2-2* mutants.

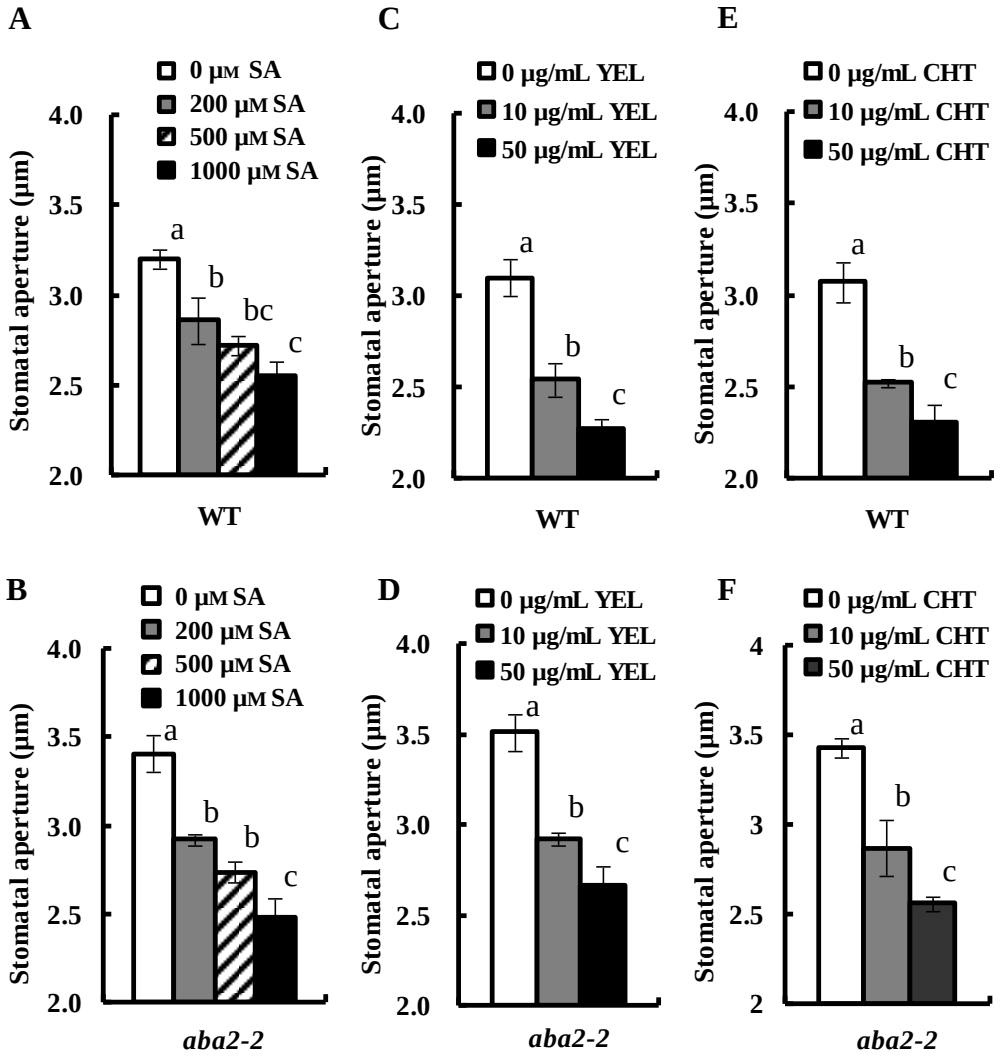

Fig. 2.1 SA-, YEL-, and CHT-Induced Stomatal Closure in the Wild-Type Plants and *aba2-2* Mutants.

A and B, Rosette leaves were treated with SA at 0 μM (white bars), 200 μM SA (ash bars), 500 μM SA (white bars with right-slanting black lines), or 1000 μM SA (black bars). C and D, Rosette leaves were treated with YEL at 0 μg/mL (white bars), 10 μg/mL (ash bars), or 50 μg/mL (black bars). E and F, Rosette leaves were treated with CHT at 0 μg/mL (white bars), 10 μg/mL (ash bars), or 50

μg/mL (black bars). Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.2 Effects of FLU on SA-, YEL-, and CHT-induced stomatal closure in the Arabidopsis wild-type plants

SA at 200 μM induced stomatal closure in wild-type plants in the absence and presence of 50 μM FLU (Fig. 2A). YEL at 50 μg/mL and CHT at 50 μg/mL induced stomatal closure in wild-type plants in the absence and presence of 50 μM FLU (Fig. 2B and C). These results suggest that the biosynthesis of ABA is not involved in SA-, YEL-, or CHT-induced stomata closure.

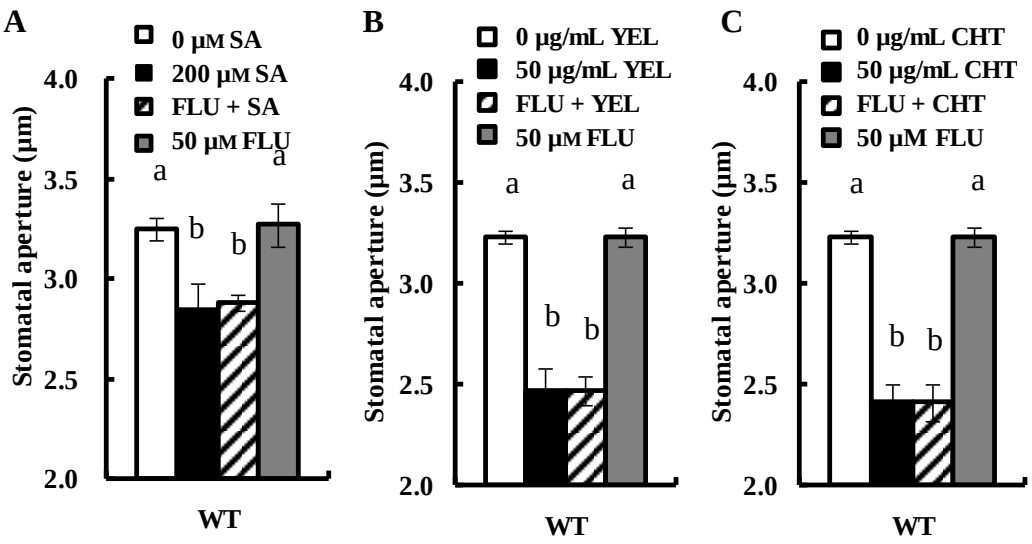

Fig. 2. Effects of FLU on SA-, YEL-, and CHT-Induced Stomatal Closure in the Wild-Type Plants.

A, Rosette leaves were treated with SA at 0 μM (white bars) or 200 μM (black bars) in the absence of FLU, or treated with SA at 0 μM (ash bars) or 200 μM (white bars with right-slanting black lines) in the presence of 50 μM FLU. B, Rosette leaves were treated with YEL at 0 μg/mL (white bars) or 50 μg/mL (black bars) in the absence of FLU, or treated with YEL at 0 μg/mL (ash bars) or 50 μg/mL (white bars with right-slanting black lines) in the presence of 50 μM FLU. C, Rosette leaves were treated with CHT at 0 μg/mL (white bars) or 50 μg/mL (black bars) in the absence of FLU, or treated with CHT at 0 μg/mL (ash bars) or 50 μg/mL (white bars with right-slanting black lines) in the presence of 50 μM FLU. FLU was added 30 min prior to SA, YEL, or CHT application. Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.4.3 SA-, YEL-, and CHT-induced stomatal closure in the *aos* mutants

SA at 200 μM, 500 μM, and 1000 μM induced stomatal closure in the *aos* mutants (Fig. 3A). There were no significant differences in SA-induced stomatal closure as between the wild-type plants and the *aos* mutants.

YEL at 10 μg/mL and 50 μg/mL and CHT at 10 μg/mL and 50 μg/mL elicited stomatal closure in the *aos* mutants (Fig. 3B and C). There were no significant differences in YEL- or in CHT-induced stomatal closure as between the wild-type plants and the *aos* mutants. SA at 200 μM, YEL at 50 μg/mL, and CHT at 50 μg/mL induced stomatal closure in *aos* mutants treated with 50 μM FLU (Fig.

3D), but 50 μM FLU alone did not induce stomatal closure in the *aos* mutants (Fig. 3D).

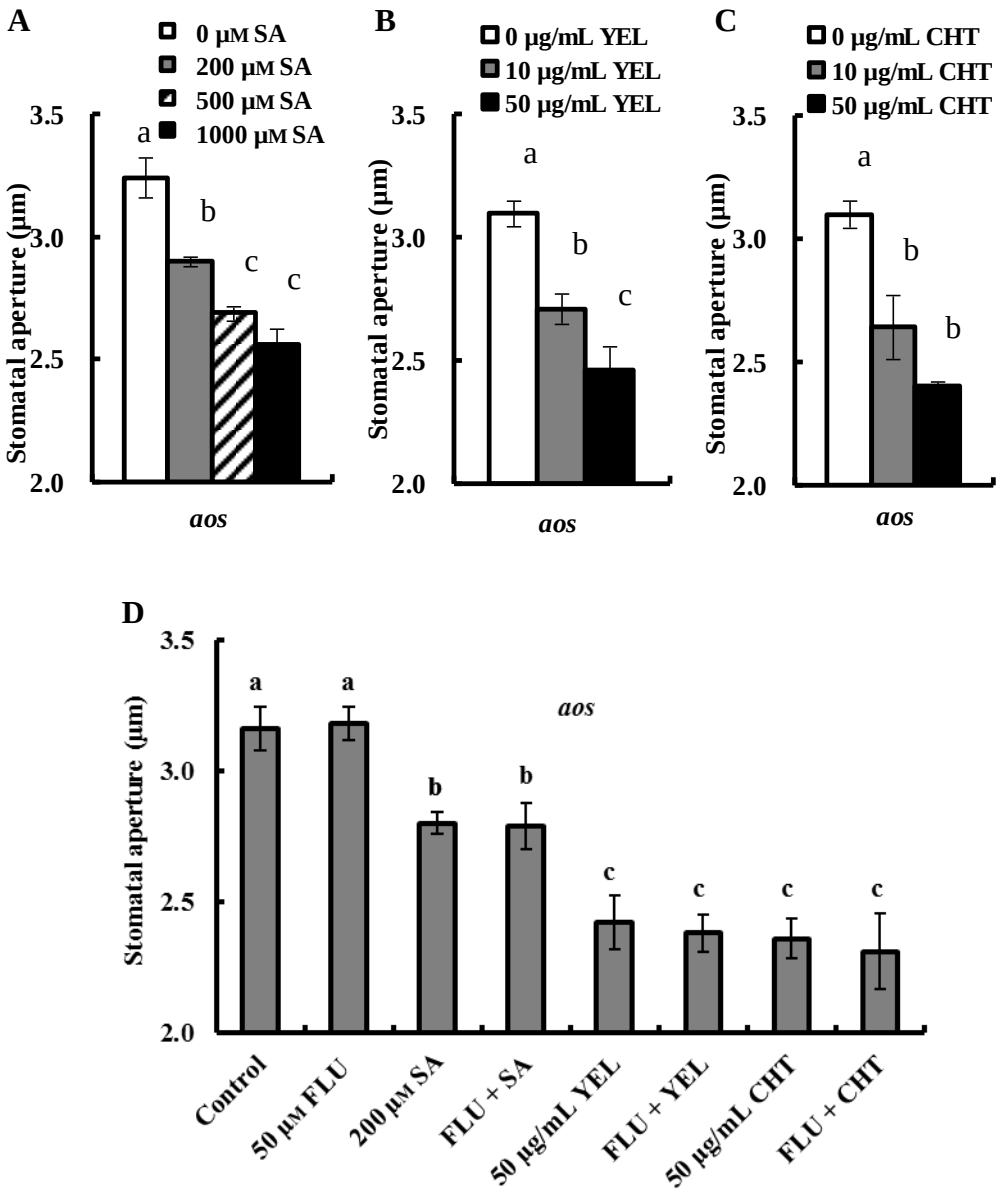

Fig. 3. SA-, YEL-, and CHT-Induced Stomatal Closure in *aos* Mutants.

A, Rosette leaves were treated with SA at 0 μM (white bars), 200 μM SA (ash bars), 500 μM SA (white bars with right-slanting black lines), or 1000 μM SA

(black bars). B, Rosette leaves were treated with YEL at 0 µg/mL (white bars), 10 µg/mL (ash bars), or 50 µg/mL (black bars). C, Rosette leaves were treated with CHT at 0 µg/mL (white bars), 10 µg/mL (ash bars), or 50 µg/mL (black bars). D, Effects of FLU on SA-, YEL-, and CHT-induced stomatal closure in the *aos* mutants. Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations. Values indicated by the same letter do not differ significantly at the 5% level, as determined by ANOVA with Tukey's test.

2.5 Discussion

2.5.1 Endogenous ABA is not required for SA-, YEL-, and CHT-induced stomatal closure

Endogenous ABA is required for MeJA-induced stomatal closure (Hossain *et al*., 2011) but not for AITC- or methylglyoxal (MG)-induced stomatal closure (Khokon *et al*., 2011B; Hoque *et al*., 2012). SA, YEL, and CHT induced stomatal closure in ABA deficient mutant *aba2-2* (Fig. 1), and in the wild-type plants treated with the ABA biosynthesis inhibitor FLU (Fig. 2). This suggests that ABA priming due to the presence of endogenous ABA is not required for SA-, YEL-, and CHT-induced stomatal closure. Moreover, SA-, YEL-, and CHT-induced stomatal closure was accompanied by the production of reactive oxygen species (ROS) mediated by salicylhydroxamic acid (SHAM)-sensitive peroxidases (Khokon *et al*., 2011a; 2010a; 2010b), whereas MeJA-induced stomatal closure was accompanied by ROS production mediated by NADPH oxidases (Munimasa *et al*., 2007). Hence, endogenous ABA might be involved in the activation of

NADPH oxidases.

2.5.2 Endogenous JAs is not required for SA-, YEL-, and CHT-induced stomatal closure

Endogenous JAs are required for AITC-induced stomatal closure (Khokon et al., 2011b) but not for MG-induced stomatal closure (Hoque *et al*., 2012). SA, YEL, and CHT induced stomatal closure in JAs-deficient mutant *aos* regardless of application of FLU (Fig. 3), suggesting that JAs priming, which is associated with endogenous JAs, is not required for SA-, YEL-, or CHT-induced stomatal closure.

2.5.3 Conclusion

From the above results it can be concluded that neither endogenous ABA nor endogenous JAs are required for SA-, YEL-, or CHT-induced stomatal closure in Arabidopsis.

CHAPTER 3

Involvement of Endogenous Salicylic Acid in Yeast Elicitor- and Chitosan-Induced Stomatal Closure in Arabidopsis

3.1 Abstract

In this study, we examined involvement of endogenous salicylic acid in yeast elicitor (YEL)- and chitosan (CHT)-induced stomatal closure using Arabidopsis (*Arabidopsis thaliana*) SA-deficient *nahG* transgenic lines. We found that YEL- and CHT-induced stomatal closures were impaired in *nahG* plants. On the other hand, SA at 50 μM after the YEL or CHT application induced stomatal closure in the *nahG* plants. We investigated YEL- and CHT-induced extracellular reactive oxygen species (ROS) production in the whole leaves and intracellular ROS accumulation in the guard cells of *nahG* plants. We observed that YEL- and CHT-induced extracellular ROS production and intracellular ROS accumulation were impaired in the *nahG* plants. However, SA at 50 μM in the presence of YEL or CHT induced ROS production and accumulation in the *nahG* plants. We also investigated YEL- and CHT-induced cytosolic alkalization and $[Ca^{2+}]_{cyt}$ elevation in the *nahG* guard cells. We observed that YEL- and CHT-induced cytosolic alkalization and $[Ca^{2+}]_{cyt}$ elevation were impaired in the *nahG* guard cells. In contrast, SA at 50 μM just after YEL or CHT application induced cytosolic alkalization and $[Ca^{2+}]_{cyt}$ elevation in the *nahG* guard cells. SA at 50 μM, which is not enough concentration to evoke SA responses in the wild-type, had no effect on the *nahG* plants. These results suggest that endogenous SA could be synthesized

by the both elicitors and is involved in YEL and CHT signal transduction leading to stomatal closure in Arabidopsis.

3.2 Introduction

Stomata are natural pores in the epidermis, and are surrounded by a pair of guard cells that regulate the size of stomatal apertures (Bergmann and Sack, 2007). Guard cells respond to a variety of external and internal stimuli such as light, drought, external Ca^{2+}, pathogen attack, phytohormones, and elicitors and regulate CO_2 uptake into leaves for photosynthesis, control of transpirational water loss, and innate immunity (Schroeder *et al*., 2001; Hetherington and Woodward, 2003; Suhita *et al*., 2004; Israelsson *et al*., 2006; Melotto *et al*., 2006; Munemasa *et al*., 2007; Shimazaki *et al*., 2007; Islam *et al*., 2009, 2010a, b; Khokon *et al*., 2010a, 2011; Salam *et al*., 2012, 2013).

Elicitors are chemical or biological molecules that mimic pathogen attack leading to physiological changes in plants (Zhao *et al*., 2005). Elicitor-induced stomatal closure is accompanied by reactive oxygen species (ROS) production (Lee *et al*., 1999; Allégre *et al*., 2009). Elicitors derived from fungal or plant cell walls induce defense responses in plants by means of complex signaling mechanisms that include ion fluxes across the plasma membrane, reversible protein phosphorylation, and the generation of reactive oxygen and nitrogen species (Lamb and Dixon, 1997; Durner and Klessig, 1999). Yeast elicitor (YEL) induces stomatal closure, hydrogen peroxide (H_2O_2) production, superoxide (O^{2-}) generation, nitric oxide (NO) production, cytosolic alkalization, and cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) oscillations in Arabidopsis (Klüsener *et al*., 2002; Khokon *et al*., 2010b; Salam *et al*., 2013). YEL

induces ROS production mediated by salicylhydroxamic acid (SHAM)-sensitive peroxidase leading to stomatal closure (Khokon *et al*., 2010b). YEL activates I_{Ca} currents and induces $[Ca^{2+}]_{cyt}$ oscillation in guard cells (Klüsener *et al*., 2002; Khokon *et al*., 2010b).

A fungal elicitor, Chitosan (CHT) (β-1,4-linked glucosamine), deacetylated derivative of chitin, induces stomatal closure in several plant species (Lee *et al*., 1999; Srivastava *et al*., 2009; Khokon *et al*., 2010a; Salam *et al*., 2012). CHT induces ROS production, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillations in Arabidopsis (Khokon *et al*., 2010a; Salam *et al*., 2012, 2013). In Arabidopsis, CHT-induced ROS production is mediated by salicylhydroxamic acid (SHAM)-sensitive peroxidase leading to stomatal closure (Khokon *et al*., 2010a). Barley mildew and its elicitor chitosan promote stomatal closure by activating S-type anion channels in guard cells (Koers *et al*., 2011). Cytosolic alkalization and $[Ca^{2+}]_{cyt}$ oscillation coordinately function in guard cell signaling in Arabidopsis (Islam *et al*., 2010b). YEL and CHT induce cytosolic alkalization and $[Ca^{2+}]_{cyt}$ oscillation in Arabidopsis guard cells (Khokon *et al*., 2010b; Salam *et al*., 2012, 2013). However, YEL and CHT signaling response to SA in guard cells are not well studied.

Salicylic acid (SA) is a phenolic plant hormone that induces stomatal closure in several plant species and plays a crucial role in plant defense (Manthe *et al*., 1992; Lee 1998; Mori *et al*., 2001; Khokon *et al*., 2011; Grant and Lamb, 2006). SA regulates various physiological processes like defense against harmful microorganisms, growth and ion uptake (Hayat *et al*., 2007). SA induces stomatal closure accompanied with extracellular ROS production and intracellular ROS

accumulation mediated by SHAM-sensitive peroxidase (Mori *et al*., 2001; Khokon *et al*., 2011). SA induces an elevation of $[Ca^{2+}]_{cyt}$ ion concentration (Kawano *et al*., 1998). An influx of extracellular Ca^{2+} to the guard cells is required for SA-induced stomatal closure (Mori *et al*., 2001). SA does not induce $[Ca^{2+}]_{cyt}$ oscillations but suppresses K^{+}_{in} channel currents (Khokon *et al*., 2011). SA is a key endogenous signal involved in plant defense responses (Dempsey *et al*., 1999). However, functions of endogenous SA in guard cells are not fully understood.

The bacterial *nahG* (*naphthalene hydroxylase G*) gene encodes a SA-degrading enzyme, salicylate hydroxylase, and its expression in transgenic plants prevents the accumulation of SA and completely eliminates the onset of systemic acquired resistance (Gaffney *et al*., 1993). Plants expressing *nahG* in the chloroplasts are unable to accumulate SA (Fragnière *et al*., 2011). *nahG* transgenic line shows SA deficiency (Abreu and Munné-Bosch, 2009). The Arabidopsis *nahG* transgenic line is intensely used to establish the essential role of SA in plant responses to abiotic stresses.

SA signaling mutant *accelerated cell death 6* (*acd6*) is an over expressed line having a high level of defenses that require SA as a sole activator as well as defenses that requires SA as a coactivator (Rate *et al*., 1999). *acd6* acts partially through a *NONEXPRESSOR OF PR1* (*NPR1*) gene-independent pathway but SA-dependent pathway that activates defenses and confers resistance to pathogen (Rate *et al*., 1999). The SA accumulating mutant *siz1* is a deficient line that causes reduction of stomatal aperture in Arabidopsis (Miura *et al*., 2013). *siz1* mutation enhances the accumulation of SA and SA-inducible gene transcripts (Lee *et al*., 2006). *SIZ1* encodes an Arabidopsis SUMO E3 ligase and regulates innate

immunity (Lee *et al*., 2006). This pathogen-induced SA biosynthesis is controlled by *SID2*, which encodes isochorismate synthase 1 (ICS1; Wildermuth et al., 2001). Arabidopsis SA induction-deficient (*sid2*) mutants are defective in pathogen-induced SA accumulation and are severely compromised in disease resistance to biotrophic pathogens (Nawrath and Metraux, 1999; Wildermuth et al., 2001). Thus, the regulation of *SID2* expression is fundamental to plant immunity.

However, there is no report whether endogenous SA requires for YEL- and CHT-induced stomatal closure in Arabidopsis. To address the physiological and biochemical functions of the plants response to YEL and CHT, we use the *Arabidopsis* mutants, *nahG, sid2, acd6, and siz1-2*, to investigate the role of endogenous SA. Here, we found that YEL and CHT did not induce stomatal closure in the *nahG* plants and from this view point we hypothesized that endogenous SA could be involve in YEL- and CHT-induced stomatal closure.

I examined (i) YEL- and CHT-induced stomatal closure in WT and *nahG* mutants. (ii) YEL- and CHT-induced H_2O_2 production in the whole leaves of the WT and *nahG* plants. (iii) YEL- and CHT-induced O^{2-} generation in the whole leaves of the WT and *nahG* plants. (iv) YEL- and CHT-induced intracellular ROS accumulation in the guard cells of the WT and *nahG* plants. (v) YEL- and CHT-induced cytosolic alkalization in the WT and *nahG* guard cells. (vi) YEL- and CHT-induced cytosolic free Ca^{2+} oscillations in the WT and *nahG* guard cells. vii) Effect of 50 μM SA on YEL- and CHT-induced stomatal closure, extracellular ROS production, intracellular ROS accumulation, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillation in *nahG* mutants. (viii) YEL- and CHT-induced stomatal closure in the *sid2* mutants. (ix) YEL- and CHT-induced stomatal closure in *acd6*

and *siz1-2* mutants. (x) SA-induced stomatal closure in WT and *nahG* mutants. Our results indicate that endogenous SA might be involved in YEL and CHT signal transduction leading to stomatal closure in Arabidopsis.

3.3 Materials and Methods

3.3.1 Plant materials and growth conditions

In this study, I used the Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia as the WT plant, *nahG* transgenic lines (Lawton *et al*., 1995) and *sid2* (stock no. CS16438 in the Arabidopsis Biological Resource Center at Ohio State University, http://abrc.osu. edu) as the SA deficient mutant, and *acd6* (Rate *et al*., 1999) and *siz1-2* (Miura *et al*., 2005) as the SA accumulating mutants. In the guard cells, cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$, was measured using a Ca^{2+}-sensing fluorescent protein, Yellow Cameleon 3.6 (YC3.6) (Nagai *et al*., 2004; Mori *et al*., 2006). To obtain YC3.6-expressing plants, *nahG* plants were crossed with a Columbia-0 plant that had previously been transformed with YC3.6. All the plants including the WT (Col-0) and *nahG* expressing Ca^{2+} reporter YC3.6 were grown on soil as previously describe (Hossain *et al*., 2011). The temperature and relative humidity in the growth chamber were 22ºC ± 2ºC and 60% ± 10%, respectively. Water was applied two to three times per week with Hyponex solution (0.1%) on the plant growth tray.

3.3.2 Measurement of stomatal aperture

Stomatal aperture measurements were performed as previously described (Murata *et al*., 2001; Munemasa *et al*., 2007). Briefly, excised rosette leaves were

floated on a stomatal assay solution containing 5 mM KCl, 50 mM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 μmol m^{-2} s^{-1}) to induce stomatal opening, followed by the addition of SA, YEL, or CHT. The pre- or post-treated SA was added 30 min before or 30 min after the SA, YEL, or CHT applications. After 2-h incubation, the leaves were shredded in a commercial blender for 30 s and the remaining epidermal tissues were collected using nylon mesh. Twenty stomatal apertures were measured per epidermal peel.

3.3.3 Measurement of Extracellular ROS Production in Whole Leaves

The Hydrogen peroxide (H_2O_2) and superoxide (O^{2-}) production in the whole leaves were visualized using 3,3′-diaminobenzidine tetrahydrochloride hydrate and nitro blue tetrazolium as previously described (Khokon *et al*., 2011; Salam *et al*., 2013). The rosette leaves of Arabidopsis plants were excised, floated on stomata assay solution supplemented with 0.05% Tween 20 and incubated for 2 h in the light. Then the leaves were transferred in the stomata assay solution supplemented with 1 mg mL^{-1} of DAB and gently infiltrated in a vacuum for 2 h. Then, 50 mg mL^{-1} YEL or 50 mg mL^{-1} CHT was added and infiltrated for 3 h. 50 μM SA was added 30 min after YEL or CHT application. After incubation, the leaves were cleared in boiling ethanol for 5 min. Localization of H_2O_2 was visualized as a reddish-brown coloration. For detection of O^{2-}, nitro blue tetrazolium (NBT) (1 mg mL^{-1}) was used in lieu of DAB. Localization of O^{2-} was visualized as blue coloration. In both cases, after clearing the leaves in ethanol, the leaves were mounted on cover glass and pictures were taken. The intensity of

coloration was quantified using Adobe Photoshop CS2 (Adobe System Inc.; Jan Jose, CA, USA) software.

3.3.4 Measurement of Intracellular ROS Accumulation in Guard Cells

ROS accumulation in guard cell was examined using 50 μM of 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma, St. Louis, MO, USA) (Murata *et al*., 2001; Suhita *et al*., 2004; Munemasa *et al*., 2007; Saito *et al*., 2008; Islam *et al*., 2010a). In case of ROS detection, epidermal tissues were incubated for 3 h in the light in a stomatal assay solution containing 5 mM KCl, 50 mM $CaCl_2$, 10 mM MES-Tris, pH 6.15 and then 50 μM H_2DCF-DA was added to this medium. The epidermal tissues were incubated for 30 min at room temperature, and then the excess dye was washed out with distilled water. The dye-loaded tissues were treated with 50 mg mL^{-1} YEL or 50 mg mL^{-1} CHT for 30 min. 50 μM SA was added just after YEL or CHT application and then fluorescence of guard cells were imaged using a fluorescence microscope (Biozero BZ-8000, Keyence, Osaka, Japan) with filter: OP-66835BZ filter GFP (excitation wavelength, 510 nm; and dichroic mirror wavelength, 505 nm). The fluorescence intensity was analyzed using ImageJ 1.42q softwere (NIH, Bethesda, MD, USA).

3.3.5 Measurement of Cytosolic Alkalization in Guard Cells

A pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Rink *et al*., 1982) were used to analyze cytosolic alkalization in guard cells as previously described (Islam *et al*.,

2010a).The epidermal tissues were collected in the assay solution containing 50 mM KCl and 10 mM MES-KOH (pH 6.5) and incubated for 3 h in light. After incubation, 20 μM BCECF-AM was added to the sample and were incubated for 30 min in the dark at room temperature to load the dye. Then the tissues were rinsed several times with the buffer solution in order to remove excess dye. The dye-loaded epidermal tissues were treated with 200 μM SA, 50 μg mL^{-1} YEL, or 50 μg mL^{-1} CHT for 20 min. 50 μM SA was added just after YEL or CHT application. Then images were captured and analyzed as described before.

3.3.6 Measurement of $[Ca^{2+}]_{cyt}$ Oscillations in Guard Cell

Four- to 6-week-old WT and *nahG* plants expressing YC3.6 were used for the measurement of guard cell $[Ca^{2+}]_{cyt}$ oscillations, as previously described (Islam *et al*., 2010a, b; Hossain *et al*., 2011). The abaxial side of the excised leaf was softly attached on a glass slide using medical adhesive (stock no. 7730; Hollister), and then adaxial epidermis and mesophyll tissues were removed carefully with a razor blade to keep the intact lower epidermis on the slide. Isolated abaxial epidermal peels were incubated in the stomatal assay solution containing 5 mM KCl, 50 mM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) under light for 2 h at 22^0C to promote stomatal opening. The turgid guard cells were used to measure the guard cell $[Ca^{2+}]_{cyt}$ oscillations. Guard cells were treated with 50 mg mL^{-1} YEL, 50 mg mL^{-1} CHT solutions by using a peristaltic pump in the 5 min after monitoring. 50 μM SA was added just after YEL or CHT application. For dual-emission ratio imaging of YC3.6, a 440AF21 excitation filter, a 445DRLP dichroic mirror, and two emission filters, 480DF30 for cyan fluorescent protein (CFP) and 535DF25 for yellow fluorescent protein (YFP),

were used. The CFP and YFP fluorescence intensities of guard cells were imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics). Note that we used the same exposure time for both CFP and YFP.

3.3.7 Chemicals

H_2DCF-DA and NBT were purchased from Sigma-Aldrich (St. Louis, Mo), SA from Wako Pure Chemical Industries (Osaka, Japan), DAB from Tokyo Chemical Industries (Tokyo, Japan), BCECF-AM from Invitrogen Molecular Probes (Eugene, Oregon, USA).

3.3.8 Accession number

Arabidopsis Genome Initiative numbers for the genes discussed in this manuscript are as follows: *NAHG* (X83926), *SID2* (AT1G74710), *ACD6* (AT4G14400), and *SIZ1-2* (AT5G60410).

3.3.9 Statistical Analysis

The significance of differences between mean values of stomatal apertures, ROS production, ROS accumulation, and cytosolic alkalization were assessed by Student's *t*-test, and the frequency of $[Ca^{2+}]_{cyt}$ oscillations was assessed by chi-squared (χ^2) test. Differences at P value ≤ 0.05 were considered significant.

3.4 Results

3.4.1 Impairment of YEL- and CHT-Induced Stomatal Closure in the *nahG* Plants

To clarify the involvement of endogenous SA in YEL- and CHT-induced stomatal closure, I examined YEL- and CHT-induced stomatal closure in the WT and *nahG* plants. Exogenous application of YEL at 50 μg mL^{-1} and CHT at 50 μg mL^{-1} induced stomatal closure in the WT plants (Fig. 1. A). On the other hand, YEL at 50 μg mL^{-1} and CHT at 50 μg mL^{-1} did not induced stomatal closures in the *nahG* plants (Fig. 1. B).

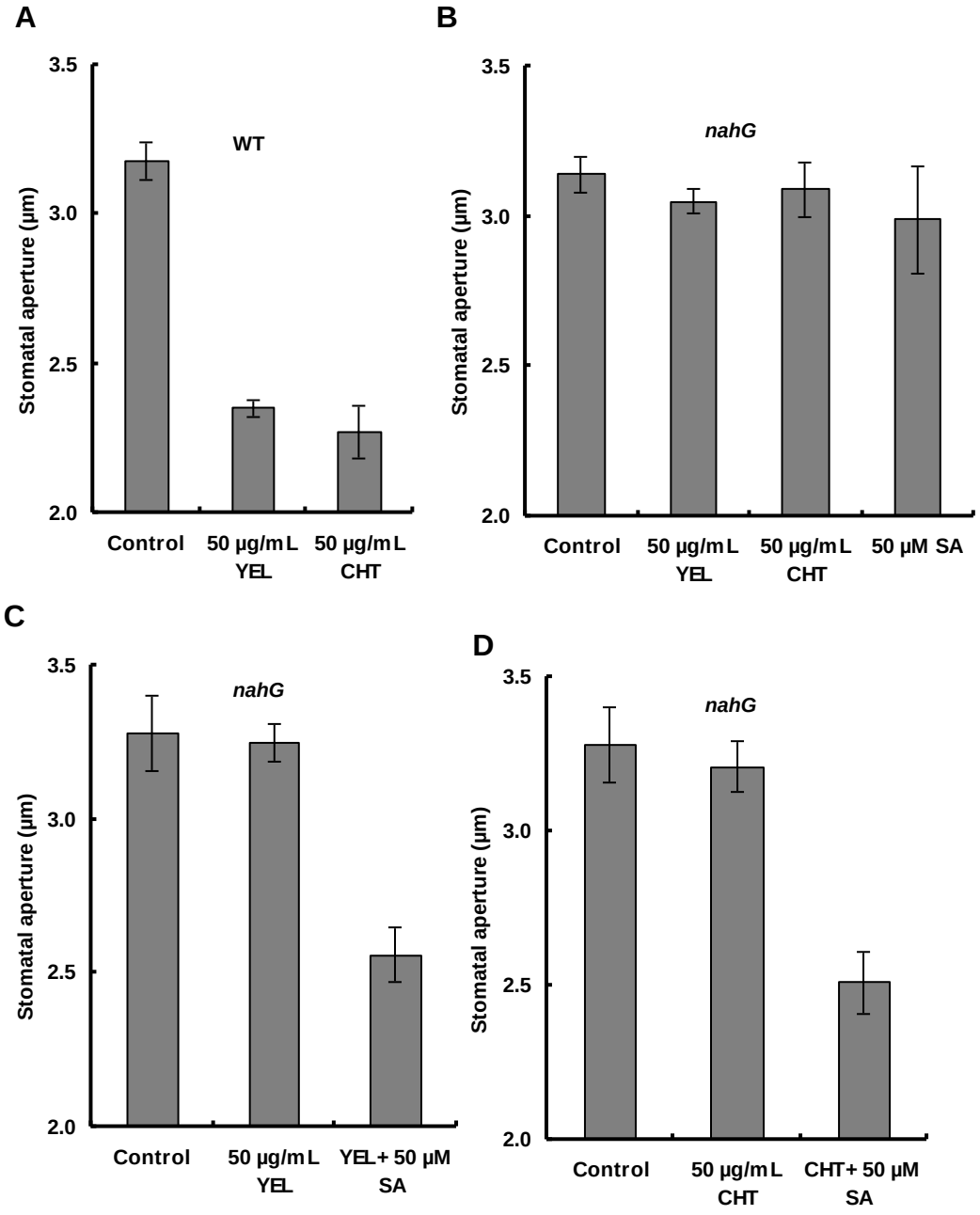

Figure 3.1 YEL- and CHT-induced stomatal closure in the Arabidopsis WT and *nahG* plants. **A**, YEL- and CHT-induced stomatal closure in the WT plants. **B**, YEL- and CHT- induced stomatal closure in the *nahG* plants. **C**, Effect of 50 μM SA on the YEL-induced stomatal closure in *nahG* plants. SA was applied 30 min after the YEL application. **D**, Effect of 50 μM SA on the CHT-induced stomatal closure in *nahG* plants. SA was applied 30 min after the CHT application. Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations.

3.4.2 Effect of 50 μM SA on YEL- and CHT-induced stomatal closure in the *nahG* plants

To obtain more clear evidence for the involvement of endogenous SA in YEL and CHT signaling, I examined effect of 50 μM SA on YEL- and CHT-induced stomatal closure in the *nahG* plants. I found that SA at 50 μM after the YEL or CHT application induced stomatal closure in the *nahG* plants (Fig. 1. C and D). On the other hand, SA at 50 μM alone did not induce stomatal closure in the WT (Figure 9 A) and *nahG* plants (Fig. 1. B). These results indicate that endogenous SA is required for YEL- and CHT-induced stomatal closure in Arabidopsis.

3.4.3 Impairment of YEL- and CHT-Induced extracellular H_2O_2 Production in the Whole Leaves of nahG plants

To confirm the involvement of endogenous SA in the YEL- and CHT-induced stomatal closure, we examined YEL- and CHT-induced extracellular H_2O_2 production in the whole leaves of WT and *nahG* plants using histochemical

approaches. Formation of H_2O_2 was visualized by using DAB staining (Thordal-Christensen et al., 1997). DAB staining demonstrated that YEL at 50 μg mL^{-1} and CHT at 50 μg mL^{-1} induced H_2O_2 production in the whole leaves of WT plants (Fig. 2. A). On the other hand, YEL at 50 μg mL^{-1} and CHT at 50 μg mL^{-1} did not induce H_2O_2 production in the whole leaves of *nahG* plants (Fig. 2. B). DAB staining also demonstrated that SA at 50 μM in the presence of YEL or CHT induced H_2O_2 production in the whole leaves of *nahG* plants (Fig. 3. B). On the other hand, SA at 50 μM alone did not induce H_2O_2 production in the whole leaves of *nahG* plants (Fig. 3. B). These results indicate that YEL- and CHT-induced extracellular H_2O_2 productions were might be due to endogenous SA.

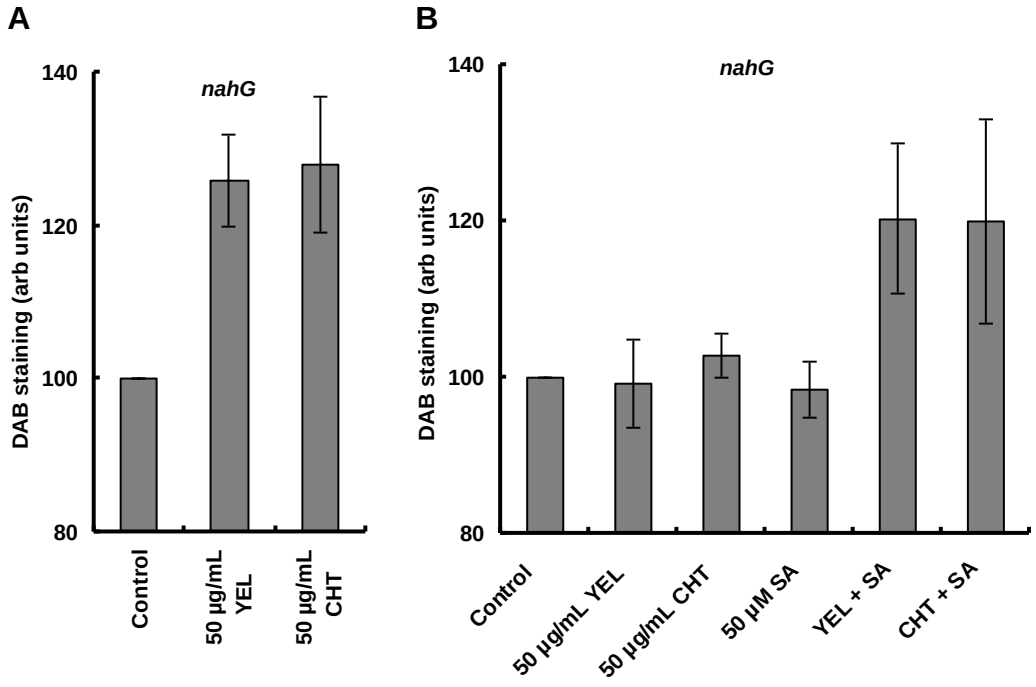

Figure 3.2 YEL- and CHT-induced H_2O_2 production in the whole leaves of WT and *nahG* plants. **A**, YEL- and CHT-induced H_2O_2 production in the whole leaves of WT *nahG* plants. **B**, YEL- and CHT-induced H_2O_2 production in the whole

leaves of *nahG* plants. DAB staining were quantified based on arbitrary value of the stained area of leaves. Effect of 50 µM SA in presence of YEL or CHT application on the H_2O_2 production are presented. Averages for three independent experiments (12 leaves in total per bar) are shown. Error bars represent standard deviations.

3.4.4 Impairment of YEL- and CHT-Induced extracellular O^{2-} generation in the Whole Leaves of nahG plants

To clarify the involvement of endogenous SA in the YEL- and CHT-induced stomatal closure, I examined YEL- and CHT-induced extracellular O^{2-} generation in the whole leaves of WT and *nahG* plants using histochemical approaches. O^{2-} generation was examined by using NBT staining (Doke, 1983). NBT staining demonstrated that YEL at 50 µg mL^{-1} and CHT at 50 µg mL^{-1} induced O^{2-} generation in the whole leaves of WT plants (Fig. 3. A). By contrast, YEL at 50 µg mL^{-1} and CHT at 50 µg mL^{-1} did not induce O^{2-} generation in the whole leaves of *nahG* plants (Fig.3. B). NBT staining also demonstrated that SA at 50 µM in the presence of YEL or CHT induced O^{2-} generation in the whole leaves of *nahG* plants (Fig.3. B). On the other hand, SA at 50 µM alone did not induce O^{2-} production in the whole leaves of *nahG* plants (Fig. 3. B). These results indicate that YEL- and CHT-induced O^{2-} generation were might be due to increment of endogenous SA, suggesting that endogenous SA is required for YEL- and CHT-induced extracellular ROS production.

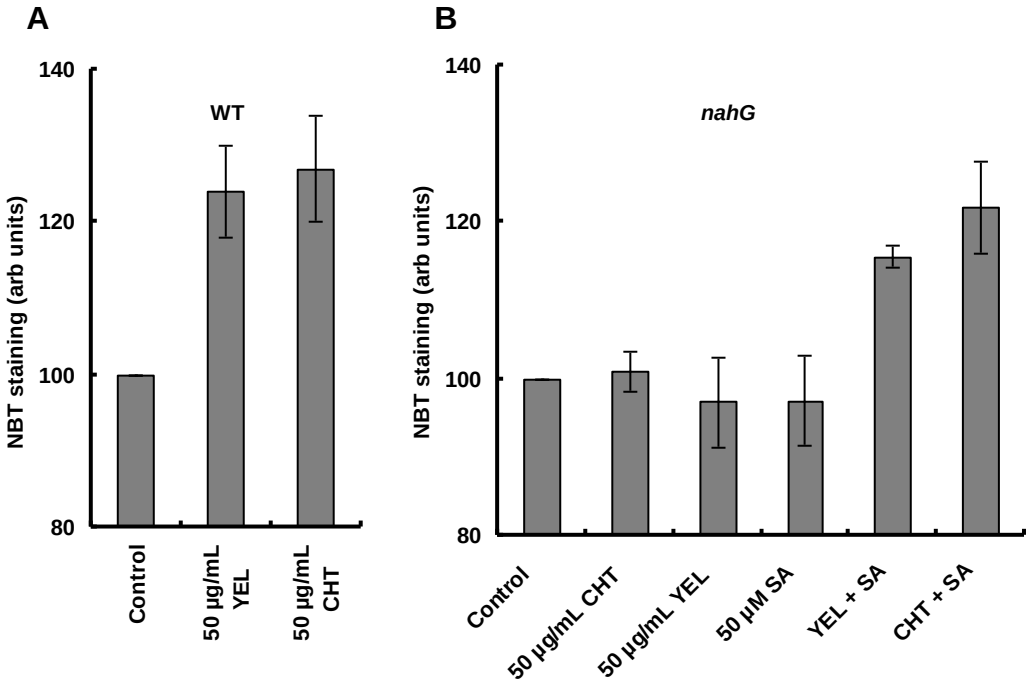

Figure 3.3 YEL- and CHT-induced O^{2-} generation in the whole leaves of WT and *nahG* plants. **A**, YEL- and CHT-induced O^{2-} generation in the whole leaves of WT plants. **B**, YEL- and CHT-induced O^{2-} generation in the whole leaves of *nahG* plants. NBT staining was quantified based on arbitrary value of the stained area of leaves. Effect of 50 µM SA in presence of YEL or CHT application on the O^{2-} generation are presented. Averages for three independent experiments (12 leaves in total per bar) are shown. Error bars represent standard deviations.

3.4.5 Impairment of YEL- and CHT-Induced intracellular ROS accumulation in the nahG guard cells

To clarify the requirement of endogenous SA in the YEL- and CHT-induced

stomatal closure in Arabidopsis, I examined YEL- and CHT-induced intracellular ROS accumulation in the WT and *nahG* guard cells. I found that YEL at 50 μg mL^{-1} and CHT at 50 μg mL^{-1} induced ROS accumulation in the WT guard cells (Fig. 4. A). By contrast, YEL at 50 μg mL^{-1} and CHT at 50 μg mL^{-1} did not induce ROS accumulation in the *nahG* guard cells (Fig. 4. B). However, SA at 50 μM in the presence of YEL or CHT induced ROS accumulation in the nahG guard cells which was completely abolished by a peroxidase inhibitor SHAM (Fig. 4. B). Moreover, SA, YEL, and CHT induced stomatal closure in the NADPH oxidase knockout mutants, *atrbohD atrbohF* (Fig. 5. B and D). On the other hand, 50 μM SA alone did not induce ROS accumulation in the *nahG* guard cells (Fig. 4. B). These results indicate that YEL- and CHT-induced ROS accumulation in the guard cells were might be due to increment of endogenous SA levels, suggesting that endogenous SA is required for YEL- and CHT-induced ROS accumulation in Arabidopsis.

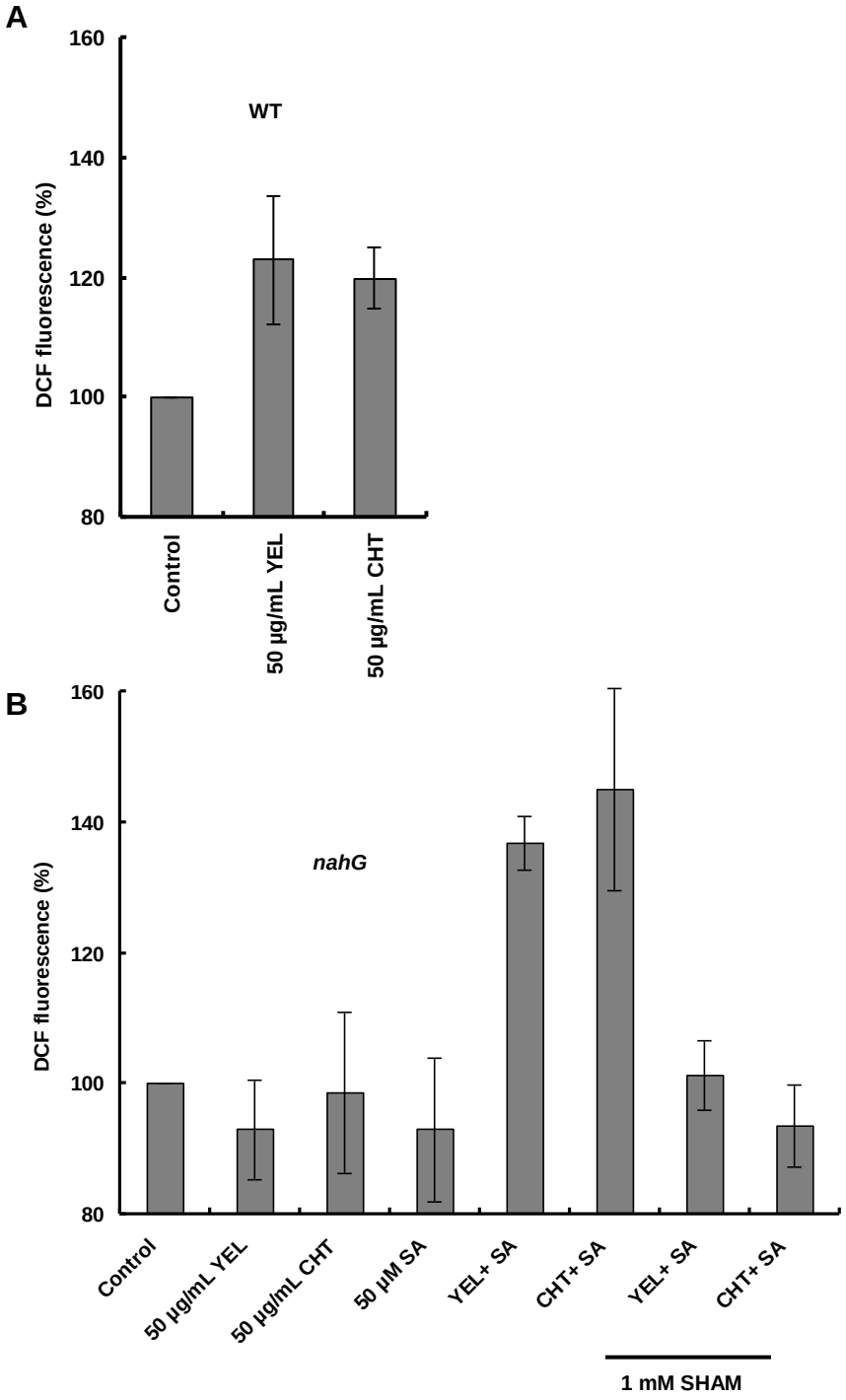

Figure 3.4 YEL- and CHT-induced ROS accumulation in the WT and *nahG* guard cells. A, YEL- and CHT-induced ROS accumulation in the WT guard cells. B, YEL- and CHT-induced ROS accumulation in the *nahG* guard cells. Effect of 50

μM SA in the presence of YEL and CHT application on the ROS accumulation are shown. Effect of 1 mM SHAM on (YEL+SA)- and (CHT+SA)-induced ROS accumulation are presented. Averages for three independent experiments (12 leaves in total per bar) are shown. Error bars represent standard deviations.

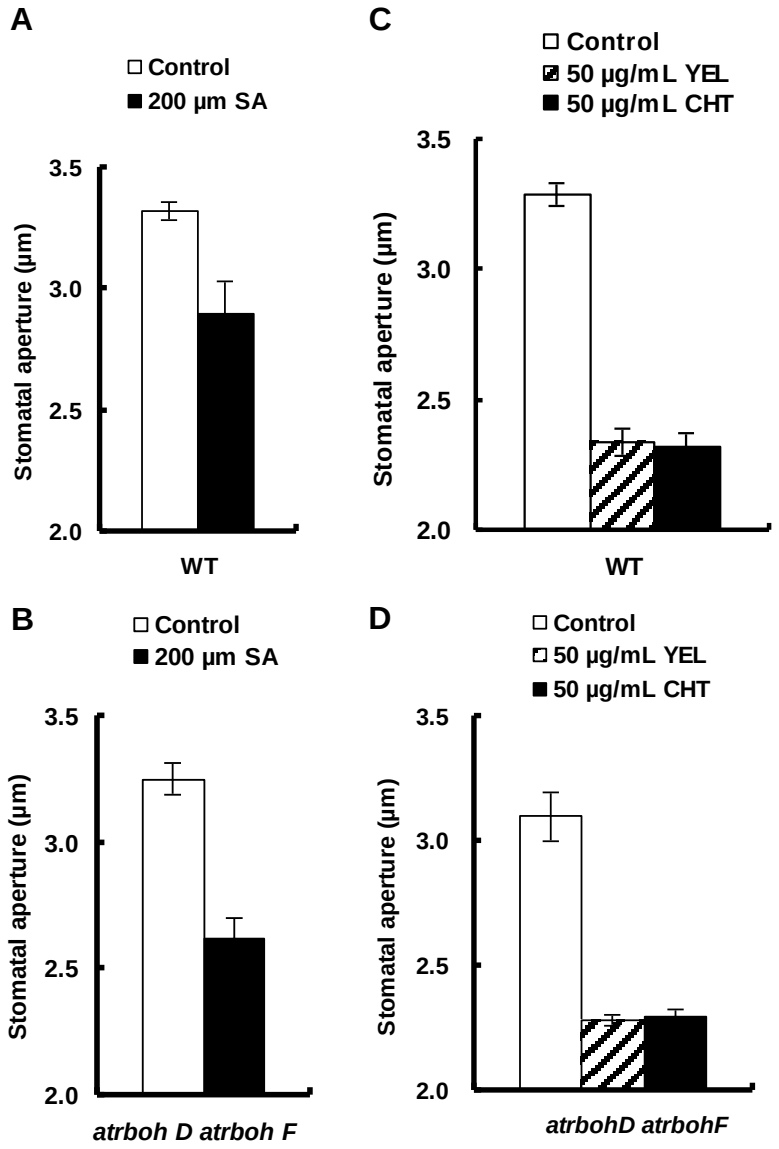

Figure 3.5 SA-, YEL-, and CHT-induced stomatal closure in the WT and *atrbohD*

atrbohF mutants. **A**, SA-induced stomatal closure in the WT plants. **B**, SA-induced stomatal closure in the *atrbohD atrbohF* mutants. **C**, YEL- and CHT-induced stomatal closure in the WT plants. **D**, YEL- and CHT-induced stomatal closure in the *atrbohD atrbohF* mutants. Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations.

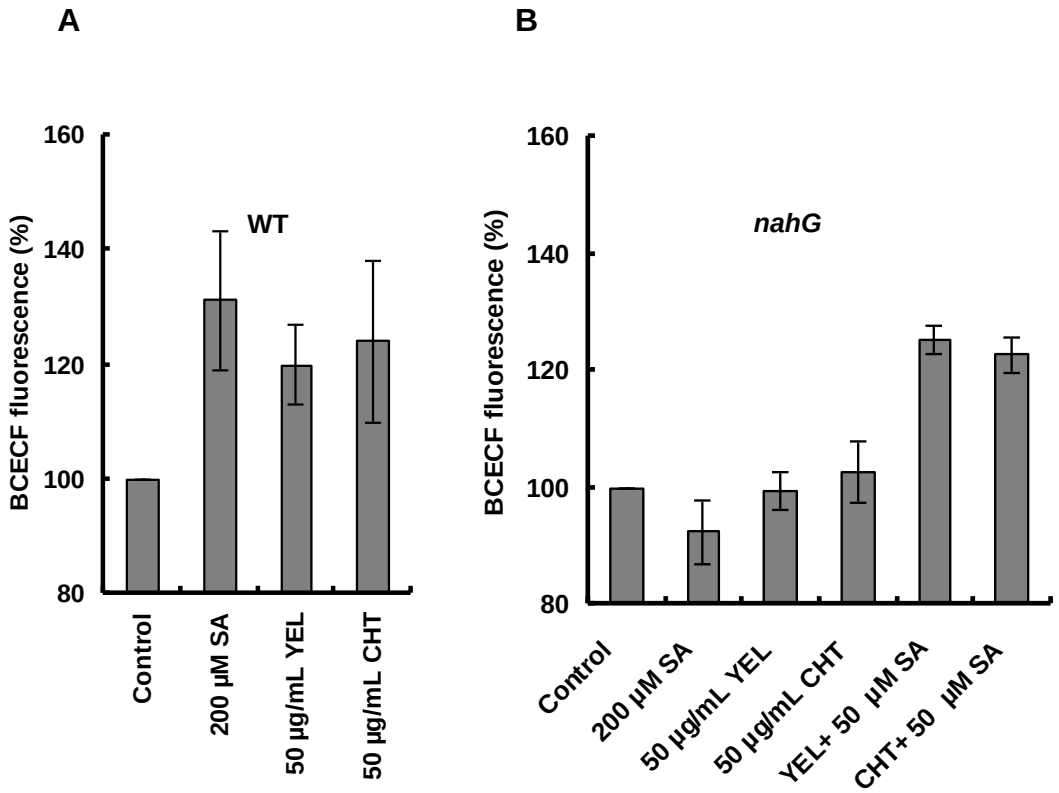

Figure 3.6 SA-, YEL-, and CHT-induced cytosolic alkalization in the Arabidopsis WT and *nahG* plants. **A**, SA-, YEL-, and CHT-induced cytosolic alkalization in the WT guard cells. **B**, SA-, YEL-, and CHT-induced cytosolic alkalization in the *nahG* guard cells. Effect of 50 µM SA in presence of YEL and CHT application on the ROS accumulation in *nahG* guard cells are presented. Averages for three

independent experiments (12 leaves in total per bar) are shown. Error bars represent standard deviations.

3.4.6 Impairment of SA-, YEL- and CHT-induced cytosolic alkalization in the *nahG* guard cells

To get more clear evidence in the involvement of endogenous SA in YEL- and CHT-induced stomatal closure, I carried out SA-, YEL-, and CHT-induced cytosolic alkalization in the WT and *nahG* guard cells. I found that SA at 200 μM, YEL at 50 μg mL^{-1}, and CHT at 50 μg mL^{-1} induced cytosolic alkalization in the WT guard cells (Fig. 6. A). By contrast, SA at 200 μM, YEL at 50 μg mL^{-1}, and CHT at 50 μg mL^{-1} did not induce cytosolic alkalization in the *nahG* guard cells (Fig. 6. B). However, application of SA at 50 μM in the presence of YEL or CHT induced cytosolic alkalization in the *nahG* guard cells. This result suggests that endogenous SA is involved in YEL- or CHT-induced cytosolic alkalization leading to stomatal closure.

3.4.7 Impairment of YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations in the nahG guard cells

In order to clarify the involvement of endogenous SA in YEL- and CHT-induced stomatal closure, we examined YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations using WT and *nahG* plants expressing the Ca^{2+} reporter Yellow Cameleon 3.6. In the WT plants, without any treatment 29% of guard cells showed $[Ca^{2+}]_{cyt}$ oscillations (n = 8 of 28 cells; three or more oscillations, 14%; one or two oscillations 15%; no oscillation 71%; Fig. 7 A and C, Control). In the *nahG* plants,

without any treatment 32% of guard cells showed $[Ca^{2+}]_{cyt}$ oscillations (n = 9 of 28 cells; three or more oscillations, 11%; one or two oscillations 21%; no oscillation 68%; Fig. 7 B and C, Control). In the WT plants, 100% of guard cells treated with 50 μg mL^{-1} YEL showed $[Ca^{2+}]_{cyt}$ oscillations (n = 28 of 28 cells; three or more oscillations, 64%; one or two oscillations 36%; Fig. 7 D and F, YEL). In the *nahG* plants, 47% of guard cells treated with 50 μg mL^{-1} YEL showed $[Ca^{2+}]_{cyt}$ oscillations (n = 17 of 36 cells; three or more oscillations, 17%; one or two oscillations 31%; no oscillation 53%; Fig. 7 E and F, YEL). In the WT plants, 80% of guard cells treated with 50 μg mL^{-1} CHT showed $[Ca^{2+}]_{cyt}$ oscillations (n = 24 of 30 cells; three or more oscillations, 37%; one or two oscillations 43%; no oscillation 20%; Fig. 7 G and I, CHT). In the *nahG* plants, 28% of guard cells treated with 50 μg mL^{-1} CHT showed $[Ca^{2+}]_{cyt}$ oscillations (n = 6 of 30 cells; three or more oscillations, 10%; one or two oscillations 10%; no oscillation 80% Fig. 7 H and I, CHT). These results indicate that endogenous SA might be required for YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations suggesting that endogenous SA is involved in YEL- and CHT-induced stomatal closure in Arabidopsis.

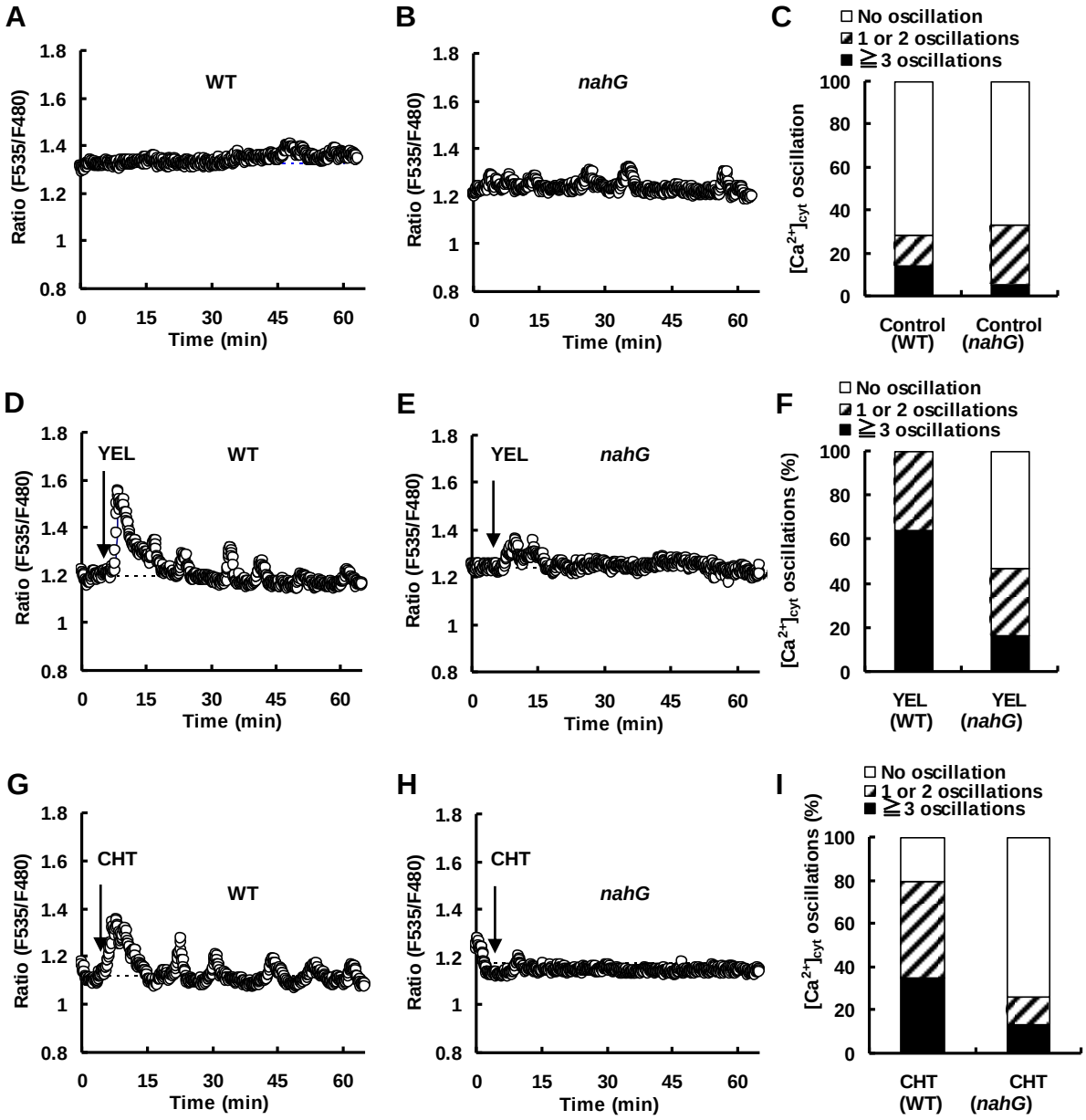

Figure 3.7 YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations in the WT and *nahG* guard cells. Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in **A,** untreated WT guard cells (n = 8 of 28 cells; 29%), **B**, untreated *nahG* guard cells (n = 9 of 28 cells; 32%), **C**, Stack column representation of untreated $[Ca^{2+}]_{cyt}$ oscillations (%) in WT guard cells (n = 28) and *nahG* guard

cells (n = 28). Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in **D,** 50 μg mL^{-1} YEL-treated WT guard cells (n = 28 of 28 cells; 100%), **E**, 50 μg mL^{-1} YEL-treated *nahG* guard cells (n = 17 of 36 cells; 47%), **F**, Stack column representation of YEL-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in WT guard cells (n = 28) and *nahG* guard cells (n = 36). Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in **G**, 50 μg mL^{-1} CHT-treated WT guard cells (n = 24 of 30 cells; 80%), **H**, 50 μg mL^{-1} CHT-treated *nahG* guard cells (n = 10 of 36 cells; 28%), **I**, Stack column representation of CHT-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in WT guard cells (n = 30) and *nahG* guard cells (n = 36).

3.4.8 Effect of 50 μM SA on the YEL- or CHT-induced $[Ca^{2+}]_{cyt}$ oscillations in the *nahG* guard cells

To get more solid evidence on the involvement of endogenous SA in YEL- and CHT-induced stomatal closure, I examined the effect of 50 μM SA on the YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations in the *nahG* guard cells. In the *nahG* plants, 100% of guard cells treated with 50 μM SA in the presence of YEL showed $[Ca^{2+}]_{cyt}$ oscillations (n = 44 of 44 cells; three or more oscillations, 75%; one or two oscillations 25%; 8 A and C). Moreover, in the *nahG* plants, 100% of guard cells treated with 50 μM SA in the presence of CHT showed $[Ca^{2+}]_{cyt}$ oscillations (n = 30 of 30 cells; three or more oscillations, 37%; one or two oscillations 63%; 8 B and C). This result suggests that endogenous SA is involved in YEL- or CHT-induced $[Ca^{2+}]_{cyt}$ oscillations leading to stomatal closure.

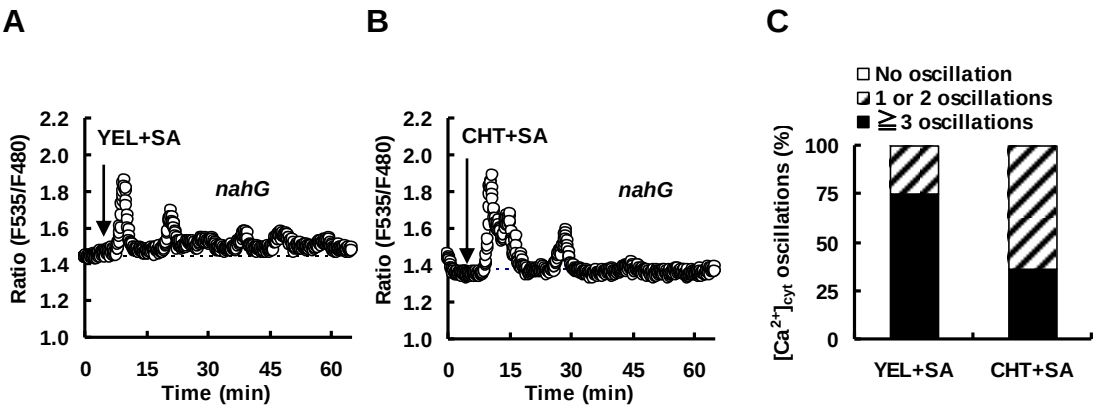

Figure 3.8 Effect of 50 μM SA on the YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations in *nahG* guard cells. A, Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 50 μM SA-treated *nahG* guard cells in the presence of 50 μg mL^{-1} YEL (n = 44 of 44 cells, 100%;). B, Representative data of fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 50 μM SA-treated *nahG* guard cells in the presence of 50 μg mL^{-1} CHT (n = 30 of 30 cells, 100%;). C, Stalk column representation of (YEL+SA)- and (CHT+SA)-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in the *nahG* guard cells. SA was applied just after YEL or CHT application.

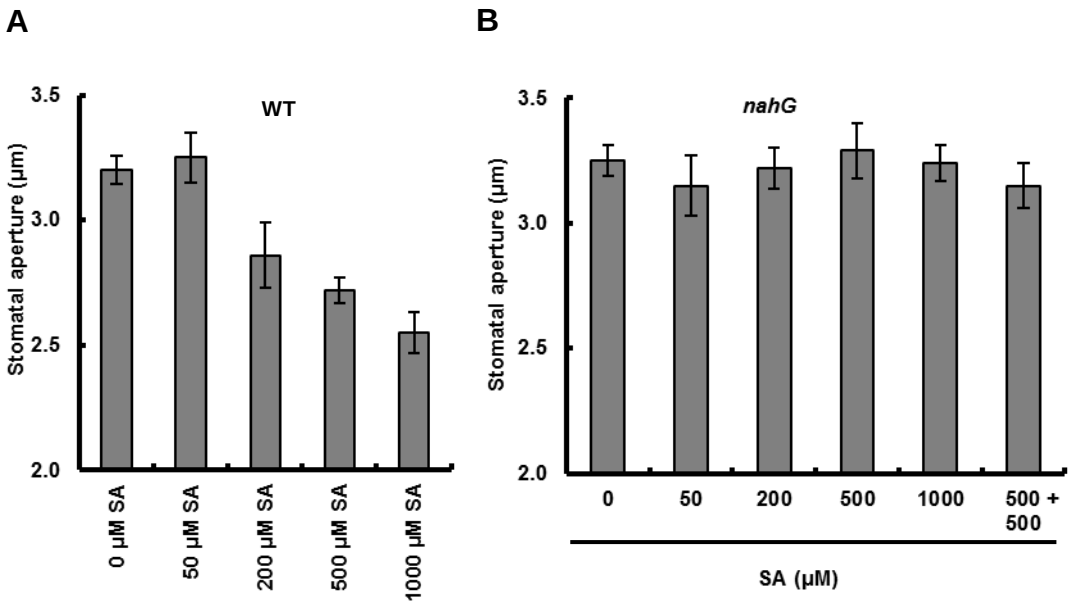

Figure 3.9 SA-induced stomatal closure in Arabidopsis WT and *nahG* plants. A, SA-inducd stomatal closure in the WT plants. B, SA-induced stomatal closure in the *nahG* plants. C, Effect of pre- and post-treated SA (500 μM) on the SA-induced stomatal closure in *nahG* plants. Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations.

3.4.9 Degradation of Exogenous SA in the *nahG* plants

To confirm the SA degradation in the *nahG* plants that express the *nahG* gene encoding salicylate hydroxylase, effect of SA on the WT and *nahG* transgenic Arabidopsis guard cells were examined. SA at 200 μM, at 500 μM, and at 1000 μM but not at 50 μM induced stomatal closure in the WT plants (Fig. 9. A). SA up to 1000 μM did not induce stomatal closure in the *nahG* plants (Fig.9. B). Even though post-treated with 500 μM SA had no effect on the SA (500 μM SA)-

induced stomatal closure indicating that *nahG* transgenic Arabidopsis could not accumulate endogenous SA.

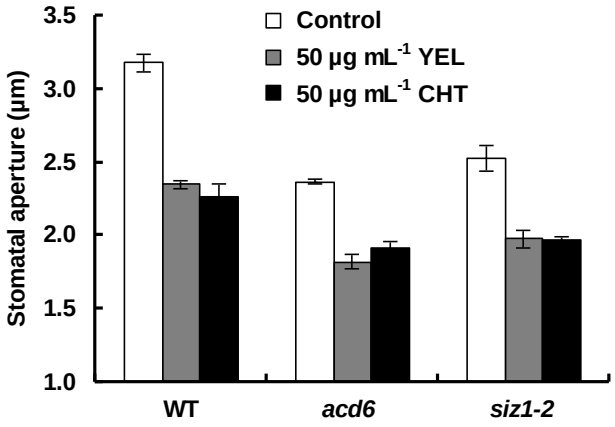

Figure 3.10 YEL- and CHT-induced stomatal closure in the *acd6* and *siz1-2* mutants. Rosette leaves of WT, *acd6*, and *siz1-2* plants were treated with 50 µg mL^{-1} YEL and 50 µg mL^{-1} CHT. Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations.

3.4.10 YEL- and CHT-induced stomatal closure in *acd6* and *siz1-2* mutants

To justify the role of YEL and CHT on endogenous SA accumulation in the guard cells in Arabidopsis, we examined YEL- and CHT-induced stomatal closure on the SA accumulating mutants, *acd6* and *siz1-2*. *acd6* and *siz1-2* mutation increased the accumulation of endogenous SA leading to stomatal closure. YEL and CHT induced stomatal closure both in the WT and in the *acd6* and *siz1-2* mutants. YEL- and CHT-induced stomatal closure in the mutants is significantly higher than the WT plants. These results suggest that SA biosynthesis

might be induced by YEL and CHT.

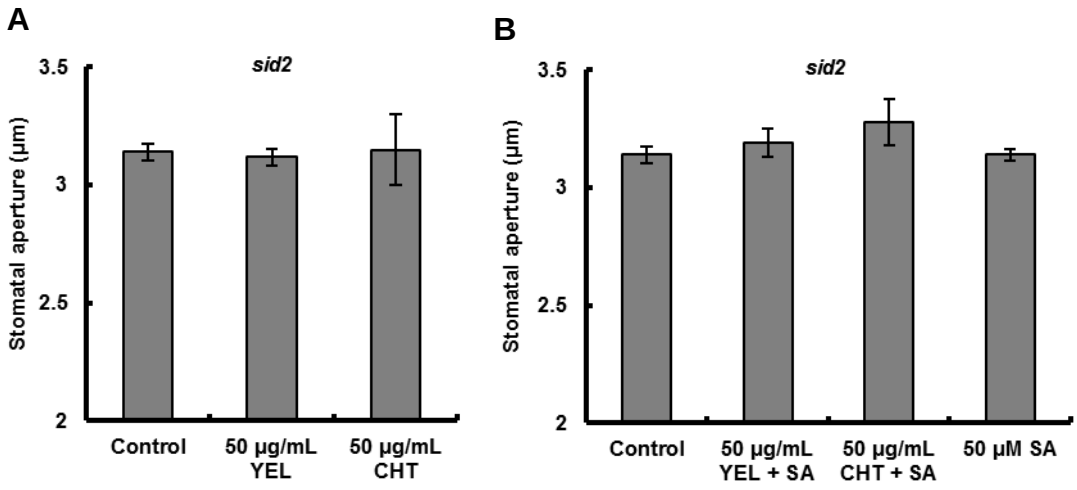

Fig. 3.11 Effect of *sid2* mutation on YEL- and CHT-induced stomatal closure. A, YEL- and CHT-induced stomatal closure in the *sid2* mutants. B, Effect of 50 μM Sa on YEL- and CHT-induced stomatal closure in the *sid2* mutants. Averages for three independent experiments (60 stomata in total per bar) are shown. Error bars represent standard deviations.

3.4.11 YEL- and CHT-induced stomatal closure in the *sid2* mutants

YEL and CHT did not induce stomatal closure in the *sid2* mutants. SA at 50 μM after the YEL and CHT application also did not induced stomatal closure in the *sid2* mutants. Moreover, 50 μM SA alone have no effect on stomatal closure in the *sid2* mutants. These results indicating that YEL and CHT might be induced SA biosynthesis.

3.5 Discussion

3.5.1 Involvement of endogenous SA in YEL- and CHT-induced

stomatal closure

Phytohormons and elicitors are regulated stomatal apertures in higher plants. SA, YEL, and CHT induce stomatal closure in Arabidopsis (Manthe et al., 1992; Lee and Raskin, 1998; Mori et al., 2001; Khokon et al., 2011a; Grant and Lamb, 2006; Klüsener *et al*., 2002; Khokon *et al*., 2010a, b; Salam *et al*., 2013). YEL- and CHT-induce stomatal closures are accompanied with ROS production mediated by salicylhydroxamic acid (SHAM)-sensitive peroxidase (Lee *et al*., 1999; Allégre *et al*., 2009; Khokon *et al*., 2010a, b). In this study, YEL and CHT did not induce stomatal closure (Fig 1 B) in the *nahG* plants. However, SA treatment after the YEL or CHT application induced stomatal closure in the *nahG* plants (Fig. 2, B), indicating that YEL- and CHT-induced stomatal closure are closely related with endogenous SA. The SA (50 μM), which was used in this study, are not enough concentration for stomatal closure in the WT plants. YEL or CHT with SA induced stomatal in the nahG plants indicating that YEL and CHT induce SA biosynthesis. These results suggest that endogenous SA is required for YEL- and CHT-induced stomatal closure in Arabidopsis.

3.5.2 Involvement of endogenous SA in YEL- and CHT-induced extracellular ROS production in the whole leaves and intracellular ROS accumulation in the guard cells

ROS production is an early event in the stomatal closure (Mori *et al*., 2001). YEL and CHT induce extracellular ROS production and intracellular ROS accumulation (Salam *et al*., 2012; Salam *et al*., 2013). YEL- and CHT-induce ROS production is mediated by salicylhydroxamic acid (SHAM)-sensitive peroxidase

(Lee *et al*., 1999; Allégre *et al*., 2009; Khokon *et al*., 2010a, b). YEL and CHT did not induce ROS production (Fig. 3, A and B) in the whole leaves and ROS accumulation (Fig. 4) in the guard cells of *nahG* plants. However, application of SA in the presence of YEL or CHT induced the production and accumulation of ROS in the *nahG* plants (Fig. 3, A, B, and Fig. 4). (YEL + SA)- and (CHT + SA)-induced ROS accumulation in the *nahG* guard cells was mediated by SHAM-sensitive peroxidase, in agreement with the SA-induced ROS accumulation (Khokon *et al*., 2011). These results indicate that the production and accumulation of ROS might be regulated by endogenous SA. These results suggest that endogenous SA is required for YEL- and CHT-induced extracellular ROS production and intracellular ROS accumulation resulting stomatal closure in Arabidopsis.

3.5.3 Involvement of endogenous SA in YEL- and CHT-induced cytosolic alkalization in guard cells

Increment of guard cells cytosolic alkalization is a common phenomenon in stomatal closure (Irving *et al*., 1992; Gehring *et al*., 1997; Suhita *et al*., 2004; Gonugunta *et al*., 2008). YEL and CHT induce cytosolic alkalization resulting to stomatal closure (Salam *et al*., 2013; Salam *et al*., 2012). Changes in cytosolic alkalization are closely involved in the modulation of ion mobilization leading to stomatal movement (Islam *et al*., 2010). Cytosolic alkalization also activates outward K^+ currents and inactivates inward K^+ currents to promote net efflux of K^+ in guard cells (Blatt and Armstrong, 1993). In this study, SA, YEL, and CHT induced cytosolic alkalization in the WT guard cells (Fig. 5, A) and expression of

the bacterial salicylate hydroxylase (*nahG*) inhibited the cytosolic alkalization (Fig. 5, B), indicating that the YEL- and CHT-induced cytosolic alkalization were closely related with endogenous SA. On the other hand, SA in the presence of YEL or CHT induced cytosolic alkalization in the *nahG* guard cells (Fig. 5, B), indicating that the YEL- or CHT-induced cytosolic alkalization might be due to endogenous SA. This result suggests that endogenous SA is required for YEL- and CHT-induced cytosolic alkalization resulting stomatal closure in Arabidopsis.

3.5.4 Involvement of endogenous SA in YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells

Changes in guard cells $[Ca^{2+}]_{cyt}$ are act as an early second messenger in signal transduction pathways that regulate stomatal aperture (Allen *et al*., 1999, 2000; Murata *et al*., 2001; Kwak *et al*., 2002; Islam *et al*., 2010a, b). $[Ca^{2+}]_{cyt}$ oscillations is attributed to Ca^{2+} influx from the extracellular space mediated by Ca^{2+} permeable channels (Pei *et al*., 2000; Siegel *et al*., 2009; Islam *et al*., 2010b). GFP-based Ca^{2+} indicator YC3.6 technique is used as a novel tool for direct monitoring of guard cell $[Ca^{2+}]_{cyt}$ oscillations (Allen *et al*., 1999, 2000). YEL and CHT induce elevations in the concentration of $[Ca^{2+}]_{cyt}$ and oscillations of $[Ca^{2+}]_{cyt}$ (Klüsener *et al*., 2002; Khokon *et al*., 2010b; Salam *et al*., 2013). YEL-induced $[Ca^{2+}]_{cyt}$ oscillation is elicited by ROS production mediated by SHAM-sensitive peroxidases (Khokon *et al*., 2010b). ROS activation of I_{Ca} channels is regulated elicitor-induced $[Ca^{2+}]_{cyt}$ increment (Klüsener *et al*., 2002). SA induces an elevation in $[Ca^{2+}]_{cyt}$ ion concentration (Kawano *et al*., 1998). An influx of extracellular Ca^{2+} to guard cells is required for SA-induced stomatal closure (Mori

et al., 2001). In this study, YEL and CHT did not show $[Ca^{2+}]_{cyt}$ oscillations in the *nahG* guard cells (Fig. 6 E, F, H, and I). On the other hand, SA treatment just after the YEL or CHT application induced $[Ca^{2+}]_{cyt}$ oscillations in the *nahG* guard cells (Fig. 7 A, B, and C), indicating that guard cell $[Ca^{2+}]_{cyt}$ elevation was induced by SA, suggesting that endogenous SA is required for YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ oscillations resulting stomatal closure.

3.6 Conclusion

Taken together, my results indicate that endogenous SA is required for YEL- and CHT-induced stomatal closure, extracellular ROS production, intracellular ROS accumulation, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillations. These findings suggest that endogenous SA might be involved in YEL and CHT signal transduction leading to stomatal closure in Arabidopsis.

General summary

Guard cells, which form stomatal pores in the leaf epidermis of higher plants, can respond to various environmental stimuli, including light, drought, and pathogen infection. To regulate carbon dioxide uptake for photosynthesis, transpirational water loss, and innate immunity adequately, plants have developed a fine-tuned signal transduction in guard cells. The volatile phytohormone SA and elicitors, YEL and CHT, induce stomatal closure similar to ABA and MeJA. Both elicitors share some signal components with ABA signaling in guard cells. YEL, CHT, and ABA induce the cytosolic alkalization and elevation of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in guard cells, resulting in stomatal closure. So far, ABA signal transduction has been extensively studied, but little is known about SA, YEL, and CHT signal transduction.

In Chapter 2, I investigated involvement of endogenous ABA and MeJA in SA, YEL, and CHT-induced stomatal closure in Arabidopsis. I examined SA-, YEL-, and CHT-induced stomatal closure in wild-type and aba2-2 mutants. SA, YEL, and CHT induced stomatal closure in the wild-type plants and aba2-2 mutants. FLU did not inhibit SA-, YEL-, and CHT-induced stomatal closure in wild-type plants. FLU alone has no effect on stomatal closure in the wild-type plants. I also investigated SA-, YEL-, and CHT-induced stomatal closure in the wild-type and aos mutants. SA, YEL, and CHT induced stomatal closure in the wild-type and aos mutants. FLU did not inhibit SA-, YEL-, and CHT-induced stomatal closure in the aos mutants. FLU alone also has no effect on stomatal closure in the aos mutants. These results suggest that neither endogenous abscisic acid nor jasmonates is involved in SA-, YEL-, and CHT-induced stomatal closure in Arabidopsis.

In Chapter 3, I examined involvement of endogenous salicylic acid in yeast elicitor (YEL)- and chitosan (CHT)-induced stomatal closure using Arabidopsis (*Arabidopsis thaliana*) SA-deficient *nahG* transgenic lines. I found that YEL and CHT did not induce stomatal closure in the *nahG* transgenic lines. On the other hand, SA at 50 μM after the YEL or CHT application induced stomatal closure in the *nahG* plants. I investigated YEL- and CHT-induced extracellular reactive oxygen species (ROS) production in the whole leaves of nahG plants. I found that YEL and CHT did not induce extracellular ROS production in the whole leaves of nahG plants. I also investigated YEL- and CHT-induced intracellular reactive oxygen species (ROS) accumulation in the nahG guard cells. I found that YEL and CHT did not induce intracellular reactive oxygen species (ROS) accumulation in the nahG guard cells. However, SA at 50 μM in the presence of YEL or CHT induced extracellular ROS production in the whole leaves and intracellular ROS accumulation in the guard cells of nahG plants. I also examined YEL- and CHT-induced cytosolic alkalization in *nahG* guard cells. I observed that YEL and CHT did not induce cytosolic alkalization in the *nahG* guard cells. In contrast, SA at 50 μM just after YEL or CHT application induced cytosolic alkalization in the *nahG* guard cells. SA at 50 μM, which is not enough concentration to evoke SA responses in the wild-type, had no effect on the *nahG* plants. Finally, I examined YEL- and CHT-induced $[Ca^{2+}]_{cyt}$ elevation in the *nahG* guard cells. I found that YEL and CHT did not induce $[Ca^{2+}]_{cyt}$ elevation in the nahG guard cells. In contrast, SA at 50 μM just after YEL or CHT application induced $[Ca^{2+}]_{cyt}$ elevation in the *nahG* guard cells. Taken together my results suggest that endogenous SA could be involved in YEL and CHT signal

transduction leading to stomatal closure in Arabidopsis.

Acknowledgements

It is the great mercy and blessings of Almighty Allah who enable me and keep me in good health to carry out my research and writing dissertation to complete my doctoral course.

I would like to express my heartiest gratitude and sincere appreciation to my benevolent research supervisor Dr. Yoshiyuki Murata, Professor, Faculty of Agriculture, Okayama University, Japan for his scholastic guidance, continuous encouragement and valuable suggestions for the successful completion of research work and preparation of the doctoral thesis.

I also express my sincere thanks to Dr. Yoshimasa Nakamura for his cordial suggestions, constructive criticism and guidance during my study. I am grateful to Dr. Izumi C. Mori for his cordial and intellectual suggestions during my research period. I am thankful to Dr. Yoshinobu Kimura to critically assess the thesis.

It is my immense pleasure to acknowledge Dr. Shintaro Munemasa for his help and valuable advice during the research period. I would like to express my sincere appreciation and thanks to all members of the lab. Special thanks to Dr. Misugi Uraji and Dr. Eiji Okuma for helping me.

I am grateful to the Ministry of Education, Sports, Science and Technology, Government of Japan for offering me the Japanese Government (Monbukagakusho) Scholarship during the entire study period in Japan.

I would like to dedicate this thesis to my beloved wife Amena Sultana for sharing my feelings, and for her special sacrifices, continuous cooperation and encouragement throughout the study period. I am also grateful to my beloved sun Labib Al-Amin for his special sacrifice to us.

Finally, I am profoundly obliged to my beloved parents, younger brothers and sisters, friends, and all the relatives for their continuous inspirations and supports in completing doctoral study.

References

Abreu ME and Munné-Bosch S. 2009. Salicylic acid deficiency in *nahG* transgenic lines and *sid2* mutants increases seed yield in the annual plant *Arabidopsis thaliana*. *J Expt Bot* **60**: 1261-1271.

Allégre M, Héloir MC, Trouvelot S, Daire X, Pugin A, Wendehenne D, Adrian M. 2009. Are grapevine stomata involved in the elicitor-induced protection against downy mildew? *Molecular Plant-Microbe Interactions* **22**: 977-986.

Allen GJ, Kuchitsu K, Chu SP, Murata Y, Schroeder JI. 1999. Arabidopsis abi1-1 and abi2-1 phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. *Plant Cell* **11**: 1785-1798.

Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF, Chori J, Schroeder JI. 2000. Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in Arabidopsis det3 mutant. *Science* **289**: 2338-2342.

Allen GJ, Murata Y, Chu SP, Nafisi M, Schroeder JI. 2002. Hypersensitivity of abscisic acid-induced cytosolic calcium increases in the Arabidopsis farnesyltransferase mutant *era1-2*. *The Plant Cell* **14,** 1649-1662.

Allan AC, Fluhr R. 1997. Two distinct sources of elicited Reactive Oxygen Species in tobacco epidermal cells. *The Plant Cell* **9:** 1559-1572.

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

Bartnicki-Garcia S. 1970. Cell wall composition and other biochemical markers in fungal phylogeny. In J.B. Harborne, ed. Phytochemical Phylogeny. Academic Press, London, PP 81-101.

Bergmann DC, Sack FD. 2007. Stomatal development. *Annu Rev plant Biol* **58**: 163-181.

Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, Denoux C, Hayes T, Gerrish C, Davies DR, Ausubel SM, Bolwell GP. 2006. Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *The Plant Journal*, **47**: 851-853.

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. 2006. ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. *The Plant Journal* **45**: 113-122.

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.

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.

Catala R, Ouyang J, Abreu IA, Hu Y, Seo H, Zhang X, Chua NH. 2007. The Arabidopsis E3 SUMO ligase SIZ1 regulates plant growth and drought responses. *Plant Cell* 19: 2952-2966.

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

Cochrane FC, Davin LB, Lewis NG. 2004. The Arabidopsis phenylalanine ammonia lyase gene family : kinetic characterization of the four PAL isoforms. *Phytochemistry* **65**: 1557-1564.

Cooper RM, Kemp B, Day R, Gomez-Vasquez R, Beeching JR. 2001. Pathogenicity and resistance in Xanthomonas blight of cassava. In: DeBoer SH, ed.

Plant pathogenic bacteria. Dortrecht: Kluwer, 319-323.

Doke N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans and to the hyphal wall components. *Physiological Plant Pathology* **23**: 345-357.

Delaney TP, Uknes S, Vernooij B, Friendrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J. 1994. A central role of salicylic acid in plant disease resistance. *Science* **266**: 1247-1250.

del Río LA, Sandalio LM, Corpas FJ, Palma JM, Barroso JB. 2006. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. *Plant Physiology* **141,** 330-335.

Dempsey D, Shah J, Klessig DF. 1999. Salicylic acid and disease resistance in plants. *Crit Rev Plant Sci* **18**: 547-575.

Dempsey DA, Volt AC, Wildermuth MC, Klessig DF. 2011. Salicylic acid biosynthesis and metabolism. *The Arabidopsis Book* **9**: 1-24.

Durner J, Klessig DF. 1999. Nitric oxide as a signal in plants. *Curr Opin Plant Biol* **2**: 369-374.

Enyedi AJ, Yalpani N, Silverman P, Raskin I. 1992. Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc Natl Acad Sci USA* **89**: 2480-2484.

Fragnière C, Serrano M, Abou-Mansour E, Métraux J, L'Haridon F. 2011. Salicylic acid and its location in response to biotic and abiotic stress. *FEBS Letters* **585**: 1847-1852.

Foreman J, Demidchik V, Bothwell JH, et al. 2003. Reactive oxygen species

produced by NADPH oxidase regulate plant cell growth. *Nature* **422,** 442-446.

Foyer CH, Noctor G. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell* **17,** 1866-1875.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* **261**: 754-756.

Gamble PE and Mullet JE. 1986. Inhibition of carotenoid accumulation and abscisic acid biosynthesis in fluridone-treated dark-grown barley. *Eur J Biochem*, **160**: 117-121.

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. *Proceedings of the National Academy of Sciences, USA* **100**: 11116-11121.

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.

Gehring CA, Irving HR, McConchie R, Parish RW. 1997. Jasmonates induce intracellular alkalization and closure of *Paphiopedilum* guard cells. *Annals of Botany* **80**: 485-489.

Gómez-vásquez R, Day R, Buschmann H, Randles S, Beeching JR, Cooper RM. 2004. Phenylpropanoids, phenylalnine ammonia lyase and peroxidases in elicitor-challenged cassava (Manihot esculenta) suspension cells and leaves. *Annals of Botany* **94**: 87-97.

Gonugunta VK, Srivastava N, Puli MR, Raghavendra AS. 2008. Nitric oxide production occurs after alkalization during stomatal closure induced by abscisic acid. *Plant Cell Env* **31**: 1717-1724.

Grant M, Lamb C. 2006. Systemic immunity. *Curr Opin Plant Biol* **9**: 414–420

Gudesblat GE, Iusem ND, Morris PC. 2007. Guard cell-specific inhibition of *Arabidopsis MPK3* expression causes abnormal stomatal responses to abscisic acid and hydrogen peroxide. *New Phytologist* **173,** 713-721.

Hayat S, Ali B, Ahmed A. 2007. Salicylic acid: Biosynthesis, metabolism and physiological role in plants. In S Hayat, A Ahmed, eds, Salicylic acid a plant hormone. Springer Inc, Dordrecht, The Netherland, pp 1-14.

Hao JH, Wang XL, Dong CJ, Zhang ZG, Shang QM. 2011. Salicylic acid induces stomatal closure by modulating endogenous hormone levels in cucumber cotyledons. *Russian J Plant Physiol* **58**: 906-913.

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

Hossain MA, Munemasa S, Uraji M, Nakamura Y, Mori IC, Murata Y. 2011. Involvement of endogenous abscisic acid in methyl jasmonate-induced stomatal closure in Arabidopsis. *Plant Physiol* **156**: 430-438.

Hoque TS, Uraji M, Ye Wenxiu, Hossain MA, Nakamura Y, and Murata Y. 2012. Methylglyoxal-induced stomatal closure accompanied by peroxidase-mediated ROS production in Arabidopsis *J Plant Physiol*, **169**: 979-986.

Issak M, Okuma E, Munemasa S, Nakamura Y, Mori IC, Murata Y. 2013. Neither endogenous abscisic acid nor endogenous jasmonate is involved in salicylic acid-, yeast elicitor-, or chitosan-induced stomatal closure in *Arabidopsis*

thaliana. *Bios Biotechnol Biochem* **77**: 1111-1113.

Islam MM, Tani C, Watanabe-Sugimoto M, Uraji M, Jahan MS, Masuda C, Nakamura Y, Mori IC, Murata Y. 2009. Myrosinases, TGG1 and TGG2, redundantly function in ABA and MeJA signaling in Arabidopsis guard cells. *Plant Cell Physiol* **50**: 1171-1175.

Islam MM, Munemasa S, Hossain MA, Nakamura Y, Mori IC, Murata Y. 2010a. Roles of AtTPC1, vacuolar two pore channel 1, in Arabidopsis stomatal closure. *Plant Cell Physiol* **51**: 302-311.

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

Israelsson M, Siegel RS, Young J, Hashimoto M, Iba K, Schroeder JI. 2006. Guard cell ABA and CO_2 signaling network updates and Ca^{2+} sensor priming hypothesis. *Curr Opin Plant Biol* **9**: 654-663.

Irving HR, Gehring CA, Parish RW. 1992. Changes in cytosolic pH and calcium of guard cells precede stomatal movements. *Proc Natl Acad Sci USA* **89**: 1790-1794.

Jammes F, Song C, Shin D, et al. 2009. MAP kinases MPK9 and MPK12 are preferentially expressed in guard cells and positively regulate ROS-mediated ABA signaling. *Proceedings of the National Academy of Sciences, USA* **106,** 20520-20525.

Jin, J.B., Jin, Y.H., Lee, J. et al. 2008. The SUMO E3 ligase, AtSIZ1, regu- lates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on FLC chromatin structure. *Plant J* **53**: 530-540.

Kawano T, Sahashi N, Takahashi K, Uozumi N, Muto S. 1998. Salicylic acid induces extracellular superoxide generation followed by an increase in cytosolic calcium ion in tobacco suspension culture: the earliest events in salicylic acid signal transduction. *Plant Cell Physiol* **39**: 721-730.

Kawano T, Takuya F, Muto SI. 2004. Controlled salicylic acid levels and corresponding signaling mechanisms in plants. *Plant Biotechnol* **21**: 319-335.

Khokon MAR, Uraji M, Munemasa S, Okuma E, Nakamura Y, Mori IC, Murata Y. 2010a. Chitosan-induced stomatal closure accompanied by peroxidase-mediated reactive oxygen species production in *Arabidopsis*. *Bios Biotechol Biochem* **74**: 2313-2315.

Khokon MAR, Hossain MA, Munemasa S, Uraji M, Nakamura Y, Mori IC, Murata Y. 2010b. Yeast-elicitor induced stomatal closure and peroxidase mediated ROS production in *Arabidopsis*. *Plant Cell Physiol* **51**: 1915-1921.

Khokon MAR, Okuma E, Hossain MA, Munemasa S, Uraji M, Nakamura Y, Mori IC, Murata Y. 2011a. Involvement of extracellular oxidative burst in salicylic acid-induced stomatal closure in *Arabidopsis*. *Plant Cell Environ* **34**: 434-443.

Khokon MAR, Jahan MS, Rahman T, Hossain MA, Muroyama D, Minami I, Munemasa S, Mori IC, Nakamura Y, and Murata Y. 2011b. Allyl isothiocyanate (AITC) induces stomatal closure in *Arabidopsis*. *Plant Cell Environ*, **34**: 1900-1906.

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. *Annual Review of Plant Biology* **61**: 561-591.

Klick S, Herrmann K. 1988. Glucosides and glucose esters of hydroxybenzoic acids in plants. *Phytochemistry* **27**: 2127-2180.

Klüsener B, Young JJ, Murata Y, Allen GJ, Mori IC, Hugouvieux V, Schroeder JI. 2002. Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in *Arabidopsis* guard cells. *Plant Physiol* **130**: 1-12.

Koers S, Guzel-Deger A, Marten I, Roelfsema MRG. 2011. Barley mildew and its elicitor chitosan promote closed stomata by stimulating guard-cell S-type anion channels. *The Plant Journal* **68**: 670-680.

Kudla J, Batistič O, Hashimoto K. 2010. Calcium signals: The lead currency of plant information processing. *The Plant Cell* **22,** 541-563.

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. *Plant Cell* **14**: 2849-2861.

Kwak JM, Mori IC, Pei ZM, 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 Journal* **22,** 2623-2633.

Lamb C, Dixon RA. 1997. The oxidative burst in plant diseases resistance. *Annu Rev Plant physiol plant Mol Biol* **48**: 251-275.

Lawton K, Weymann K, Friedrich L, Vernooij B, Uknes S, Ryals J. 1995. Systemic-acquired resistance in Arabidopsis requires salicylic acid but not ethylene. *Mol Plant Microbe Interact* **8**: 863-870.

Lee JS. 1998. The mechanism of stomatal closing by salicylic acid in *Commelina communis* L. *J Plant Biol* **41:** 97-102.

Lee H and Raskin I. 1998. Glucosylation of salicylic acid in Nicotiana tabacum

cv. Xanthi-nc. *Phytopathol*, **88**: 692-697.

Lee S, Choi H, Suh S, Doo I, Oh K, Choi EJ, Taylor ATS, Low PS, Lee Y. 1999. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol* **121**: 147-152.

Lee J, Nam J, Park HC, Na G, Miura K, Jin JB, Yoo CY, Baek D, Kim DH, Jeong JC, Kim D, Lee SY *et al.* 2007. Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase. *Plant J* **49**: 79-90.

Leung J, Bouvier-Durand M, Morris P-C, Guerrier D, Chefdor F, Giraudat J. 1994. Arabidopsis ABA response gene ABl7-features of a calcium-modulated protein phosphatase. *Science* **264,** 1448-1452.

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

Liechti R, Farmer EE. 2002. The jasmonate pathway. *Science* **296,** 1649-1650.

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.

Malamy J, Hennig J, Klessig DF. 1992. Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. *Plant Cell* **4**: 359-366.

Manthe B, Schulz M, Schnabl H. 1992 Effects of salicylic acid on growth and stomatal movements of *Vicia faba* L.: Evidance for salicylic acid metabolization. *J Chem Ecol* **18**: 1525-1539.

Mateo A, Mühlenbock P, Ruste´ rucci C, Chang CCC, Miszalski Z, Karpinska B, Parker JE, Mullineaux PM, Karpinski S. 2004. *LESION SIMULATING DISEASE 1* is required for acclimation to conditions that promote excess excitation energy. *Plant Physiol* **136**: 2818-2830.

Mauch F., Mauch-Mani B. & Boller T. 1988. Antifungal hydrolases in pea tissue. II Inhibition of fungal growth by combination of chitinase and β-1,3-glucanase. *Plant Physiology* **88**: 936-942.

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 Physiology* **111**: 1031-1042.

Melotto M, Underwood W, Koczan J, Nomura K, He SY. 2006. Plant stomata function in innate immunity against bacterial invasion. *Cell* **126**: 969-980.

Messner B, Schroder P. 1999. Burst amplifying system in cell suspension cultures of spurs (*Pices abies*). Modulation of elicitor-induced release of hydrogen peroxidase (oxidative burst) by ionophores and salicylic acid. *J Appl Bot* **73**: 6-10.

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

Miura K, Rus A, Sharkhuu A, Yokoi S, Karthikeyan AS, Raghothama KG, Baek D, Koo YD, Jin JB, Bressan RA, Yun D, Hasegawa PM. 2005. The Arabidopsis SUMO E3 ligase *SIZ1* controls phosphate deficiency responses. *Proc. Natl Acad. Sci. USA* **102**: 7760-7765

Miura K., Jin JB, Hasegawa PM. 2007a. Sumoylation, a post-translational regulatory process in plants. *Curr. Opin. Plant Biol.* **10**: 495-502.

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun DJ, Hasegawa PM. 2007b. SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in Arabidopsis. *Plant Cell* 19: 1403-1414.

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM. 2009. Sumoylation of ABI5 by the Arabidopsis SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. *Proc. Natl Acad. Sci. USA* **106:** 5418-5423.

Miura K, Hasegawa PM. 2009. Sumoylation and abscisic acid sig- naling. *Plant Signal Behav* **4**: 1176-1178.

Miura K, Hasegawa PM. 2010. Sumoylation and other ubiquitin- like post-translational modifications in plants. *Trends Cell Biol* **20**: 223-232.

Miura K, Lee J, Gong Q, Ma S, Jin JB, Yoo CY, Miura T, Sato A, Bohnert HJ, Hasegawa PM. 2011a. SIZ1 regulation of phosphate starvation-induced root architecture remodeling involves the control of auxin accumulation. *Plant Physiol* **155**: 1000-1012.

Miura K, Sato A, Ohta M, Furukawa J. 2011b. Increased tolerance to salt stress in the phosphate-accumulating Arabidopsis mutants siz1 and pho2. *Planta* 234: 1191-1199.

Miura K, Okammoto H, Okuma E, Shiba H, Kamada H, Hasegawa PM, Murata Y. 2013. *SIZ1* deficiency causes reduced stomatal aperture and enhanced tolerance via controlling salicylic acid-induced accumulation of reactive oxygen species in Arabidopsis. *The Plant Journal* **73**: 91-104.

Mori IC, Pinontoan R, Kawano T, Muto S. 2001. Involvement of superoxide generation in salicylic acid-induced stomatal closure in *Vicia faba*. *Plant Cell*

Physiol **42**: 1383-1388.

Mori IC, Murata Y, Yang Y, Munemasa S, Wang Y, 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* **6**:1749-1762.

Moore R and Smith JD. 1984. Growth, graviresponsiveness and abscisic-acid content of Zea *mays* seedlings treated with fluridone. *Planta* **164**: 342-344.

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.

Munemasa S, Hossain MA, Nakamura Y, Mori IC, Murata Y. 2011. The *Arabidopsis* calcium-dependent protein kinase, CPK6, functions as a positive regulator of methyl jasmonate signaling in guard cells. *Plant Physiology* **155**: 553-561.

Murata Y, Pei ZM, Mori IC, Schroeder JI. 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. *The Plant Cell* **13**: 2513-2523.

Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A. 2004. Expanded dynamic range of fluorescent indicators for $Ca(^{2+})$ by circularly permuted yellow fluorescent proteins. *Proc Natl Acad Sci USA* **101**: 10554-10559.

Nambara E, Kawaide H, Kamiya Y, and Naito S. 1998. Characterization of an

Arabidopsis thaliana mutant that has a defect in ABA accumulation: ABA-dependent and ABA-independent accumulation of free amino acids during dehydration. *Plant Cell Physiol* **39**: 853-858.

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.

Ohkuma K, Lyon JL, Addicott FT, Smith OE. 1963. Abscisin II, an abscission accelerating substance from young cotton fruit. *Science* **142**: 1592-1593.

Outlaw WHJr. 2003. Integration of cellular and physiological functions of guard cells. *Critical Reviews in Plant Sciences* **22**: 503-529.

Park J, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, and Feyereisen R. 2002. A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. *Plant J* **31**: 1-12.

Park SY, Fung P, Nishimura N, et al. 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324,** 1068-1071.

Pei ZM, Murata Y, Benning G, Thomine S, Klu¨sener B, AllenGJ, Grill E, Schroeder JI. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406**: 731-734.

Rate DN, Cuenca JV, Bowman GR, Guttman DS, Greenberg JT. 1999. The gain-of-function Arabidopsis *acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. *Plant Cell* **11**: 1695-1708.

Rink TJ, Tsien RY, Pozzan T. 1982. Cytoplasmic pH and free Mg^{2+} in lymphocytes. *The J Cell Biol* **95**: 189-196.

Rodriguez PL, Benning G, Grill E. 1998. ABI2, a second protein phosphatase 2C involved in abscisic acid signal transduction in *Arabidopsis*. *FEBS Letters* **421,** 185-190.

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 signalling. *The Plant Journal* **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.

Salam MA, Jammes F, Hossain MA, Ye W, Nakamura Y, Mori IC, Kwak JM, Murata Y. 2013. Two guard cell-preferential MAPKs, MPK9 and MPK12, regulate YEL signaling in Arabidopsis guard cells. *Plant Biol* **15**: 436-442.

Salam MA, Jammes F, Hossain MA, Ye W, Nakamura Y, Mori IC, Kwak JM, Murata Y. 2012. MAP kinases, MPK9 and MPK12, regulate chitosan-induced stomatal closure. *Bios Biotechnol Biochem* **76**: 1785-1787.

Santiago J, Dupeux F, Round A, Antoni R, Park S-Y, Jamin M, Cutler SR, Rodriguez PL, Márquez JA. 2009. The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature* **462**: 665-668.

Sanders D, Brownlee C, Harper JF. 1999. Communicating with calcium. *The Plant Cell* **11**: 691-706.

Sanders D, Pelloux J, Brownlee C, Harper JF. 2002. Calcium at the crossroads of signaling. *The Plant Cell* **14**: 401-417.

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D. 2001*a*. Guard cell signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 627-658.

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

Shimazaki K, Doi M, Assmann SM, Kinoshita T. 2007. Light regulation of stomatal movement. *Annu Rev Plant Biol* **58**: 219-247.

Siegel RS, Xue S, Murata Y, Yang Y, Nishimura N, Wang A, Schroeder JI. 2009. Calcium elevation-dependent and attenuated resting calcium-dependent abscisic acid-induced enhancement of calcium sensitivities of S-type anion and inward-rectifying K^+ channels in Arabidopsis guard cells. *The Plant Journal* **59**: 207-220.

Srivastava N, Gonugunta VK, Puil MR, Raghavendra AS. 2009. Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of *Pisum sativum*. *Planta* **229**: 757-765.

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.

Sun LR, Hao FS, Lu BS, Ma LY. 2010. AtNOA1 modulates nitric oxide accumulation and stomatal closure induced by salicylic acid in Arabidopsis. *Plant*

Signal Behav **5**: 1022-1024.

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *The Plant Journal* **11**: 1187-1194.

Torres MA, Dangl JL, Jones JDG. 2002. *Arabidopsis* gp91phox homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proceedings of the National Academy of Sciences, USA* **99,** 517-522.

Turner JG, Ellis C, Devoto A. 2002. The jasmonate signal pathway. *The Plant Cell* **14,** S153-164.

Yamamoto S, Katagiri M, Maeno H, Hayaishi O. 1965. Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. *J Biol Chem* **240**: 3408-341.

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 & Molecular Biology* **16,** 1230-1236.

Yoo CY, Miura K, Jin JB, Lee J, Park HC, Salt DE, Yun DJ, Bres- san RA, Hasegawa PM. 2006. SIZ1 small ubiquitin-like modifier E3 ligase facilitates basal thermotolerance in Arabidopsis independent of salicylic acid. *Plant Physiol* **142**: 1548-1558.

Young JJ, Mehta S, Isrealsson M, Godoshki J, Grill E, Schroeder JI. 2006. CO_2 signaling in guard cells: calcium sensitivity response modulation, a Ca^{2+}-independent phase, and CO_2 sensitivity of the *gca2* mutant. *Proceedings of the*

National Academy of Sciences, USA **103,** 7506-7511.

Wees SCM and Glazebrook J (2003) Loss of non-host resistance of Arabidopsis nahG to Pseudomonas syringae pv. phaseolicola is due to degradation products of salicylic acid. *The Plant Journal* **33**: 733-742.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochoris- mate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**: 562-565.

Wu SJ, Siu KC, Wu JY. 2011. Involvement of anion channels in mediating elicitor-induced ATP efflux in *Salvia miltiorrhiza* hairy roots. *Journal of Plant Physiology*, **168**: 128-132.

Zhang Y, Xu W, Li Z, Deng XW, Wu W, Xue W. 2008. F-Box Protein DOR functions as a novel inhibitory factor for abscisic acid-induced stomatal closure under drought stress in Arabidopsis guard cell signaling. *Plant Physiology* **148**:2121-2133.

Zhao J, Davis LC, Verpoorte R. 2005. Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol Adv* **23**: 283-333.

Zhu SY, Yu XC, Wang XJ, et al. 2007. Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. *The Plant Cell* **19**: 3019-3036.

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