

# **Roles of Glutathione in Abscissic Acid- and Methyl Jasmonate-Induced Stomatal Closure**

**2012, 9**

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(Doctor's Course)  
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# **Roles of Glutathione in Abscissic Acid- and Methyl Jasmonate-Induced Stomatal Closure**

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

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## Abbreviations used

|                                    |                                                                    |
|------------------------------------|--------------------------------------------------------------------|
| ABA                                | abscisic acid                                                      |
| ANOVA                              | analysis of variance                                               |
| BCECF-AM                           | 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein<br>acetomethylester |
| [Ca <sup>2+</sup> ] <sub>cyt</sub> | cytosolic free calcium concentration                               |
| CFP                                | cyan fluorescent protein                                           |
| EA                                 | ethacrynic acid                                                    |
| FRET                               | fluorescent resonance energy transfer                              |
| GPX                                | glutathione peroxidase                                             |
| GSB                                | glutathione <i>S</i> -bimane                                       |
| GSH                                | glutathione                                                        |
| GSHmee                             | glutathione monoethylester                                         |
| GST                                | glutathione <i>S</i> -transferase                                  |
| H <sub>2</sub> DCF-DA              | 2',7'-dichlorodihydrofluorescein diacetate                         |
| H <sub>2</sub> O <sub>2</sub>      | hydrogen peroxide                                                  |
| I <sub>Ca</sub> channel            | plasma membrane Ca <sup>2+</sup> permeable channel                 |
| IDM                                | iodomethane                                                        |
| MCB                                | monochlorobimane                                                   |
| MeJA                               | methyl jasmonate                                                   |
| PNBC                               | <i>p</i> -nitrobenzyl chloride                                     |
| PP2C                               | Protein phosphatase 2C                                             |
| pH <sub>cyt</sub>                  | cytosolic pH                                                       |
| ROS                                | reactive oxygen species                                            |
| YC                                 | yellow cameleon                                                    |
| YFP                                | yellow fluorescent protein                                         |

# **CHAPTER 1**

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## **General background**

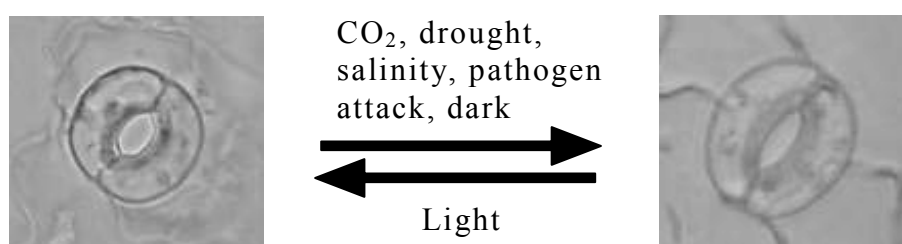
### **1.1. Stress and plant responses in plants**

Stress can be refers as the external conditions that adversely affect plant growth, development and productivity. Stresses trigger a wide range of plant responses such as changes in growth rates and crop yields, cellular metabolism, altered gene expression etc. Stress can be biotic and abiotic. Biotic stress can be imposed by other organisms, while abiotic stress can be arising from an excess or deficit in the physical and chemical environment. A stress response is initiated when plants recognizes stress at the cellular level. Stress recognition activates signal transduction pathways that transmit information within the individual plant and throughout the plant. Changes in gene expression may modify growth and development. To survive with these challenges, plant defense responses rely on signaling mechanisms of hormones and other plant specific substances (Zhang et al., 2008).

### **1.2. Stomata**

Stomata are tiny pores on the leaf epidermis of higher plants that regulate the uptake of carbon dioxide for photosynthesis and the loss of water vapor by transpiration (Hetherington & Woodward 2003). The aperture of the pore is controlled by the state of turgor of the two guard cells. Guard cells have complex sensory and signal transduction machinery that allows them to respond to a variety of factor such as light, CO<sub>2</sub>, and water stress. In drought condition guard cells reduce stomatal aperture,

thereby protecting the plant from transpirational water loss. Guard cells regulate stomatal pore apertures *via* integration of endogenous hormonal stimuli and environmental signals, and guard cells have been highly developed as a model system to dissect the dynamics and mechanisms of plant cell signaling (Kim et al., 2010). Several key physiological stimuli regulate stomatal aperture, including light, CO<sub>2</sub> concentration, humidity, temperatures, plant hormones like abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA) etc. (Schroeder et al., 2001; Murata et al., 2001; Hetherington & Woodward 2003; Suhita et al., 2004; Munemasa et al., 2007; Akter et al., 2010; Islam et al., 2010a, b; Khokon et al., 2011a).



**Figure 1.1.** Open and closed stoma of Arabidopsis.

### 1.3. Absciscic acid

The plant hormone abscisic acid (ABA) regulates a range of physiological processes, including seed maturation, control of vegetative growth, and promotion of dormancy, as well as tolerance of plants to adverse environmental conditions such as drought, cold, and salinity (Koornneef et al., 1998; Leung & Giraudat 1998). In response to drought, ABA causes closing of stomatal pores, which are formed by a pair of guard cells in the epidermis of leaves and other aerial tissues. Stomatal closing

results in a reduction of plant transpirational water loss. ABA induces an increase in cytosolic  $\text{Ca}^{2+}$  in guard cells, which precedes the reduction in stomatal aperture (McAinsh et al., 1990).

The effects of ABA on stomatal aperture have been well documented (Cummins, Kende & Raschke 1971; Willmer, Don & Parker 1978; Mansfield, Hetherington & Atkinson 1990; Assmann & Shimazaki 1999). ABA binds to receptor proteins in the guard cells' plasma membrane and cytosol, which first raises the pH of the cytosol of the cells and cause the concentration of free  $\text{Ca}^{2+}$  to increase in the cytosol due to influx from outside the cell and release of  $\text{Ca}^{2+}$  from internal stores such as the endoplasmic reticulum and vacuoles (McAinsh & Pittman, 2008). Receptors for ABA have not yet been identified, and the location, number, and nature of ABA binding proteins in plant cells are poorly understood. Action and metabolism of ABA is modulated not only by environmental signals but also by endogenous signals. Previous studies have demonstrated that G proteins, phospholipases, protein kinases, and protein phosphatases participate in early events in ABA signaling (Griloy et al., 1990; Pie et al., 1997; Assmann & Shimazaki 1999; Meyer et al., 1994; Li et al., 2000; Wang et al., 2001).

#### **1.4. Methyl jasmonate**

Methyl jasmonate (MeJA) is a volatile organic compound used in plant defense and many diverse developmental pathways such as seed germination, root growth, flowering, fruit ripening, and senescence. MeJA is derived from jasmonic acid and the reaction is catalyzed by S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase. Plants produce jasmonic acid and MeJA in response to many biotic and abiotic

stresses (herbivory and wounding), which build up in the damaged parts of the plant.

Previous studies have demonstrated that MeJA induces stomatal closure in many plant species, including *Arabidopsis thaliana* (Suhita et al., 2004; Munemasa et al., 2007; Saito et al., 2008, Akter et al., 2012b), *Hordeum vulgare* (Tsonev et al., 1998), *Commelina benghalensis* (Raghavendra & Reddy, 1987), *Vicia faba* (Liu et al., 2002), and *Paphiopedilum tonsum* (Gehring et al., 1997). These findings suggest that jasmonate-induced stomatal closure is one of the fundamental physiological responses in plants. MeJA failed to induce stomatal closure and GSH depletion in *atrbohD atrbohF* mutant (Akter et al., 2012 a). Moreover, MeJA did not induce stomata closure in *jar1*, *coil* but these mutants are sensitive to ABA (Suhita et al., 2004; Islam et al., 2010b). In contrast, the mutant *ost1-2* is insensitive to ABA but not to MeJA, suggesting a cross talks of ABA and MeJA signaling on stomatal movements (Suhita et al., 2004; Munemasa et al., 2007).

## **1.5. Second messengers in guard cell ABA and MeJA signaling**

### **1.5.1. Reactive oxygen species**

Reactive oxygen species (ROS) is a collective term that broadly describes O<sub>2</sub>-derived free radicals such as superoxide anions (O<sub>2</sub><sup>-</sup>), hydroxyl radicals (HO•), peroxy (RO<sub>2</sub>•), alkoxy (RO•), as well as O<sub>2</sub><sup>-</sup>-derived non-radical species such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Takahashi & Asada, 1988, Mittler, 2002). ROS, including <sup>1</sup>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup> and HO• are toxic molecules capable of causing oxidative damage to proteins, DNA and lipids (Apel & Hirt 2004). Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as

chloroplast, mitochondria, apoplast and peroxisomes. However, during stress condition the rate of ROS production is drastically increased. Under physiological steady state conditions these molecules are scavenged by different antioxidative defense components that are often confined to particular compartments (Alscher et al., 1997). The equilibrium between production and scavenging of ROS may be perturbed by a number of adverse environmental factors (Mittler et al., 2004). Antioxidants such as ascorbic acid (AsA), glutathione (GSH) and ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR) are essential for ROS detoxification during normal metabolism, and particularly during stress (Takahashi & Asada 1988; Apel & Hirt 2004; Mittler et al., 2004).

Different mechanisms have been proposed for ROS generation in plants. Of these mechanisms, NADPH oxidases are linked to produce  $O_2^-$  in response to pathogen attack and wounding (Sagi & Fluhr 2001; Razem & Bernards 2003). In addition to NADP(H)-oxidases, other classes of oxidases also play role in ROS generation. Amine oxidases release  $H_2O_2$  by oxidizing various forms of amines (Walters 2003). Cell wall peroxidases may also play role in ROS generation in response to pathogen attacks (Lamb & Dixon 1997; Bolwell et al., 2002; Mika et al., 2004). Reactive oxygen species (ROS) function as second messenger in ABA and MeJA signaling 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). Protein phosphatase *abil*, and *rcn1*, protein kinases *ost1* mutant is impaired ABA-induced guard cells ROS production and stomatal closure (Munemasa et al., 2007; Saito et al., 2008). So far, biochemical and

pharmacological approach on ABA and MeJA signal transduction has been well studied, but little is known about histochemical approach on ABA and MeJA signal transduction.

### **1.5.2. Calcium**

$\text{Ca}^{2+}$  functions as a second messenger in guard cell signaling and stomatal movements (Schroeder and Hagiwara, 1989; McAinsh et al., 1995). The cytosolic  $\text{Ca}^{2+}$  concentration is modulated in response to many physiological stimuli and is delicately balanced by ‘ $\text{Ca}^{2+}$  stores’, like vacuoles, endoplasmic reticulum (ER), mitochondria, nucleus, chloroplast and cell wall (McAinsh & Pittman, 2008).  $\text{Ca}^{2+}$  enters the cytoplasm through channels located in plasma membrane and ER, increasing the cytoplasmic, mitochondrial, and nucleoplasmic  $\text{Ca}^{2+}$  concentration that trigger physiological responses. Both ABA and MeJA activate plasma membrane  $\text{I}_{\text{Ca}}$  currents to increase  $[\text{Ca}^{2+}]_{\text{cyt}}$  in guard cells, which leads to stomatal closure (Murata et al., 2001; Munemasa et al., 2007, Islam et al., 2010).  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation activates S-type and R-type anion channels (Schroeder and Hagiwara, 1989; Hedrich et al., 1990) and inhibits  $\text{K}_{\text{in}}$  channel (Schroeder and Hagiwara, 1989). By using a  $\text{Ca}^{2+}$  indicator, yellow cameleon, Allen et al. revealed that in guard cells, duration and frequency of “spiky”  $\text{Ca}^{2+}$  signals encode important factors to induce long-lasting stomatal closure (Allen et al., 2000).

### **1.5.3. Cytosolic pH**

Intracellular pH changes have long been proposed to function in signaling and transport control in plant cells, such as plant defense responses and response to hormone activity such as abscisic acid and methyl

jasmonate (MeJA) (Suhita et al., 2004). Stomatal closure is associated with  $K^+$  release from the vacuoles and efflux across the plasma membrane (MacRobbie 1998), and closure can be induced by elevated level of cytoplasmic  $Ca^{2+}$ . ABA-induced increase in cytoplasmic  $Ca^{2+}$  can be achieved without  $Ca^{2+}$  influx, by release from cytoplasmic stores, but do not rule out ABA-induced  $Ca^{2+}$  influx when external  $Ca^{2+}$  is present. ABA-induced changes in  $[Ca^{2+}]_{\text{cyt}}$  and  $pH_{\text{cyt}}$  affect inward- ( $I_{K\text{in}}$ ) and outward-rectifying ( $I_{K\text{out}}$ )  $K^+$  channels and of  $Cl^-$  channels ( $I_{Cl}$ ) to bias the membrane for  $KCl$  efflux and a loss of cell turgor (Blatt 2000). Blatt & Armstrong (1993) showed that acidification of the cytoplasm by external sodium butyrate inhibited the outward  $K^+$  channel, prevented the ABA-induced alkalization of the cytoplasm, and blocked the ABA-induced activation of the outward  $K^+$  channel, arguing for regulation by cytoplasmic pH, and positioning pH in the signaling chain leading from ABA to this channel. Like ABA, MeJA also suppresses  $I_{K\text{in}}$  current in *Arabidopsis* (Saito et al., 2008) and simultaneously activates  $I_{K\text{out}}$  current in *Vicia* (Evans 2003). These results indicate that elevation of  $[Ca^{2+}]_{\text{cyt}}$  and  $pH_{\text{cyt}}$  function in ion mobilization.

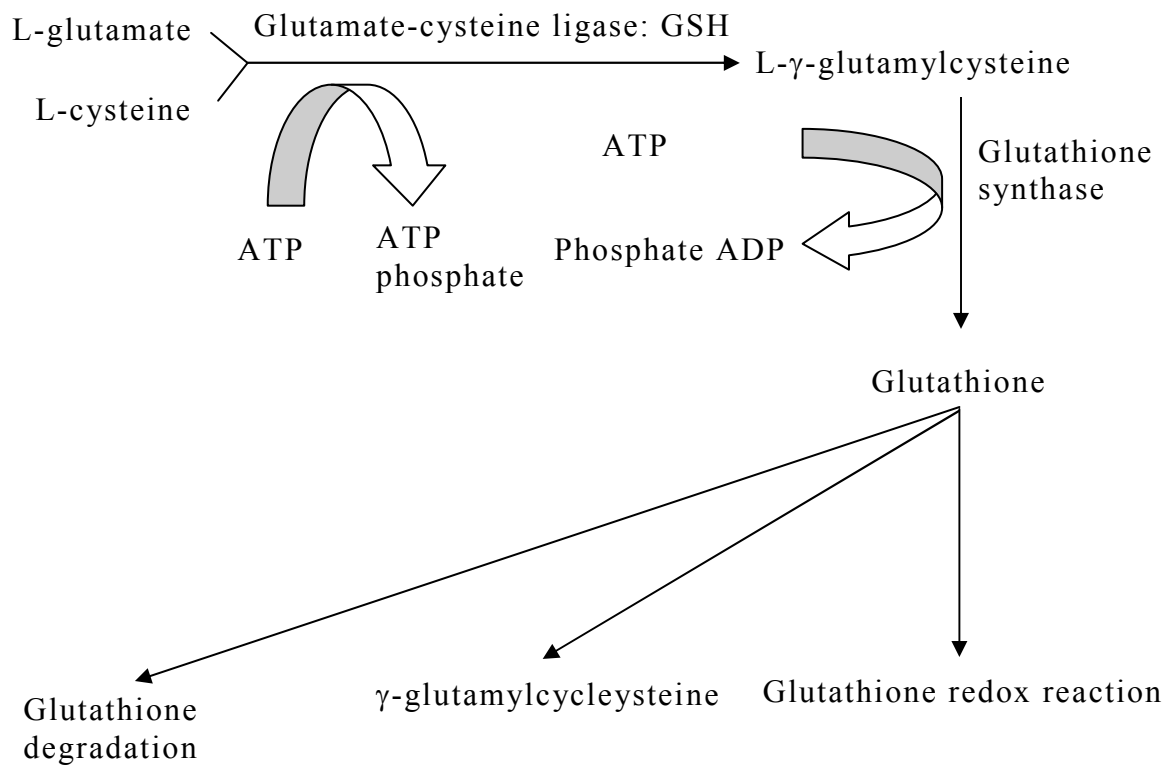
## 1.6. Glutathione

Glutathione is a simple sulfur compound composed of three amino acids and the major non-protein thiol in many organisms, including plants. The functions of glutathione are manifold but notably include redox-homeostatic buffering. Glutathione status is modulated by oxidants as well as by nutritional and other factors, and can influence protein structure and activity through changes in thiol-disulfide balance. For these reasons, glutathione is a transducer that integrates environmental information into

the cellular network. Moreover, GSH (or a functionally homologous thiol) is an essential metabolite with multiple functions in plants.

GSH is one of the most important reducing equivalents in plants. It participates in the maintenance of cellular homeostasis in plants during normal and adverse environmental conditions. GSH exists in two forms; reduced glutathione and oxidized glutathione. Generally plants maintain higher levels of reduced glutathione and it is required for sustainable growth of plants as well.

GSH is synthesized in plants from its constituent amino acids by two ATP-dependent steps (Meister 1988; Noctor et al., 2002a). Glutathione biosynthesis occurs through a two-step pathway found in all organisms. In the first reaction,  $\gamma$ -glutamylcysteine is synthesized from L-glutamate and cysteine *via* the enzyme  $\gamma$ -glutamylcysteine synthetase (glutamate cysteine ligase, GCL). This reaction is the rate-limiting step in glutathione synthesis. Next, glycine is added to the C-terminal of  $\gamma$ -glutamylcysteine via the enzyme glutathione synthetase (Fig. 1.2). Nucleophilic attack on the acylphosphate intermediate by glycine leads to formation of with release of ADP and inorganic phosphate (Meister & Anderson 1983; Nishimura et al., 1964).



**Fig. 1.2.** Schematic diagram of the glutathione synthesis

## 1.7. Arabidopsis mutants

### 1.7.1. *chlorinal-1* mutant

The chlorophyll *b*-less (*chl*) mutant's defective in a light-harvesting antenna in photosystem II showed reduced GSH levels with accumulation of the GSH precursor cysteine, and receive photosynthetic light by the limited number of chlorophyll *a* in the PSII core complex. Introduction of the  $\gamma$ -ECS gene *GSH1* under the control of the cauliflower mosaic virus 35S promoter (*35S-GSH1*) into the *chl* mutant altered the GSH level in response to the  $\gamma$ -ECS mRNA level. These indicate that photosynthesis limits the  $\gamma$ -ECS reaction to regulate GSH biosynthesis. The *chl-1* mutation retarded plant growth, which led to absolute delay in

flowering time under weak light conditions, and this late-flowering phenotype was rescued by supplementation of GSH.

### **1.7.2. *cad2-1* mutant**

The glutathione (GSH)-deficient mutant, *cad2-1*, of *Arabidopsis* is deficient in the first enzyme in the pathway of GSH biosynthesis,  $\gamma$ -glutamylcysteine synthetase (GCS). The mutant accumulates a substrate of GCS, cysteine, and is deficient in the product,  $\gamma$ -glutamylcysteine. The mutant *cad2-1* was originally isolated in a mutant screen on  $cd^{2+}$  (Howden et al., 1995) and contains mutation in the gene At4g23100, which encodes glutathione-cysteine ligase (GSH1, EC 6.3.2.2), the first enzyme of GSH biosynthesis (Cobbett et al., 1998). The late flowering phenotype of the *cad2-1* mutant defective in the first enzyme  $\gamma$ -ECS of GSH biosynthesis (Cobbett 2000, Ogawa et al., 2001) was strong at a low light intensity. This mutation lead to a reduced overall level of GSH of only 30% compared to wild-type plants. The mutant plants grow and develop normally with little deviation from the wild-type phenotype, but show significantly increased sensitivity under conditions of stress.

### **1.7.3. *Atrboh* mutants**

The NAD(P)H oxidase, also known as the respiratory burst oxidase (RBO), *Arabidopsis* (*Arabidopsis thaliana*) has 10 *Atrboh* (*Arabidopsis* RBO homolog) genes homologous to *gp91<sup>phox</sup>* (Torres & Dangl 2005). NAD(P)H oxidases are plasma membrane protein (Keller et al., 1998) that may produce ROS in the vicinity of the plasma membrane ion channels. Guard cell expressed NAD(P)H oxidase catalytic subunit gene *gp91<sup>phox</sup>* homologous sequence (Torres et al., 1998) were identified using degenerate

oligomers. A guard cell expressed *gp91<sup>phox</sup>* homologous gene, *AtrbohD* was identified from guard cell cDNA libraries. Genechip experiments followed to identify another *gp91<sup>phox</sup>* homologous gene *AtrbohF*. *atrbohD atrbohF* double mutant were obtained from crosses between homozygous *atrbohD* and *atrbohF* mutants. MeJA- and ABA-induced stomatal closure, ROS production and cytosolic calcium increases were strongly impaired in *atrbohD atrbohF* mutant (Kwak et al., 2003; Suhita et al., 2004; Munemasa et al., 2007). Exogenously applied H<sub>2</sub>O<sub>2</sub> can rescue wild type stomatal responses in the *atrbohD atrbohF* mutant (Kwak et al., 2003).

### **1.8. Purpose of the study**

The aim of this study is to investigate the involvement of intracellular GSH in methyl jasmonate and abscisic acid signaling in the model plant *Arabidopsis thaliana*, GSH deficient mutants, *cad2-1* and *chl-1*; rboh mutants, *atrbohD*, *atrbohF*, and *atrbohD atrbohF* mutant; protein phosphatases 2C (PP2Cs) *abi1-1* and *abi2-1* mutants. Genetical and histochemical studies were performed to elucidate the roles of glutathione in ABA- and MeJA-induced stomatal closure.

Specific aims were as follows-

1. To investigate the involvement of intracellular glutathione in methyl jasmonate signaling in *Arabidopsis*.
2. To elucidate the negative regulation of methyl jasmonate-induced stomatal closure by glutathione in *Arabidopsis*.
3. To investigate the effects of depletion of glutathione on abscisic acid- and methyl jasmonate-induced stomatal closure in *Arabidopsis*.

## CHAPTER 2

---

### **The involvement of intracellular glutathione in methyl jasmonate signaling in Arabidopsis guard cells**

#### **2.1. Abstract**

We examined involvement of intracellular glutathione (GSH) in methyl jasmonate (MeJA) signaling. The *chlorinal-1* (*chl-1*) mutation decreased GSH in guard cells and narrowed stomatal aperture. GSH monoethyl ester increased intracellular GSH to diminish this phenotype. GSH did not affect MeJA-induced reactive oxygen species production and cytosolic  $\text{Ca}^{2+}$  oscillation, suggesting GSH modulates MeJA signaling downstream of the production and the oscillation.

## 2.2. Introduction

Stomatal pores, formed by a pair of symmetric guard cells, play a physiological role in regulating gaseous fluxes across leaf epidermis (Taiz et al., 2006). Methyl jasmonate (MeJA) is ubiquitous plant signaling compound to mediate plant defense responses (Creelman et al., 1997) and induces stomatal closure (Islam et al., 2010b). Reactive oxygen species (ROS) and cytosolic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) function as signaling components in MeJA signal transduction in guard cells during MeJA-induced stomatal closure (Islam et al., 2010b). Elevation of  $[\text{Ca}^{2+}]_{\text{cyt}}$  in guard cells occurs during stomatal closure (McAinsh et al., 1995) and application of MeJA, ABA and  $\text{H}_2\text{O}_2$  elicits  $I_{\text{Ca}}$  currents and  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillation in guard cells (Allen et al., 1999; Hamilton et al., 2000; Pie et al., 2000; Munemasa et al., 2007; 2011).

GSH regulates many physiological functions *via* redox state of GSH pools in plants (Noctor et al., 1998; Jahan et al., 2008). Control of intracellular GSH may, therefore, be expected to have important consequence for the cells, through modification of the metabolic functions associated with GSH-regulated genes. GSH level are known to vary during plant development and in response to numerous stimuli such as atmospheric pollutants, biotic and abiotic stress, and light (Alscher 1989; Sánchez-Fernández et al., 1997).

GSH is synthesized in two sequential reactions catalyzed by  $\gamma$ -glutamylcysteine synthetase (GSH1 gene product) and glutathione synthetase (GSH2 gene product). However, the photosynthetic electron flow regulates the rate of GSH biosynthesis through the production of ATP. The *chl-1* mutant lacks the light-harvesting protein of photosystem II (PSII), and receives photosynthetic light by a limited number of chlorophyll *a* in

the PSII core complex, and provides lower GSH levels in guard cells and narrowed stomatal aperture (Ogawa et al., 2004; Jahan et al., 2008).

GSH content has been investigated in roots (Sánchez-Fernández et al., 1997; Meyer & Fricker 2001), seedlings (Ogawa et al., 2001), suspension cells (Howden et al., 1995; Meyer et al., 2001) and trichome cells (Gutierrez-Alcalá et al., 2000). It has been reported that ABA-induced stomata closure accompanied by decreasing intracellular GSH and that intracellular GSH regulates stomatal movement (Jahan et al., 2008; Okuma et al., 2011).

In order to clarify whether GSH acts as a signal molecule or signal regulator in MeJA signaling in guard cells, we investigated the involvement of intracellular GSH on MeJA-induced stomatal closure in *Arabidopsis*.

## **2.3. Materials and Methods**

### **2.3.1. Plant materials and growth conditions**

*Arabidopsis thaliana* wild type (ecotype Columbia) and the *chl-1*, wild type expressing  $\text{Ca}^{2+}$  reporter yellow cameleon 3.6 (YC3.6) (Columbia accession) plants were used in this study. These plants were grown in plastic pots filled with 70% (v/v) vermiculite (Asahi-Kogyo, Okayama, Japan) and 30% (v/v) Kureha soil (Kureha Chemical, Tokyo, Japan) in a growth chamber ( $80 \mu\text{mol m}^{-2} \text{s}^{-1}$  under a 16-h-light/8-h-dark regime). Temperature and relative humidity in the growth chamber were controlled at  $22 \pm 2^\circ\text{C}$  and  $60 \pm 10\%$ .

### **2.3.2. Measurement of stomatal aperture**

Width of stomatal apertures was measured as described previously (Murata et al. 2001). Excised rosette leaves from 4- to 6-week-old plants

were floated on the medium containing 5 mM KCl, 50  $\mu$ M CaCl<sub>2</sub>, and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) to induce stomatal opening. To increase GSH content in guard cells, leaves were treated with GSHmee for 2 h under light. After 2-h incubation in the presence of MeJA under light, the leaves were shredded in a blender for 30 s and remaining epidermal tissues were collected using nylon mesh (pore size: 30  $\mu$ m). The epidermal tissues were mounted on a slide glass and images of stomatal apertures were captured using an Olympus IX71S87 microscope equipped with a CS230 digital imaging color camera and were analyzed with image analysis software (WinROOF V3.61, MITANI Corporation, Fukui, Japan). For each sample, twenty stomatal apertures were measured.

### **2.3.3. Measurement of GSH in guard cells**

Contents of GSH in guard cells were examined using monochlorobimane (MCB) as previously described by Akter et al. (2010). The abaxial side of excised leaf was gently mounted on a glass slide with a medical adhesive (Hollister Inc., Libertyville, Illinois, USA), followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade in order to keep the abaxial epidermis intact on the slide. The abaxial epidermis was incubated in a staining solution containing 100  $\mu$ M of MCB for 2 h at room temperature. Monochlorobimane reacts with intracellular GSH to form fluorescent glutathione *S*-bimane (GSB) in guard cells. After incubation, the excess dye was washed out with deionized water and covered with a cover slip using a drop of deionized water. The fluorescence intensity of GSB in guard cells was observed under a fluorescent microscope (Biozero BZ-8000, Keyence, Osaka, Japan) with a filter:

OP-66834 BZ filter DAPI-BP (excitation wavelength 360/40 nm, absorption wavelength 460/50 nm, and dichroic mirror wavelength 400 nm). The fluorescence intensity of guard cells was quantified using ImageJ 1.42q software (National Institutes of Health, US).

#### **2.3.4. Measurement of ROS production in guard cells**

Production of ROS in guard cells was evaluated using 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) (Sigma, St. Louis, MO, USA) as previously described by Munemasa et al. (2007). Epidermal peels were collected from leaves of 4- to 6-week-old plants with a commercial blender for 40 s, incubated for 3 h in medium containing 5 mM KCl, 50  $\mu$ M CaCl<sub>2</sub>, and 10 mM MES-Tris (pH 6.15) in the light, were incubated with 50  $\mu$ M H<sub>2</sub>DCF-DA for 30 min at room temperature to load the dye into the guard cells, rinsed with distilled water to remove excess dye using a nylon mesh, were treated with 10  $\mu$ M GSHmee for 20 min and then treated with 10  $\mu$ M MeJA for another 20 min. Fluorescence of guard cells was imaged using the fluorescence microscope (BZ-8000) with a filter (OP-66835 BZ filter GFP at excitation wavelength 480/30 nm, absorption wavelength 510 nm, and dichroic mirror wavelength 505 nm). DCF fluorescence intensity of guard cells was quantified using ImageJ 1.42q software.

#### **2.3.5. Measurement of [Ca<sup>2+</sup>]<sub>cyt</sub> oscillations in guard cells**

Rosette leaves of Arabidopsis Yellow Cameleon 3.6 (YC3.6)-expressing plants were used to examine [Ca<sup>2+</sup>]<sub>cyt</sub> oscillations in guard cells as previously described (Islam et al., 2010b). The abaxial side of excised leaf was gently mounted on a glass slide by using a medical

adhesive, followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade in order to keep the lower epidermis intact on the slide. The abaxial epidermis was kept in the incubation solution containing 5 mM KCl, 50  $\mu$ M CaCl<sub>2</sub> and 10 mM MES-Tris (pH 6.15) under light (80  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) for 2 h at 22° C. The turgid guard cells were used to measure [Ca<sup>2+</sup>]<sub>cyt</sub>. Then, the incubation solution supplemented with 10  $\mu$ M MeJA was applied to GSHmee-untreated or 10  $\mu$ M GSHmee-treated guard cells with a peristaltic pump 5 min after starting of measurement. Fluorescence of YC3.6 was measured by dual-emission ratio imaging using a 440DF20 excitation filter, 445DRLP dichroic mirror, and two emission filters 480DF30 for CFP and 535DF25 for YFP. The CFP and YFP fluorescence intensity of guard cells was imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics, Hamamatsu, Japan).

#### **2.3.6. Statistical analysis**

Significance of differences between mean values was assessed using analysis of variance (ANOVA) with Student-Neuman-Keuls multiple-range test, or  $\chi^2$ -text analysis. We regarded differences at the level of  $p < 0.05$  as significant.

#### **2.3.7. Accession number**

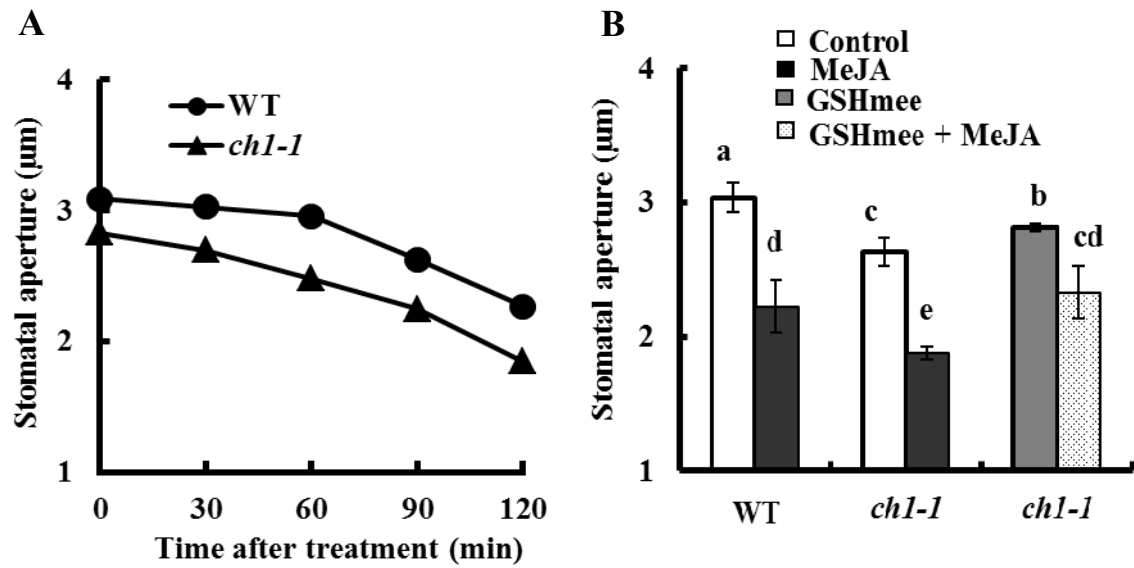
Arabidopsis genome initiative number for CH1 gene is (At1g44446).

## **2.4. Results**

### **2.4.1. Effect of intracellular GSH on MeJA-induced stomatal closure**

A time course of MeJA-induced stomatal closure shows that 10  $\mu$ M MeJA reduced stomatal apertures by 16% at 30 min and by 36% at 120 min in *chl-1* mutant and by 13% at 30 min and by 21% at 120 min in wild type plants (Fig. 2.1 A). MeJA-induced stomatal closure in wild-type plants and decreased GSH by 20% in wild-type guard cells (Fig. 2.1 B) like ABA (Jahan et al., 2008). The stomatal apertures of *chl-1* mutants were narrower than those of wild-type plants regardless of MeJA application (Fig. 2.1 B).

In order to confirm that depletion of GSH is involved in enhancing of MeJA sensitivity to stomatal closure in the *chl-1* mutant (Fig. 2.2 B), we investigated the effects of exogenous GSHmee on stomatal aperture of the *chl-1* mutant. GSHmee can permeate cell membranes and is hydrolyzed by cytosolic esterases to release free intracellular GSH (Puri & Meister 1983). Treatment with GSHmee widened the stomatal apertures of *chl-1* regardless of MeJA application (Fig. 2.1 B), indicating that supplement of intracellular GSH suppresses stomatal closure. These results also suggest that intracellular GSH is involved in MeJA-induced stomatal closure as well as ABA-induced stomatal closure.

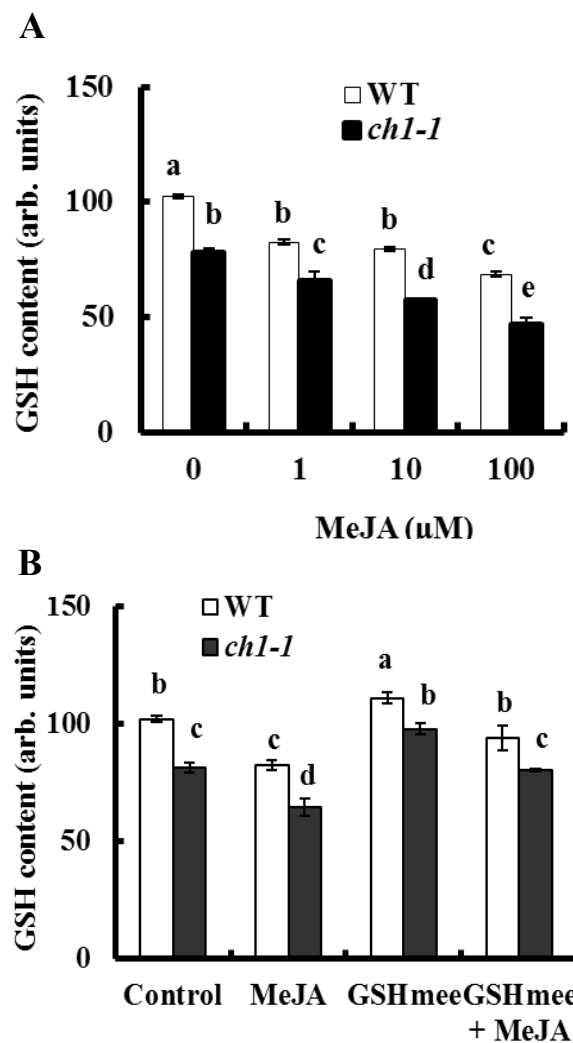


**Fig. 2.1.** Stomatal apertures in wild-type and *chl-1* mutant plants. (A) The time course of stomatal apertures in the *chl-1* plants treated with 10 μM MeJA. (B) Effects of 10 μM MeJA and 10 μM GSHmee on stomatal aperture in wild-type and *chl-1* mutant plants untreated (black bars) and treated (dotted bar) with 10 μM GSHmee. Error bars represent standard deviation (60 stomata per bar, n = 3).

#### 2.4.2. Effect of intracellular GSH on MeJA-induced GSH depletion in guard cells

Effect of MeJA on GSH contents in guard cells of the *chl-1* plants was measured using MCB (Fig. 2.2 A). Application of 1, 10 and 100 μM MeJA decreased GSH contents by 16% ( $P < 10^{-3}$ ), 23% ( $P < 10^{-3}$ ) and 29% ( $P < 10^{-3}$ ) in wild type and by 21% ( $P < 0.007$ ), 30% ( $P < 10^{-5}$ ) and 39% ( $P < 10^{-5}$ ) in *chl-1* mutant plants, respectively, and showed a dose-dependent manner (Fig. 2.2 A), which is similar to the previous results in ABA-induced GSH depletion in guard cells of *Arabidopsis* (Jahan et al., 2008). Treatment with 10 μM GSHmee significantly increases GSH content

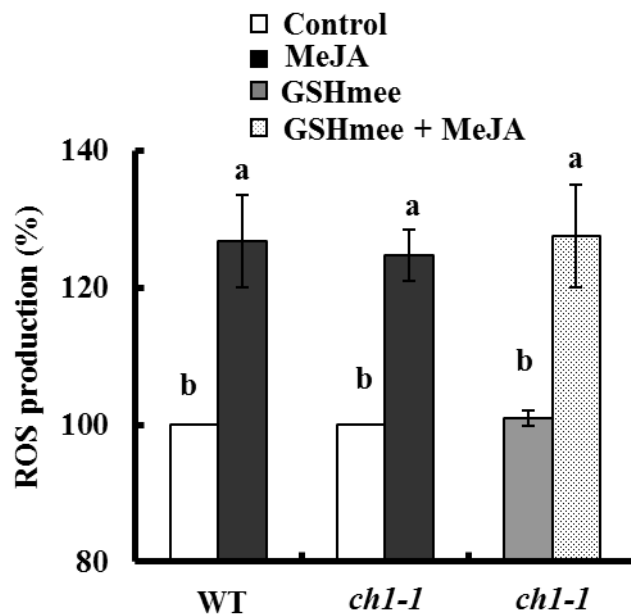
in *chl-1* mutant guard cells and complements the stomatal phenotype of the *chl-1* mutant (Fig. 2.2 B).



**Fig. 2.2.** Glutathione (GSH) content in wild-type and *chl-1* mutant plants. A, Effect of 1, 10, and 100  $\mu$ M MeJA on GSH content in guard cells of wild-type (white bars) and *chl-1* (black bars) mutant plants. B, Effect of 10  $\mu$ M MeJA and 10  $\mu$ M GSHmee on GSH contents in guard cells of wild-type (white bars) and *chl-1* mutant plants (black bars). The vertical scale represents arbitrary unit of GSH contents.

#### 2.4.3. Effect of intracellular GSH on MeJA-induced ROS production in guard cells

ROS production in guard cells is required by MeJA-induced stomatal closure as well as ABA-induced stomatal closure (Murata et al., 2001; Munemasa et al., 2007). Application of MeJA significantly induced ROS production in guard cells of the *chl-1* mutant ( $p < 10^{-3}$ ) as well as wild type ( $p < 0.002$ ) (Fig. 2.3 A). Even when epidermal tissues were treated with GSHmee, MeJA significantly increased ROS accumulation in the *chl-1* mutant ( $p < 0.002$ ) (Fig. 2.3 A). These results indicate that increment of GSH by treatment with GSHmee does not affect MeJA-induced ROS production.

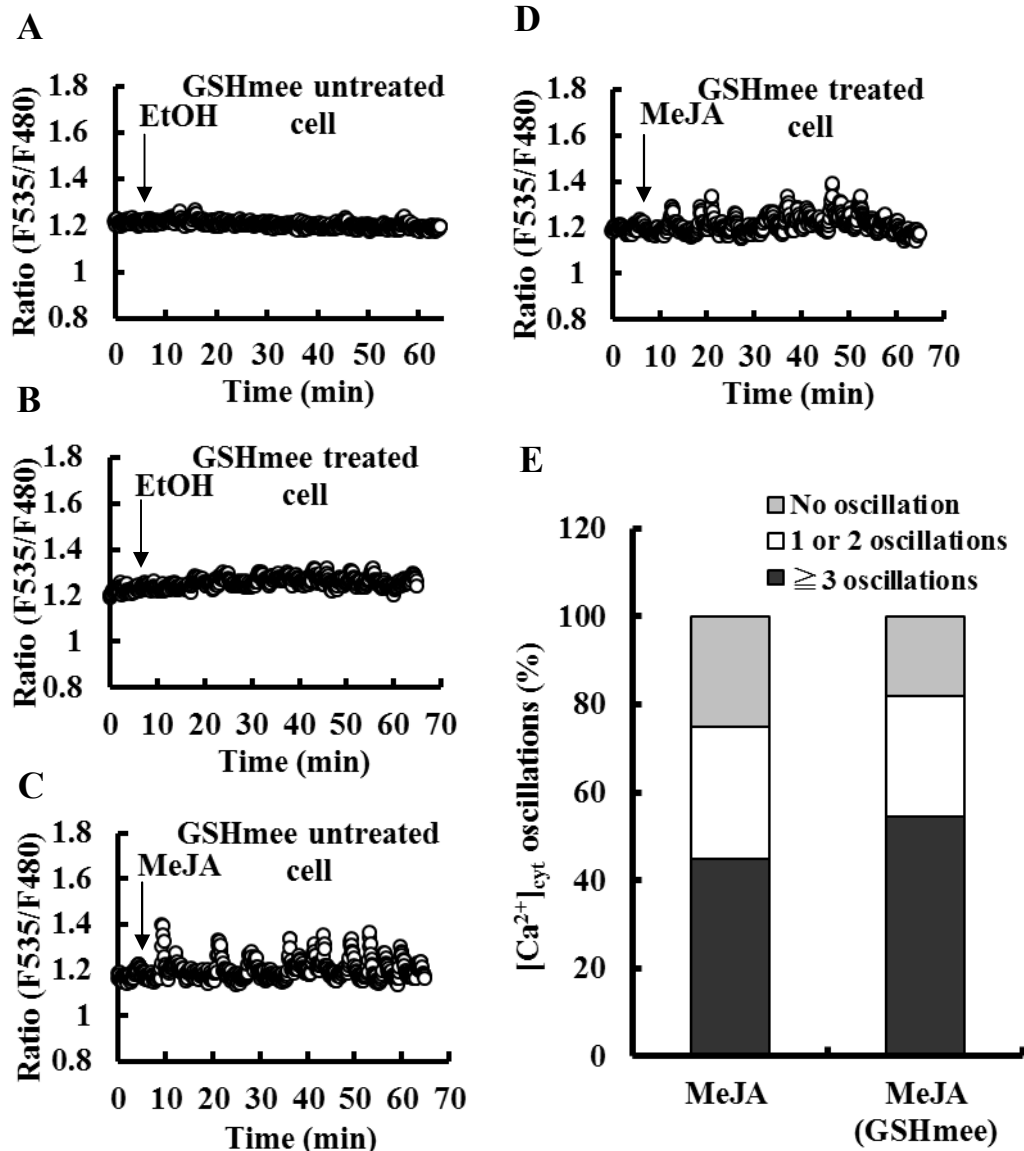


**Fig. 2.3.** ROS production in *chl-1* and wild-type guard cells. A, MeJA-induced (10  $\mu$ M) ROS production in guard cells of wild-type and *chl-1* mutant plants untreated (black bars,  $n = 3$ ) and treated (dotted bar,  $n = 6$ ) with 10  $\mu$ M GSHmee. The vertical scale represents the percentages of DCF fluorescent levels when the fluorescent intensities of MeJA-treated

cells were normalized to the control value taken as 100% for each experiment. Each data was obtained from at least 60 guard cells. Error bars represent standard deviation.

#### **2.4.4. Effect of intracellular GSH on MeJA-induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells**

Calcium signaling in guard cells is one of the major pathways to regulate stomatal movements (Yang et al., 2001). To evaluate the role of GSH in MeJA-induced  $[Ca^{2+}]_{cyt}$  in the wild-type guard cells was investigated. MeJA-induced stomatal closure is accompanied with  $[Ca^{2+}]_{cyt}$  oscillation in guard cells (Islam et al., 2010b). The guard cells untreated and treated with GSHmee did not show  $[Ca^{2+}]_{cyt}$  oscillation (Fig. 2.4 A and B). The application of MeJA induced  $[Ca^{2+}]_{cyt}$  oscillation in 15 of the 20 guard cells untreated with GSHmee (Fig. 2.4 C and E) and in 18 of 22 guard cells treated with GSHmee (Fig. 2.4 D and E). In sum, there was no significant difference in  $[Ca^{2+}]_{cyt}$  oscillation between the GSHmee-untreated guard cells and the GSHmee-treated cells ( $\chi^2 = 0.28$ ,  $p = 0.70$ ), suggesting that intracellular GSH does not affect MeJA-induced  $[Ca^{2+}]_{cyt}$  oscillation in guard cells.



**Fig. 2.4.**  $[Ca^{2+}]_{cyt}$  oscillations in wild-type guard cells. A, B, C and D,  $[Ca^{2+}]_{cyt}$  oscillations (changes in fluorescence emission ratio, 535/480 nm) in YC3.6 expressing guard cells which were untreated or treated with GSHmee for 30 min in the (B,C) absence and (D, E) presence of MeJA. E, Frequencies of MeJA-elicited  $[Ca^{2+}]_{cyt}$  oscillation with GSHmee untreated ( $n = 20$ ) and treated ( $n = 22$ ) guard cells.

## 2.5. Discussion

Glutathione (GSH) has many functions in sulphur metabolism, growth, development, cell defense, redox signaling, and regulation of gene expression. GSH is closely concerned with ROS homeostasis and redox status. In guard cells, ROS is one of the important signaling components in MeJA signal cascades (Suhita et al., 2004; Munemasa et al., 2007; 2011). Previously Jahan et al. (2011) found that MCB staining indicates that GSH accumulates with high contents in guard cells than in other cells of leaves, mesophyll cells and epidermal cells. Therefore, GSH is expected to play a role in stomatal movements. These prompted us to investigate the function of GSH on MeJA signaling in guard cells.

The guard cells of *chl-1* plants show higher sensitivity to MeJA than wild-type plants (Fig. 2.1 and 2.2). These results suggest that the function of ATP on stomatal movements, that is, *chl-1* mutant receives photosynthetic light by a limited number of chlorophyll a in the PSII core complex due to lack of light harvesting antenna and low levels of GSH and ATP. This study shows that MeJA induces stomatal closure with decreasing GSH content in guard cells (Fig. 2.1 and 2.2) and our previous study reported that ABA-induced stomatal closure is accompanied by decreasing GSH content in guard cells (Jahan et al., 2008). Moreover, oxidative condition made by MeJA could be eliminated by exogenously application of GSHmee (Fig. 2.1 B) due to increase intracellular GSH level in guard cells (Fig. 2.2 B). These results indicate that GSH modulates MeJA signaling and ABA signaling to lead to stomatal closure.

MeJA as well as ABA induce ROS production *via* activation of NADPH oxidases and induce  $[Ca^{2+}]_{\text{cyt}}$  oscillation *via* activation of  $I_{Ca}$  channels, to lead to stomatal closure (Allen et al., 1999; Kwak et al., 2003;

Suhita et al., 2004; Munemasa et al., 2007, 2011). In this study, supplement of GSH by GSHmee or *chl-1* mutation did not affect MeJA-induced ROS production and  $[Ca^{2+}]_{cyt}$  oscillation *via*  $Ca^{2+}$  channel activation, suggesting that GSH modulates signal components downstream of ROS production and  $[Ca^{2+}]_{cyt}$  oscillation in MeJA signaling in guard cells. One of the possible signal components is the guard-cell ion channel, since GSH modulates slow vacuolar (SV) channel activity, which is regulated by the cytosolic pH in carrot vacuoles (Scholz-Starke et al., 2004).

Glutathione peroxidases (GPXs) are key enzymes in the scavenging the oxyradicals in animal cells (Arthur 2000). However, in Arabidopsis, glutathione peroxidases, AtGPX1, 2, 3, 5 and 6, function to reduce  $H_2O_2$  and organic hydroperoxides using thioredoxin, but not glutathione as an electron donor (Arthur 2000; Iqbal et al., 2006). Taking this together with our results, it is suggested that GPX-mediated ROS scavenging with GSH as a substrate is not involved in the MeJA-induce stomatal closure.

In conclusion, MeJA-induced stomatal closure as well as ABA-induced stomatal closure is accompanied with decreasing GSH in guard cells and GSH also is involved in MeJA signaling as well as ABA signaling in Arabidopsis guard cells.

## CHAPTER 3

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### Negative regulation of methyl jasmonate-induced stomatal closure by glutathione in *Arabidopsis*

#### 3.1. Abstract

Glutathione (GSH) has been shown to negatively regulate methyl jasmonate (MeJA)-induced stomatal closure. Here, we investigated the roles of GSH in MeJA signaling in guard cells using an *Arabidopsis* mutant, *cad2-1*, that is deficient in the first GSH biosynthesis enzyme,  $\gamma$ -glutamylcysteine synthetase. MeJA induced stomatal closure and decreased GSH contents in guard cells. Decreasing GSH by the *cad2-1* mutation enhanced MeJA-induced stomatal closure. Depletion of GSH by the *cad2-1* mutation or increment of GSH by glutathione monoethyl ester (GSHmee) did not affect either MeJA-induced production of reactive oxygen species (ROS) or MeJA-induced cytosolic alkalization in guard cells. MeJA and abscisic acid (ABA) induced stomatal closure and GSH depletion in *atrbohD* and *atrbohF* single mutants but not in the *atrbohD atrbohF* double mutant. In addition, MeJA did not induce stomatal closure and GSH depletion in *abi1-1* and *abi2-1* mutants. Moreover, exogenous hydrogen peroxide ( $H_2O_2$ ) induced stomatal closure but did not deplete GSH in guard cells. These results indicate that GSH affects MeJA signaling as well as ABA signaling and that GSH negatively regulates a signal component other than ROS production and cytosolic alkalization in MeJA signal pathway of *Arabidopsis* guard cells.

### 3.2. Introduction

Stomatal pores are surrounded by pairs of guard cells in the leaf epidermis of higher plants. Stomata control CO<sub>2</sub> uptake, transpirational water loss, and innate immunity, in response to a variety of external and internal stimuli (Schroeder et al., 2001; Hetherington & Woodward 2003; Munemasa et al., 2007; Shimazaki et al., 2007).

Methyl jasmonate (MeJA), a linolenic acid derivative, regulates many processes of plant growth and development and mediates various plant defense responses (Liechti & Farmer 2002; Turner et al., 2002). MeJA as well as abscisic acid (ABA) elicits stomatal closure in several plant species (Gehring et al., 1997; Tsonev et al., 1998; Liu et al., 2002; Suhita et al., 2004; Munemasa et al., 2007, 2011; Saito et al., 2008).

Glutathione (GSH) regulates growth, development, defense systems, signaling, and gene expression (May et al., 1998; Noctor & Foyer 1998). These functions depend on the amounts and/or redox state of GSH pools (May et al., 1998; Noctor & Foyer 1998) but some of the functions cannot be simply explained by the antioxidant activity of GSH (Foyer & Noctor 2005; Ogawa 2005). The GSH levels in plant cells vary during plant development and in response to a wide variety of stimuli such as atmospheric pollutants, biotic and abiotic stress, and light (Alscher 1989; Sánchez-Fernández et al., 1997).

We found that both MeJA- and ABA-induced stomatal closure are enhanced in the *chlorinal-1* (*chl-1*) mutant, which accumulates less GSH due to impairment of the light-harvesting protein in photosystem II, and that both MeJA- and ABA-induced stomatal closure are also enhanced in wild-type plants treated with a GSH-decreasing chemical, 1-chloro-2,4-dinitrobenzene (CDNB) (Jahan et al., 2008; Akter et al., 2010;

Okuma et al., 2011). A GSH-increasing agent, glutathione monoethyl ester (GSHmee) restored the GSH level in the GSH deficient mutant guard cells and complemented the stomatal phenotype of the mutant (Jahan et al., 2008; Akter et al., 2010; Okuma et al., 2011). We recently demonstrated using an Arabidopsis mutant, *cad2-1* (cadmium-sensitive), that is deficient in the first GSH biosynthesis enzyme,  $\gamma$ -glutamylcysteine synthetase, that GSH negatively regulates ABA signaling in Arabidopsis (Okuma et al., 2011). However, how GSH negatively regulates MeJA signaling and ABA signaling in guard cells is not understood.

Production of reactive oxygen species (ROS) mediated by NADPH oxidases accompanies ABA- and MeJA-induced stomatal closure (Pei et al., 2000; Kwak et al., 2003; Suhita et al., 2004; Munemasa et al., 2007) and exogenous hydrogen peroxide ( $H_2O_2$ ) elicits stomatal closure in a number of species including Arabidopsis, Vicia, and Pisum (Pei et al., 2000; Zhang et al., 2001; Neill et al., 2002; Kwak et al., 2003; Munemasa et al., 2007).  $H_2O_2$  activates plasma membrane  $Ca^{2+}$ -permeable channel ( $I_{Ca}$ ) currents (Hamilton et al., 2000; Pei et al., 2000). The *atrbohD atrbohF* mutation impairs ABA and MeJA signaling upstream of ROS production (Kwak et al., 2003; Suhita et al., 2004). However, it is unclear whether this mutation suppresses the decrease in GSH contents in guard cells by MeJA.

Both MeJA- and ABA-induced stomatal closure are accompanied by cytosolic alkalization in guard cells of Arabidopsis (Islam et al., 2010b) and Pisum (Suhita et al., 2004). Cytosolic alkalization activates outward  $K^+$  currents ( $K^+_{out}$ ) and inactivates inward  $K^+$  currents ( $K^+_{in}$ ) in Vicia guard cells (Blatt & Armstrong 1993), which is favorable to stomatal closure. Cytosolic alkalization occurs by both MeJA signaling and ABA signaling.

In order to determine whether GSH is involved in MeJA-induced stomatal closure, we examined the effect of the *cad2-1* mutation on stomatal closure, ROS production, cytosolic alkalization in response to MeJA and the effects of the *atrbohD*, *atrbohF*, and *atrbohD atrbohF* mutations on MeJA-induced GSH depletion in guard cells in Arabidopsis.

### **3.3. Materials and Methods**

#### **3.3.1. Plant materials and growth conditions**

*Arabidopsis thaliana* wild type (ecotype Columbia) and the *cad2-1*, *atrbohD*, *atrbohF*, and *atrbohD atrbohF*, *abi1-1* and *abi2-1* (Columbia accession) plants were used in this study. These plants were grown in a growth chamber described previously in chapter 2.

#### **3.3.2. Measurement of stomatal aperture**

Stomatal aperture measurements were performed as described previously (Murata et al., 2001) in chapter 2.

#### **3.3.3. Measurement of GSH in guard cells**

Contents of GSH in guard cells were examined using monochlorobimane (MCB) as previously described (Akter et al., 2010) in chapter 2.

#### **3.3.4. Measurement of ROS production in guard cells**

Production of ROS in guard cells was evaluated using 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) (Sigma, St. Louis, MO, USA) as previously described (Munemasa et al., 2007) in chapter 2.

### **3.3.5. Measurement of cytosolic pH**

Cytosolic pH ( $\text{pH}_{\text{cyt}}$ ) in guard cells was analyzed using 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetomethylester (BCECF-AM) as previously described by Islam et al. (2010b). Epidermal tissues were isolated from 4- to 6-week-old plants with a commercial blender. The epidermal tissues were incubated for 3 h under light ( $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) in incubation buffer (50 mM KCl, 10 mM MES-KOH, pH 6.5). After this incubation, epidermal tissues were incubated in incubation buffer containing 20  $\mu\text{M}$  BCECF-AM for 30 min in the dark at room temperature to load the dye into the guard cells. Loading was terminated by rinsing the epidermal tissues three times in incubation buffer lacking the dye. The epidermal tissues were treated with 10  $\mu\text{M}$  GSHmee for 20 min and then treated with 10  $\mu\text{M}$  MeJA for another 20 min. Fluorescence of guard cells was imaged using the fluorescence microscope (BZ-8000) with a filter (OP-66835 BZ filter GFP at excitation wavelength 480/30 nm, absorption wavelength 510 nm, and dichroic mirror wavelength 505 nm). BCECF fluorescence intensity of guard cells was quantified using ImageJ 1.42q software (NIH, USA).

### **3.3.6. Statistical analysis**

Significance of differences between mean values was assessed using Student *t*-test and analysis of variance (ANOVA) with Student-Neuman-Keuls multiple-range test. We regarded differences at the level of  $p < 0.05$  as significant.

### **3.3.7. Accession numbers**

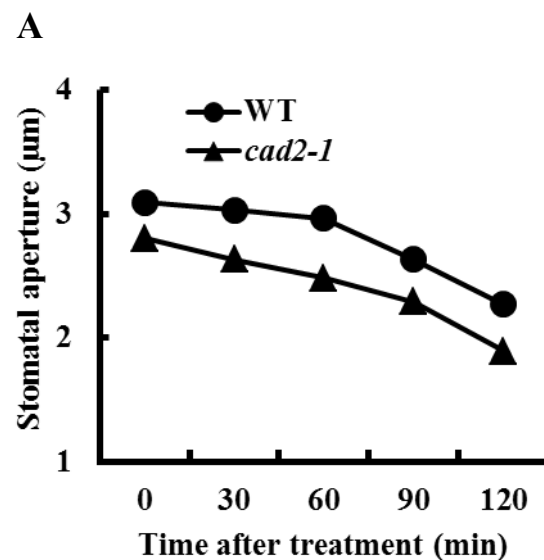
Arabidopsis Genome Initiative numbers for the genes discussed in

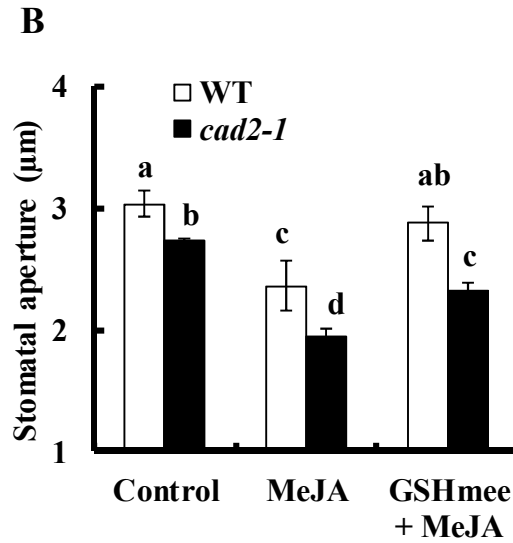
this article are as follows: CAD2 (At4G23100), AtRBOH genes are AtRBOHD (At5g47910) and AtRBOHF (At1g64060), ABI1 (At4g26080) and ABI2 (At5g57050).

### 3.4. Results

#### 3.4.1. GSH modulation of MeJA-induced stomatal closure

A time course of MeJA-induced stomatal closure shows that 10  $\mu$ M MeJA reduced stomatal apertures by 15% at 30 min and by 29% at 120 min in *cad2-1* mutant and by 13% at 30 min and by 21% at 120 min in wild type plants (Fig. 3.1 A). Application of 10  $\mu$ M MeJA narrowed stomatal apertures by 22% ( $p < 0.007$ ) in wild-type plants and by 28% ( $p < 10^{-5}$ ) in *cad2-1* plants (Fig. 3.1 B). Stomatal apertures of *cad2-1* plants were narrower than those of wild-type plants regardless of MeJA treatment ( $p < 0.009$  without MeJA,  $p < 0.03$  with MeJA) (Fig. 3.1 B). These results suggest that the genetic depletion of GSH in guard cells enhances MeJA-induced stomatal closure.

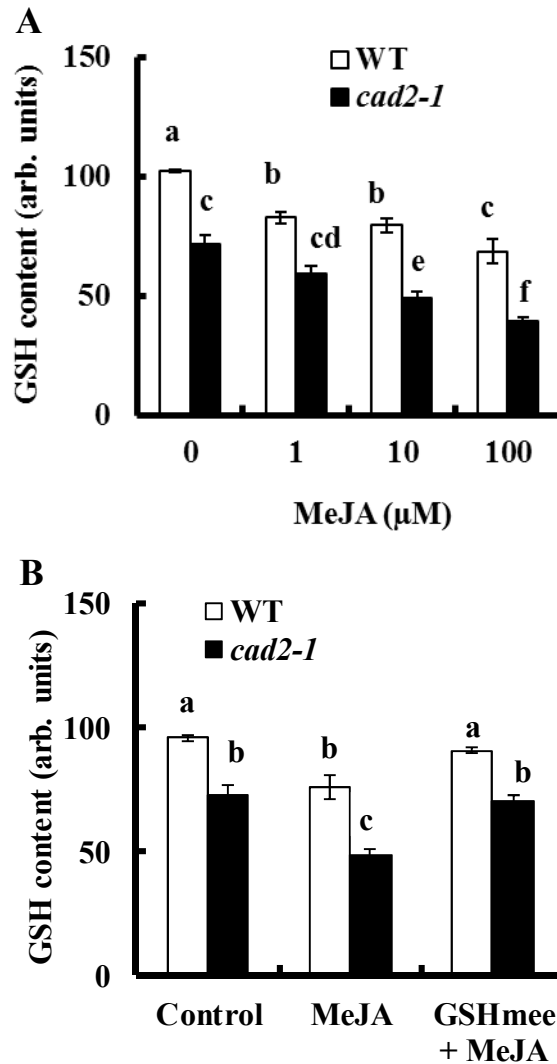




**Fig. 3.1.** Stomatal aperture in *cad2-1* mutant plants. (A) The time course of stomatal apertures in the *cad2-1* plants treated with 10  $\mu$ M MeJA. (B) Effects of GSHmee and MeJA on stomatal aperture in wild-type plants (white bars) and *cad2-1* plants (black bars). Rosette leaves were pre-treated with 10  $\mu$ M GSHmee for 30 min were treated with 10  $\mu$ M MeJA for 2 h. Averages from three independent experiments (60 total stomata per bar) are shown. Bars showing the same letter are not significantly different at  $p < 0.05$ . Error bars represent standard errors.

Effect of MeJA on GSH contents in guard cells of the *cad2-1* plants was measured using MCB staining (Fig. 3.2). The amounts of GSH in the *cad2-1* plants were less than those in wild type at all concentrations of MeJA that we evaluated in this study. MeJA decreased GSH contents in mutant guard cells as well as wild type plants in a concentration-dependent manner (Fig. 3.2 A). MCB staining showed that MeJA application decreased the amounts of GSH in guard cells of the wild-type plants ( $p < 0.002$ ) and the *cad2-1* plants ( $p < 0.001$ ) (Fig. 3.2 B). The *cad2-1* mutant accumulates lower levels of GSH than the wild type ( $p < 10^{-3}$ ) (Fig. 3.2 B),

which is consistent with previous results (Howden et al., 1995; Okuma et al., 2011).



**Fig. 3.2.** Effects of MeJA and GSHmee on GSH contents in guard cells. **A** Effects of MeJA on GSH contents in guard cells. **B** Effects of GSHmee and MeJA on GSH content in guard cells of wild-type plants (white bars) and *cad2-1* plants (black bars). Abaxial epidermal leaf tissues pre-treated with 10 μM GSHmee for 30 min were treated with 10 μM MeJA. GSH contents in guard cells were measured using 100 μM MCB. The vertical scale represents the arbitrary unit of GSH contents/GSB fluorescence intensity.

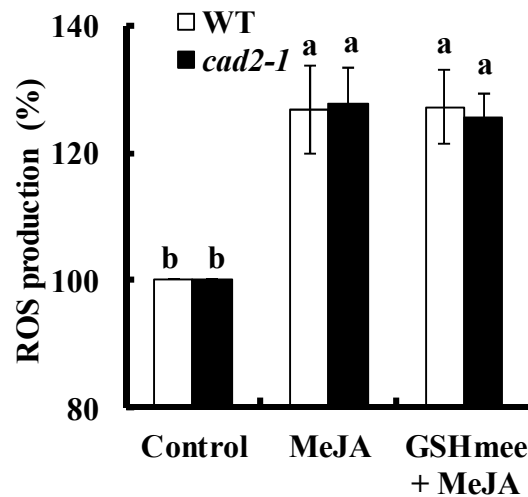
Each data was obtained from at least 60 guard cells ( $n = 3$ ). Bars showing the same letter are not significantly different at  $p < 0.05$ . Error bars represent standard errors.

To confirm that GSH depletion enhanced MeJA-induced stomatal closure in the *cad2-1* mutant (Fig. 3.1 B), we investigated the effects of exogenous GSHmee on stomatal aperture of the *cad2-1* mutant. GSHmee can permeate cell membranes and is hydrolyzed by cytosolic esterases to release free intracellular GSH (Puri & Meister 1983). Treatment with 10  $\mu$ M GSHmee significantly increases GSH content in *cad2-1* mutant guard cells and complements the stomatal phenotype of the *cad2-1* mutant (Okuma et al., 2011). The stomatal aperture in the *cad2-1* mutant after treatment with 10  $\mu$ M GSHmee was not significantly different from that in the wild type in the presence of MeJA ( $p = 0.78$ ) (Fig. 3.1 B), which is similar to our previous results using *Arabidopsis chl-1* mutants (Akter et al., 2010). Extracellular application of free GSH, unlike GSHmee, did not affect either GSH contents or stomatal apertures in the *cad2-1* mutant (data not shown).

#### **3.4.2. Intracellular GSH does not affect ROS production and cytosolic alkalization**

MeJA induces ROS production leading to stomatal closure (Suhita et al., 2004; Munemasa et al., 2007, 2011). To elucidate how GSH functions in MeJA-induced stomatal closure, MeJA-induced ROS production was measured using H<sub>2</sub>DCF-DA (Munemasa et al., 2007). As shown in Fig. 3.3, 10  $\mu$ M MeJA induced ROS production in wild-type guard cells untreated ( $p < 0.002$ ) and treated ( $p < 10^{-3}$ ) with 10  $\mu$ M GSHmee and in *cad2-1* mutant guard cells untreated ( $p < 10^{-4}$ ) and treated ( $p < 10^{-4}$ ) with GSHmee. There

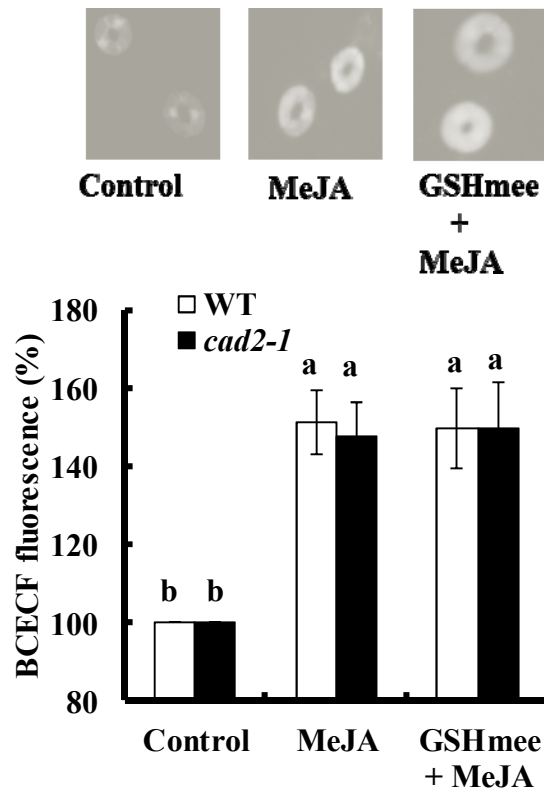
was no significant difference in MeJA-induced ROS production between the wild-type guard cells and the *cad2-1* mutant guard cells regardless of GSHmee treatment ( $p = 0.83$  without GSHmee,  $p = 0.70$  with GSHmee) (Fig. 2A). Increasing intracellular GSH by GSHmee did not affect MeJA induced ROS accumulation in the *cad2-1* mutant guard cells ( $p = 0.57$ ) (Fig. 3.3). These results indicate that MeJA-induced ROS production did not affect by the amount of GSH in guard cells.



**Fig. 3.3.** Production of ROS in MeJA-treated *cad2-1* guard cells. Effects of GSHmee and MeJA on ROS production in guard cells of wild-type plants (white bars) and *cad2-1* mutant plants (black bars). Epidermal leaf tissues were pre-treated with 10  $\mu$ M GSHmee for 30 min were treated with 10  $\mu$ M MeJA. The vertical scale represents the percentage of H<sub>2</sub>DCF-DA fluorescence levels (ROS) when fluorescent intensities of MeJA-treated cells are normalized to control value taken as 100% for each experiment. Each data was obtained from at least 60 guard cells ( $n = 3$ ). Bars showing the same letter are not significantly different at  $p < 0.05$ . Error bars

represent standard errors.

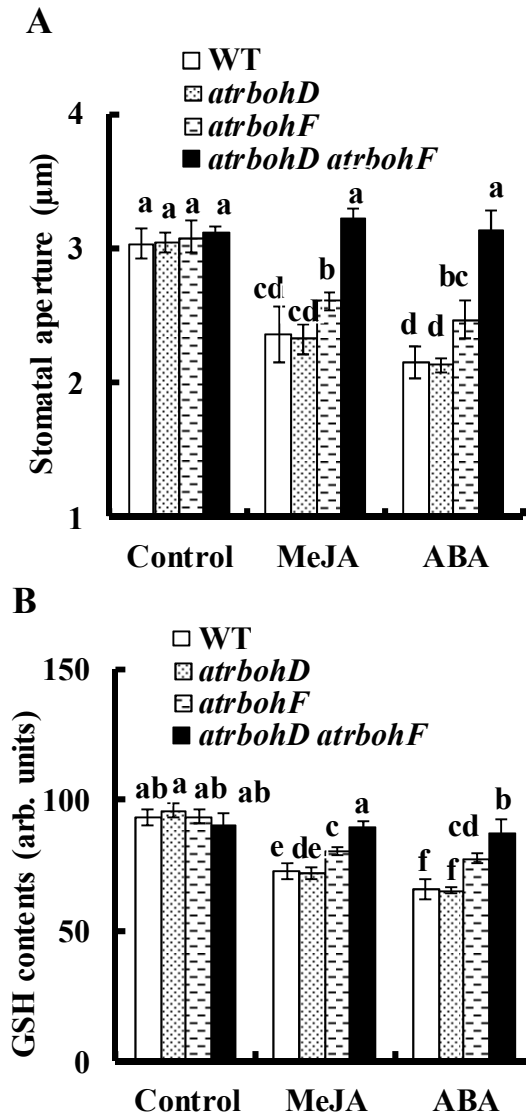
MeJA-induced stomatal closure is accompanied by cytosolic alkalization in guard cells (Suhita et al., 2004; Gonugunta et al., 2009; Islam et al., 2010b). MeJA-induced cytosolic alkalization was examined using BCECF-AM (Suhita et al., 2004; Islam et al., 2010b). Application of 10  $\mu$ M MeJA induced cytosolic alkalization in the wild-type guard cells untreated with 10  $\mu$ M GSHmee ( $p < 10^{-3}$ ) (Fig. 3.4), in agreement with our previous result (Islam et al., 2010b), and in the wild-type guard cells treated with 10  $\mu$ M GSHmee ( $p < 0.001$ ) (Fig. 3.4). MeJA at 10  $\mu$ M induced cytosolic alkalization in the *cad2-1* mutant guard cells regardless of GSHmee treatment ( $p < 10^{-3}$  without GSHmee,  $p < 0.001$  with GSHmee). There was no significant difference in MeJA-induced cytosolic alkalization between the wild-type guard cells and the *cad2-1* mutant guard cells ( $p = 0.64$  without GSHmee,  $p = 0.99$  with GSHmee) (Fig. 3.4). Increasing intracellular GSH did not affect MeJA-induced cytosolic alkalization in the *cad2-1* mutant guard cells ( $p = 0.81$ ) (Fig. 3.4). These results suggest that intracellular GSH did not affect MeJA-induced cytosolic alkalization in *Arabidopsis* guard cells.



**Fig. 3.4.** Cytosolic alkalization in MeJA-treated *cad2-1* guard cells. Effects of GSHmee and MeJA cytosolic pH ( $\text{pH}_{\text{cyt}}$ ) in guard cells of wild-type plants (white bars) and *cad2-1* mutant plants (black bars). Epidermal leaf tissues were pre-treated with 10  $\mu\text{M}$  GSHmee for 30 min were treated with 10  $\mu\text{M}$  MeJA. The vertical scale represents the percentage of BCECF-AM fluorescence levels ( $\text{pH}_{\text{cyt}}$ ) when fluorescent intensities of MeJA-treated cells are normalized to control value taken as 100% for each experiment. Each data was obtained from at least 60 guard cells ( $n = 3$ ). Bars showing the same letter are not significantly different at  $p < 0.05$ . Error bars represent standard errors.

### 3.4.3. MeJA- and ABA-induced stomatal closure and GSH depletion in *atrboh* mutants

Application of 10  $\mu\text{M}$  MeJA and 10  $\mu\text{M}$  ABA induced stomatal



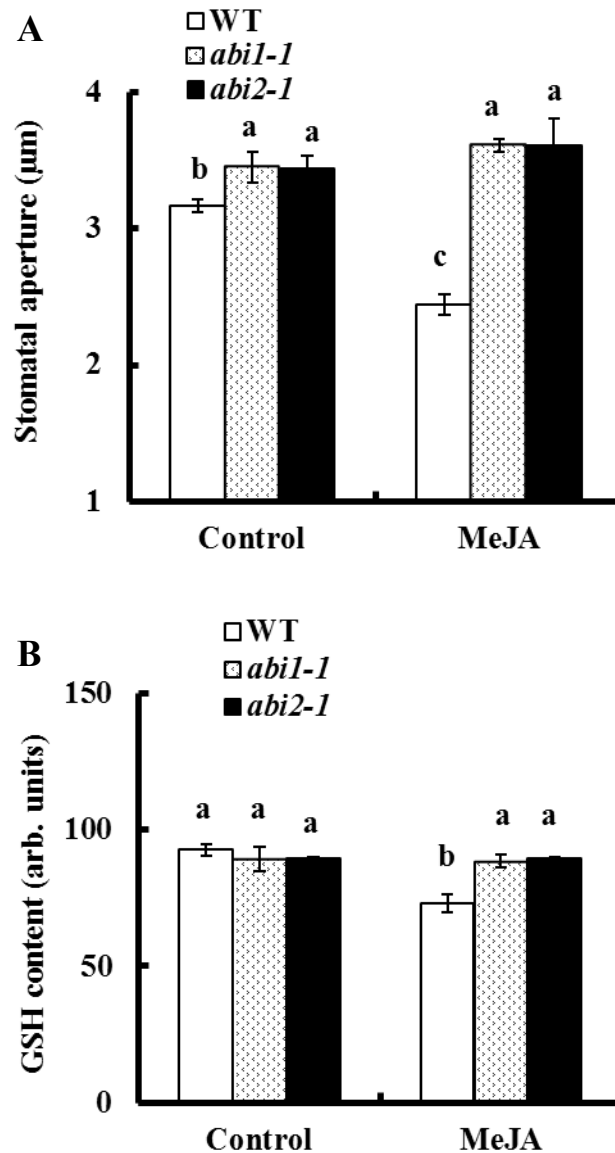
**Fig. 3.5.** Stomatal aperture and intracellular GSH in *atrboh* mutants.

(A) Effects of 10  $\mu\text{M}$  MeJA stomatal aperture of *atrbohD*, *atrbohF*, and *atrbohD atrbohF* plants. Averages from three independent experiments (60 total stomata per bar) are shown. (B) Effects of 10  $\mu\text{M}$  MeJA and 10  $\mu\text{M}$  ABA on GSH content in guard cells of *atrbohD*, *atrbohF*, and *atrbohD atrbohF* plants. The vertical scale represents the arbitrary unit of GSH contents/GSB fluorescence intensity. Each data was obtained from at least 60 guard cells ( $n = 3$ ). Bars showing the same letter are not significantly different at  $p < 0.05$ . Error bars represent standard errors.

closure in the *atrbohD* ( $p < 10^{-5}$  for MeJA;  $p < 10^{-5}$  for ABA) and *atrbohF* ( $p < 0.005$  for MeJA;  $p < 0.004$  for ABA) single mutants but not in the *atrbohD atrbohF* double mutant ( $p = 0.09$  for MeJA;  $p = 0.83$  for ABA) (Fig. 3.5 A), in agreement with the results of (Suhita et al., 2004). Application of 10  $\mu$ M MeJA and 10  $\mu$ M ABA decreased the GSH level in *atrbohD* ( $p < 0.001$  for MeJA;  $p < 0.001$  for ABA) and *atrbohF* ( $p < 0.003$  for MeJA;  $p < 10^{-3}$  for ABA) single mutants (Fig. 3.5 B) but not in the double mutant ( $p = 0.74$  for MeJA;  $p = 0.21$  for ABA) (Fig. 3.5 B).

#### **3.4.4. MeJA-induced stomatal closure and GSH depletion in *abi1-1* and *abi2-1* PP2C mutants**

Protein phosphatase 2C activity is necessary for normal increases in  $[Ca^{2+}]_{\text{cyt}}$  activation of calcium-dependent anion channels, and inactivation of inward  $K^+$  channels in guard cells in response to ABA (Grabov et al., 1997; Pei et al., 1997; Allen et al., 1999). We, therefore, tested whether PP2C enzyme involves in MeJA-induced stomatal closure and GSH depletion in guard cells, stomatal apertures and GSH contents were measured in *abi1-1* and *abi2-1* PP2C mutants. Application of 10  $\mu$ M MeJA failed to induce stomatal closure in the *abi1-1* ( $p = 0.08$  for MeJA) and *abi2-1* ( $p = 0.23$  for MeJA) mutant plants (Fig. 3.6 A), in agreement with the results of (Munemasa et al., 2007). Application of 10  $\mu$ M MeJA failed to decrease the GSH level in *abi1-1* ( $p = 0.77$  for MeJA) and *abi2-1* ( $p = 0.71$  for MeJA) mutant plants (Fig. 3.6 B). These results suggest that PP2C enzyme involved in mediating MeJA-induced stomatal closure and GSH depletion.

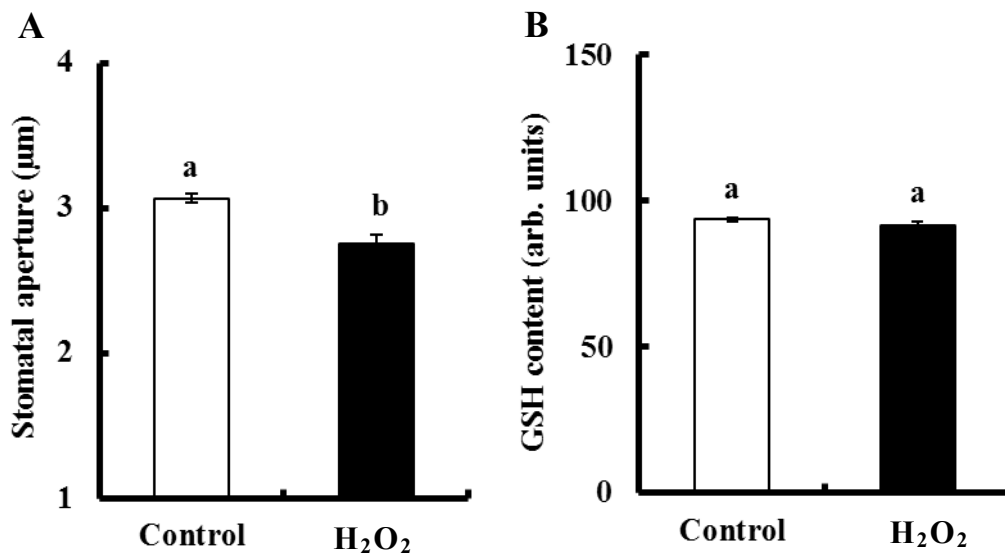


**Fig. 3.6.** Stomatal aperture and intracellular GSH in *abil-1* and *abi2-1* mutant plants. (A) Effects of 10  $\mu\text{M}$  MeJA on stomatal aperture of *abil-1* and *abi2-1* mutant plants. Averages from three independent experiments (60 total stomata per bar) are shown. (B) Effects of 10  $\mu\text{M}$  MeJA on GSH content in guard cells of *abil-1* and *abi2-1* mutant plants. The vertical scale represents the arbitrary unit of GSH contents/GSB fluorescence intensity. Each data was obtained from at least 60 guard cells ( $n = 3$ ). Bars showing

the same letter are not significantly different at  $p < 0.05$ . Error bars represent standard errors.

### 3.4.5. H<sub>2</sub>O<sub>2</sub> induces stomatal closure but not GSH depletion in wild-type guard cells

We examined whether exogenous ROS-induced stomatal closure is accompanied by GSH depletion in wild-type guard cells. In the wild-type plants, 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> induces stomatal closure ( $p < 0.001$ ) (Fig. 3.7 A) but did not deplete GSH in guard cells ( $p = 0.09$ ) (Fig. 3.7 B). These results suggest that intracellular GSH is not involved in ROS scavenge during stomatal closure in Arabidopsis.



**Fig. 3.7.** Stomatal aperture and intracellular GSH in wild-type plants.

(A) Effect of 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> on stomatal aperture of wild-type plants. Averages from three independent experiments (60 total stomata per bar) are shown. (B) Effect of 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> on GSH content in guard cells of

wild-type plants. The vertical scale represents the arbitrary unit of GSH contents/GSB fluorescence intensity. Each data was obtained from at least 60 guard cells ( $n = 3$ ). Bars showing the same letter are not significantly different at  $p < 0.05$ . Error bars represent standard errors.

### 3.5. Discussion

#### 3.5.1. Depletion of GSH in guard cells during MeJA-induced stomatal closure

Glutathione functions in scavenging ROS as well as degradation of toxic chemicals (Alscher et al., 1997; Blum et al., 2007; Foyer & Halliwell 1976; Noctor & Foyer 1998). Despite the importance of GSH in MeJA signaling of guard cells, we are aware of only one related study (Akter et al., 2010), in which MeJA-induced stomatal closure was found to be attenuated in the *chlorinal-1* (*chl-1*) mutant, which accumulates less GSH. The present results also show that MeJA-induced stomatal closure was enhanced in the GSH-deficient mutant, *cad2-1*, which is consistent with our previous conclusion that GSH negatively regulates MeJA-induced stomatal closure (Akter et al., 2010).

Hartmann et al. (2003) reported that conjugation of GSH with MCB occurs in the cytosol but not in chloroplasts, and Joo et al. (2005) reported that the *atrbohD atrbohF* mutant exhibits oxidative stress in chloroplasts. Moreover, this study shows that MeJA- and ABA-induced GSH depletion was impaired in the *atrbohD atrbohF* double mutant. Taken together, these results suggest that cytosolic GSH depletion in guard cells is related to cytosolic ROS accumulation but not chloroplastic ROS accumulation. Our previous studies (Jahan et al., 2008; Akter et al., 2010; Okuma et al., 2011) and this study have found that supplementing cytosolic GSH by GSHmee

did not affect MeJA- and ABA-induced ROS accumulation, suggesting that depletion of GSH follows ROS production in MeJA and ABA signaling. In addition, the GSH depletion was impaired in the *atrbohD atrbohF* double mutant but not in either single mutant, suggesting that AtRBOHD and AtRBOHF redundantly regulate GSH depletion induced by MeJA and ABA in guard cells.

Protein phosphatase 2C proteins, ABI1 and ABI2, are also crucial for ABA-mediated stomatal regulation (Gosti et al., 1999; Leung et al., 1997; Merlot et al., 2001). The dominant negative mutant *abi1-1* shows ABA-insensitive stomatal conductance (Koornneef et al., 1989; Leung et al., 1994), whereas revertant and loss-of-function recessive mutants of ABI1 show hypersensitivity in ABA-mediated stomatal response, leading to the conclusion that ABI1 is a negative regulator of ABA signaling. This study shows that MeJA-induced stomatal closure and GSH depletion was impaired in the *abi1-1* and *abi2-1* mutants (Fig. 3.6). Based on these observations it has been proposed that ABI1 and ABI2 redundantly regulate stomatal closure and GSH depletion induced by MeJA in guard cells.

MeJA induces cytosolic alkalization in guard cells, which is closely related to cytosolic  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) oscillation (Islam et al., 2010b). The present study shows that neither depletion nor supplement of GSH affects either ROS production or cytosolic alkalization by MeJA (Fig. 3.3 and 3.4), suggesting that GSH negatively regulates MeJA signaling downstream of ROS production and cytosolic alkalization. These results are consistent with our previous result that GSH does not affect MeJA-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillation (Akter et al., 2010) and ABA-induced activation of  $\text{I}_{\text{Ca}}$  currents (Okuma et al., 2011).

Vacuolar-type (V-type) ATPase contributes to the  $\text{pH}_{\text{cyt}}$  homeostasis

(Sze et al., 1992) but is not modulated by GSH in the leaf vacuole (Dietz et al., 1998). In Arabidopsis, GSH does not contribute to scavenging of ROS *via* glutathione peroxidases (GPXs) (AtGPX1, 2, 3, 5 and 6) because the GPXs scavenge H<sub>2</sub>O<sub>2</sub> and organic hydroperoxides using thioredoxin as an electron donor rather than GSH (Iqbal et al., 2006; Miao et al., 2006). These results are also consistent with the results in this study that GSH does not affect either cytosolic alkalization or ROS accumulation in guard cells. Moreover, extracellular H<sub>2</sub>O<sub>2</sub> had no effect on depletion of GSH in guard cells but induces stomatal closure (Fig. 3.6). These results also support the conclusion that GSH is not involved in scavenging ROS which is produced during MeJA-induced stomatal closure.

### **3.5.2. Sharing signal components between MeJA signaling and ABA signaling**

MeJA failed to induce stomatal closure in a number of mutants, including ABA-insensitive mutants, the *atrbohD atrbohF* mutant (Suhita et al. 2004), the *abi1* and the *abi2* (protein phosphatase 2C) (PP2C) mutants (Munemasa et al., 2007), the *rcn1* (regulatory subunit of protein phosphatase 2A) (PP2A) mutant (Saito et al., 2008), and the *tgg1 tgg2* (myrosinase) mutant (Islam et al., 2009). Therefore, MeJA recruits many ABA signaling components to induce stomatal closure. Our previous results suggest that GSH is a common negative signaling factor between the MeJA and ABA signal cascades (Jahan et al., 2008; Akter et al., 2010; Okuma et al., 2011), and this study showed that GSH negatively regulates MeJA signaling. These results indicate that GSH is involved both MeJA signaling and ABA signaling.

MeJA signaling as well as ABA signaling share a number of characteristics, including mobilization of ions, activation of slow (S-type) anion channels,  $I_{Ca}$  channels and  $K^{+}_{out}$  channels, and inactivation of  $K^{+}_{in}$  channels in Arabidopsis guard cells (Evans 2003; Munemasa et al., 2007; Saito et al. 2008). Because GSH modulates slow vacuolar (SV) channel activity in cultured carrot cells (Scholz-Starke et al., 2004), it may negatively regulate certain ion channels related to stomatal closure. However, Arabidopsis Two Pore Channel 1 (AtTPC1) encoding the SV channel is not involved in either ABA or MeJA signaling in guard cells (Islam et al., 2010a). Moreover, GSH does not affect  $I_{Ca}$  channels (Okuma et al., 2011). Taken together, these results suggest that GSH regulates signal components other than ion channels.

Allyl isothiocyanate (AITC) induces stomatal closure accompanied by GSH depletion, and AITC-induced stomatal closure is suppressed by GSHmeee treatment, which increases GSH in guard cells (Khokon et al., 2011). Isothiocyanates (ITCs) can enzymatically and non-enzymatically react with GSH because of their electrophilicity (Zhang et al., 1995). However, MeJA and ABA are unlikely to react with GSH because MeJA and ABA are not as electrophilic as ITCs.

In conclusion, GSH negatively regulates MeJA- and ABA-induced stomatal closure *via* modulation of common signal components other than early signal components, ROS production,  $[Ca^{2+}]_{cyt}$  elevation, and cytosolic alkalization.

## CHAPTER 4

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### **Effects of depletion of glutathione on abscisic Acid- and methyl jasmonate-induced stomatal closure in *Arabidopsis thaliana***

#### **4.1. Abstract**

Glutathione (GSH) is involved in abscisic acid (ABA)- and methyl jasmonate (MeJA)-induced stomatal closure in *Arabidopsis thaliana*. In this study, we examined the effects of GSH-decreasing chemicals, *p*-nitrobenzyl chloride (PNBC), iodomethane (IDM), and ethacrynic acid (EA) on ABA- and MeJA-induced stomatal closure in *Arabidopsis*. Treatments with PNBC, IDM, and EA decreased GSH contents in guard cells. Depletion of GSH by PNBC and IDM enhanced ABA- and MeJA-induced stomatal closure and inhibition of light-induced stomatal opening by ABA, whereas EA did not enhance either ABA- and MeJA-induced stomatal closure or inhibition of light-induced stomatal opening by ABA. Depletion of GSH did not significantly increase the production of the reactive oxygen species (ROS), cytosolic alkalization, or cytosolic  $\text{Ca}^{2+}$  oscillation induced by ABA and MeJA. These results indicate that depletion of GSH enhances ABA- and MeJA-induced stomatal closure without affecting ROS production, cytosolic alkalization, or cytosolic  $\text{Ca}^{2+}$  oscillation in guard cells of *Arabidopsis*.

## 4.2. Introduction

A stoma, which consists of a pair of guard cells in the epidermis of the above-ground parts of higher plants, responds to various external and internal stimuli (Schroeder et al., 2001; Hetherington et al., 2003). Absciscic acid (ABA) functions in abiotic and biotic stress responses and in regulation of many developmental processes, including maintenance of seed dormancy and development, (Hetherington et al., 1991; Koornneef et al., 1998) and methyl jasmonate (MeJA), a linolenic acid derivative, is involved in plant development and defense responses (Creelman et al., 1997; Liechti & Farmer, 2002). ABA and MeJA induce stomatal closure in many plant species (Suhita et al., 2004; Munemasa et al., 2007) and both ABA- and MeJA-induced stomatal closure are accompanied by ROS production mediated by NADPH oxidase and cytosolic alkalization and cytosolic  $\text{Ca}^{2+}$  oscillation ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) (Suhita et al., 2004; Munemasa et al., 2007; Kwak et al., 2003; Islam et al., 2010b).

Glutathione (GSH) regulates plant growth and development *via* control of redox state (Noctor et al., 1998; May et al., 1998), and GSH levels in plant cells vary in response to various stresses (Alscher et al., 1989; Sánchez-Fernández et al., 1997). ABA and MeJA decrease the GSH contents in guard cells. ABA-induced stomatal closure is enhanced by a GSH-decreasing chemical, 1-chloro-2,4-dinitrobenzene (CDNB), in *Arabidopsis thaliana* (Okuma et al., 2011), and GSH monoethyl ester (GSHmee), which increases GSH in guard cells of *Arabidopsis*, complements the stomatal phenotype of GSH-deficient mutants, *chl-1* and *cad2-1* (Okuma et al., 2011; Akter et al., 2010; Jahan et al., 2008). Previous studies have found that ABA inhibits light-induced stomatal opening

(Shimazaki et al., 2007; Uraji et al., 2012). In addition, deficient glutathione facilitates ABA- and MeJA-induced stomatal closure but does not effect on light-induced stomatal opening (Akter et al., 2010; Jahan et al., 2008). These results suggest that GSH is a common negative signaling factor between ABA and MeJA signal cascades (Okuma et al., 2011; Akter et al., 2010). We recently found that allyl isothiocyanate (AITC) induces stomatal closure accompanied by GSH depletion and that AITC-induced stomatal closure is suppressed by GSHmex (Khokon et al., 2011). Hence, GSH might be a key factor in stomatal closure in Arabidopsis, but evidence for this has been limited.

Like CDNB, *p*-nitrobenzyl chloride (PNBC), iodomethane (IDM), and ethacrynic acid (EA) act as substrates of glutathione *S*-transferase (GST) to form a conjugate with GSH, resulting in depletion of intracellular GSH (Habig et al., 1974; Schafer et al., 2001). To elucidate the roles of GSH in ABA- and MeJA-induced stomatal closure, we investigated the effects of PNBC, IDM, and EA on GSH levels, stomatal movement, ROS production, and cytosolic alkalization in response to ABA and MeJA in Arabidopsis.

### **4.3. Materials and Methods**

#### **4.3.1. Chemicals**

ABA was purchased from Sigma-Aldrich (St. Louis, MO). MeJA, PNBC, IDM and 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and EA was from Tokyo Chemical Industry (Tokyo). PNBC and EA were dissolved in dimethyl sulfoxide (DMSO) and IDM was in distilled water to yield stock solutions. Monochlorobimane (MCB) was purchased from Fluka

(Switzerland) and 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetomethylester (BCECF-AM) was from Invitrogen (Carlsbad, CA).

#### **4.3.2. Plant materials and growth conditions**

*Arabidopsis thaliana* wild-type (ecotype Columbia) plants were used in this study. Plants were grown in a growth chamber described previously in chapter 2.

#### **4.3.3. Measurement of stomatal aperture**

Width of stomatal apertures was measured as described previously (Murata et al., 2001) in chapter 2. For stomatal opening, excised rosette leaves were floated on a medium containing 30 mM KCl, 50  $\mu$ M CaCl<sub>2</sub> and 10 mM MES-Tris (pH 6.15) for 2 h in the dark to induce stomatal closure. These leaves were transferred in the light for 2.5 h in the presence of ABA. PNBC, IDM and EA at 10  $\mu$ M were added 30 min prior to ABA application.

#### **4.3.4. Measurement of GSH in guard cells**

The levels of GSH in guard cells were examined using MCB, as previously described (Akter et al., 2010) in chapter 2. Note that GSH decreasing chemicals were added 30 min prior to ABA or MeJA application.

#### **4.3.5. Measurement of ROS production in guard cells**

Production of ROS in guard cells was evaluated using H<sub>2</sub>DCF-DA, as previously described (Munemasa et al., 2007).

#### 4.3.6. Measurement of cytosolic pH

Cytosolic pH ( $\text{pH}_{\text{cyt}}$ ) in guard cells was analyzed using BCECF-AM as previously described (Islam et al., 2010b).

#### 4.3.7. Statistical analysis

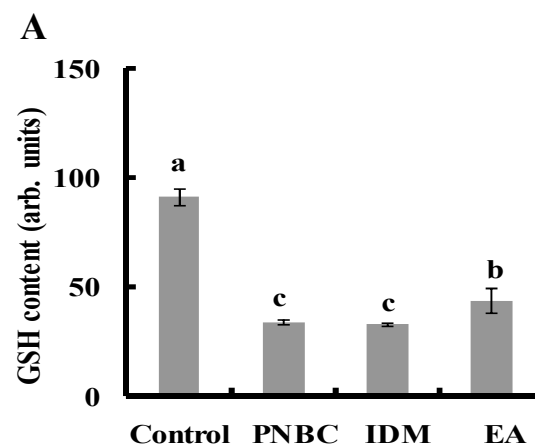
Significance of differences between mean values was assessed using analysis of variance (ANOVA) with Student-Neuman-Keuls multiple-range test, or  $\chi^2$ -test. Differences at  $p < 0.05$  were considered significant.

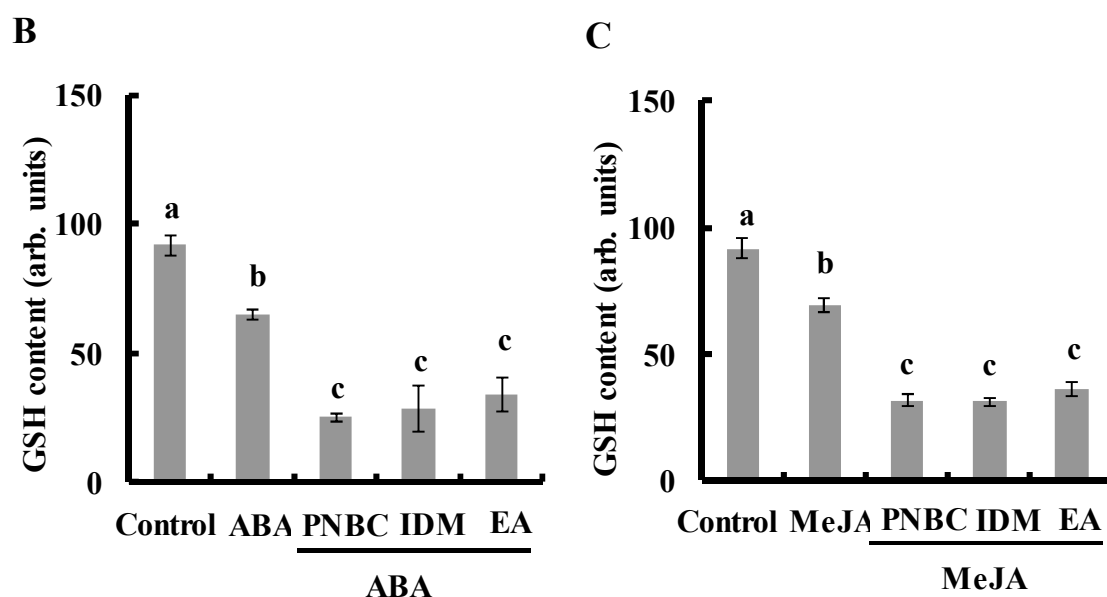
### 4.4. Results

#### 4.4.1. GSH contents in guard cells treated with GSH-decreasing chemicals

Treatments with PNBC, IDM, and EA at 10  $\mu\text{M}$  significantly decreased intracellular GSH contents in guard cells untreated with ABA or MeJA (Fig. 4.1 A).

ABA and MeJA at 1  $\mu\text{M}$  significantly decreased intracellular GSH in guard cells (Fig. 4.1 B and C). Pretreatment of the guard cells with PNBC, IDM, and EA significantly enhanced ABA- and MeJA-induced GSH depletion in the guard cells (Fig. 4.1 B and C).



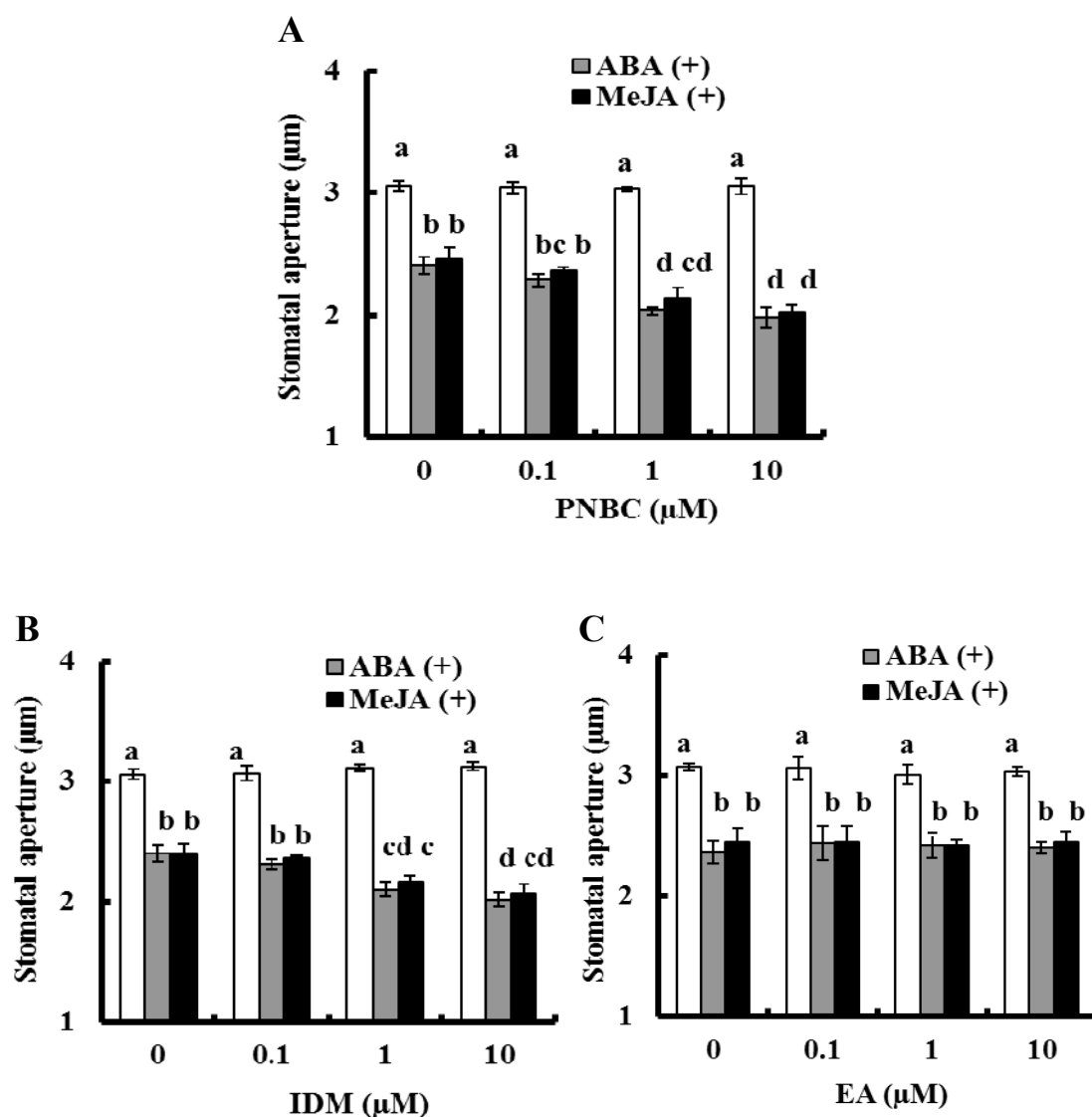


**Fig. 4.1.** Effects of GSH-decreasing chemicals on GSH content in guard cells. A, Glutathione content in guard cells of wild-type plants treated with PNBC, IDM, and EA. B and C, Effects of PNBC, IDM, and EA on ABA- and MeJA-induced GSH depletion in guard cells of wild-type plants. Abaxial epidermal leaf tissues were pre-treated with PNBC, IDM and EA at 10  $\mu$ M for 30 min and then were treated with 1  $\mu$ M ABA or MeJA for 2 h. The vertical scale represents the GSB fluorescence intensity. Each data was obtained from at least 120 guard cells ( $n = 4$ ). Error bars represent standard errors. The different letters indicate the statistically significant difference at 5% level.

#### 4.4.2. Effects of GSH-decreasing chemicals on ABA- and MeJA-induced stomatal closure

Treatment with PNBC, with IDM, and with EA did not affect stomatal apertures in the absence of ABA and MeJA (Fig. 4.2). However,

treatment with PNBC and with IDM enhanced ABA- and MeJA-induced stomata closure in a dose-dependent manner (Fig. 4.2 A and B). This accords with our previous result that CDN B enhanced ABA-induced stomatal closure in *Arabidopsis* (Okuma et al., 2011). In contrast, treatment with EA did not enhance ABA- and MeJA-induced stomatal closure (Fig. 4.2 C).

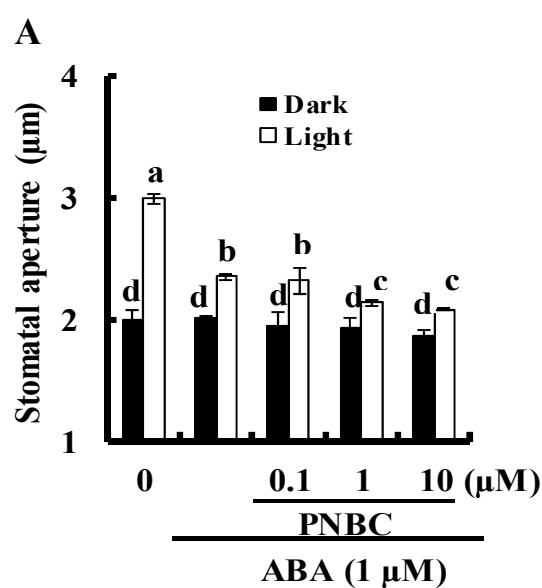


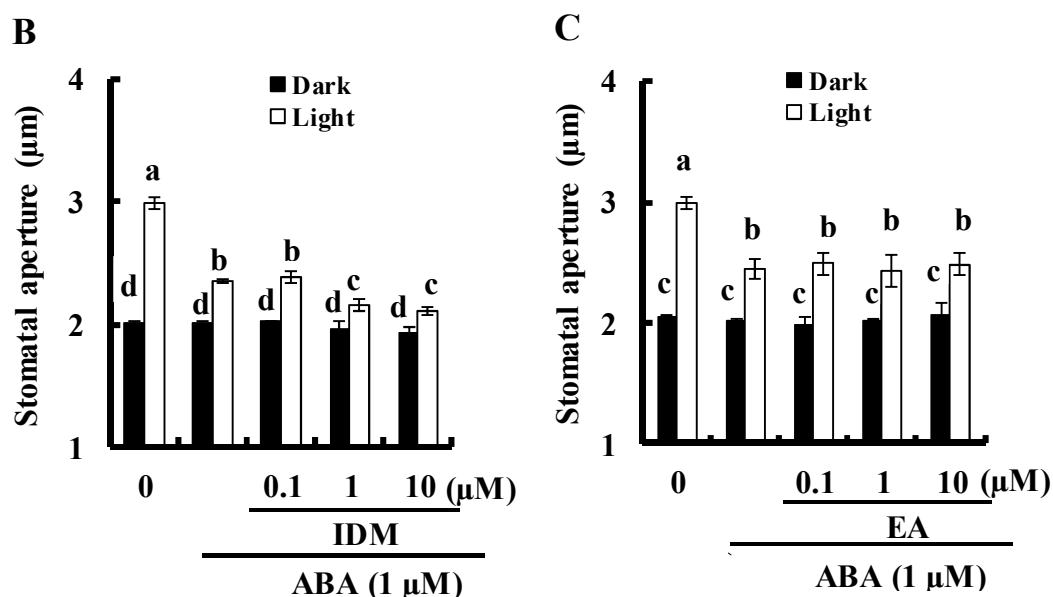
**Fig. 4.2.** Effects of GSH-decreasing chemicals on stomatal aperture in wild-type plants. A, B, and C, Effects of PNBC, IDM and EA on ABA- and

MeJA-induced stomatal closure in wild-type plants. Excised rosette leaves were pre-treated with PNBC, IDM and EA at 10  $\mu$ M for 30 min, followed by 1  $\mu$ M ABA (Ash bars) or MeJA (black bars) treatment for 2 h. The data are averages of 3 independent experiments (60 stomata observations per bar). Error bars represent standard errors. The different letters indicate the statistically significant difference at 5% level.

#### 4.4.3. Effects of GSH-decreasing chemicals on inhibition of light-induced stomatal opening by ABA

ABA at 1  $\mu$ M significantly inhibited light-induced stomatal opening in the wild type (Fig. 4.3). On the other hand, treatment with PNBC and IDM enhanced inhibition of light-induced stomatal opening by ABA in a dose-dependent manner (Fig. 4.3 A and B). However, treatment with EA did not enhance inhibition of light-induced stomatal opening by ABA (Fig. 4.3 C).



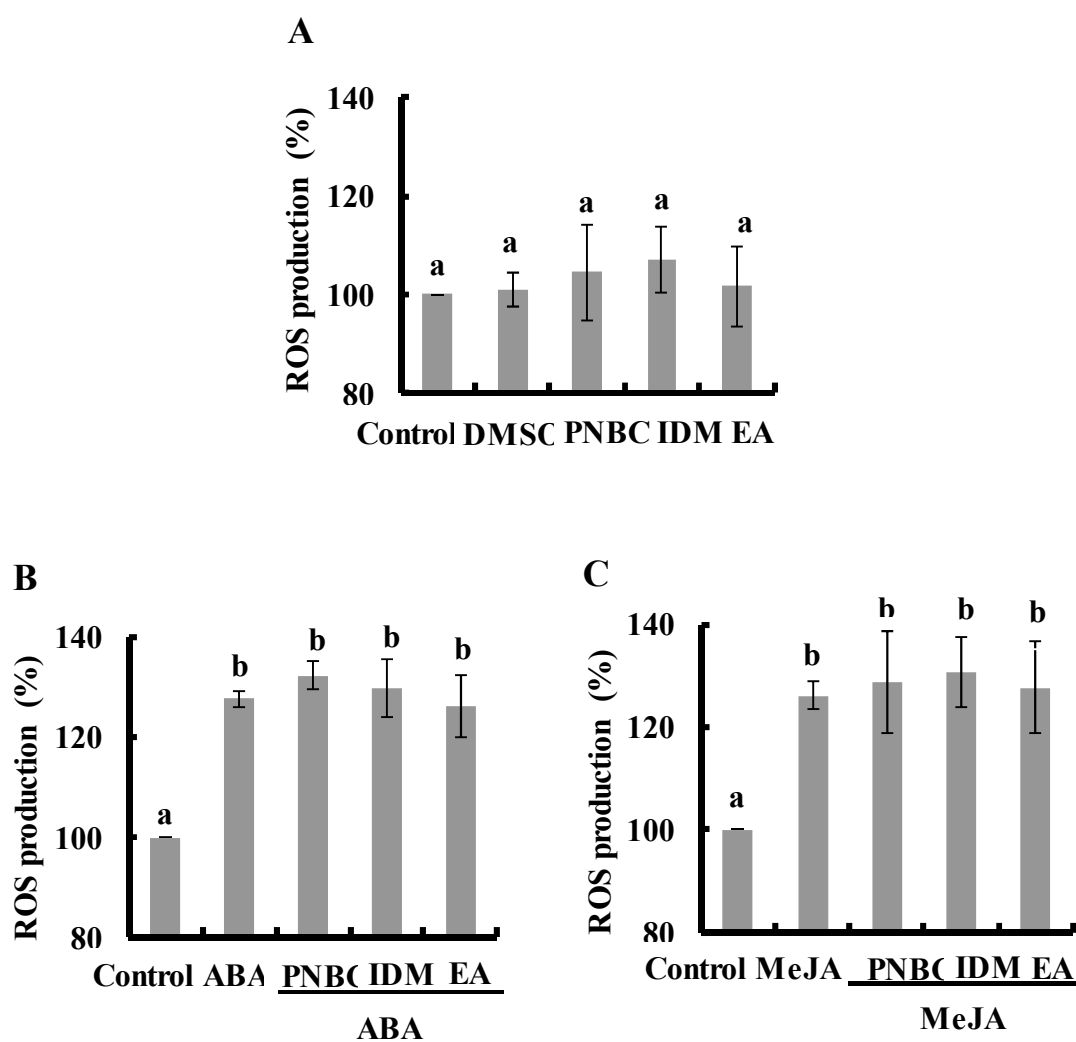


**Fig. 4.3.** Effects of GSH-decreasing chemicals on stomatal opening in wild-type plants. A, B, and C, Effects of PNBC, IDM, and EA on inhibition of light-induced stomatal opening by ABA in wild-type plants. Rosette leaves were placed in the dark for 2 h. These leaves were replaced in the light in the absence or presence of  $1 \mu\text{M}$  ABA. PNBC, IDM and EA at  $10 \mu\text{M}$  were added 30 min prior to ABA application. The data are averages of 3 independent experiments (60 stomata observations per bar) are shown. Error bars represent standard errors. The different letters indicate the statistically significant difference at 5% level.

#### 4.4.4. Effects of GSH depletion on ABA- and MeJA-induced ROS production in guard cells

Depletion of GSH by PNBC, by IDM and by EA at  $10 \mu\text{M}$  did not induce ROS production in guard cells in the absence of ABA and MeJA (Fig. 4.3 A). ABA and MeJA at  $1 \mu\text{M}$  significantly induced ROS production (Fig.

4.3 B and C), in agreement with our previous results (Saito et al., 2008). Depletion of GSH by PNBC, IDM and EA did not significantly affect ABA- and MeJA-induced ROS production in guard cells (Fig. 4.3B and C), in accord with our previous results that CDNB did not affect ABA-induced ROS production in *Arabidopsis* guard cells (Okuma et al., 2011).

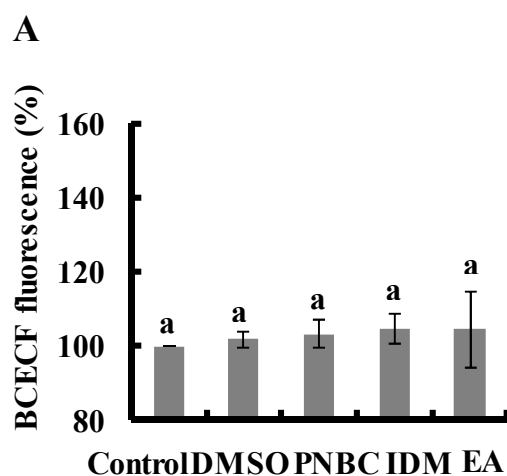


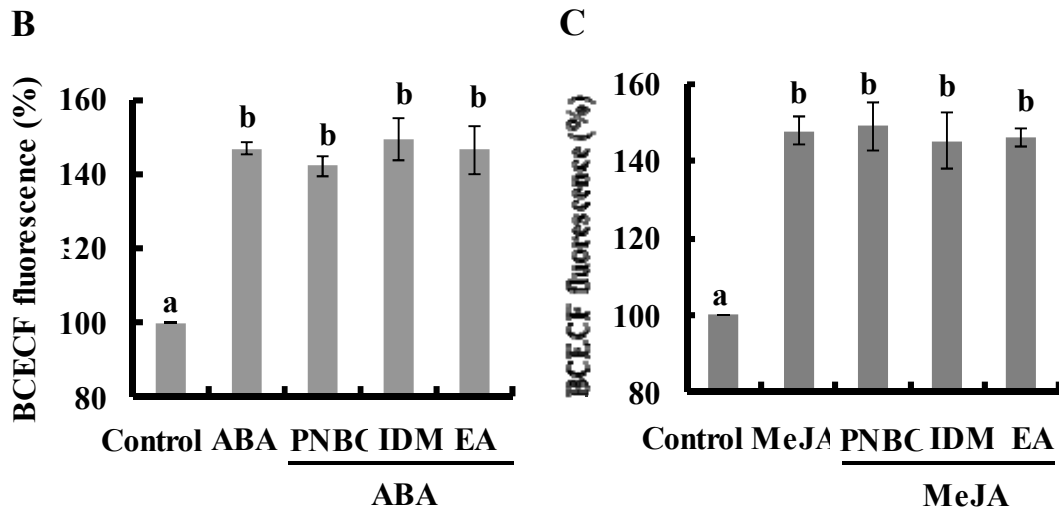
**Fig. 4.4.** Production of ROS in PNBC-, IDM-, and EA-treated guard cells. A, Effect of PNBC, IDM, and EA on ROS production in guard cells of wild-type plants. B, C, Effects of PNBC, IDM, and EA on ABA- and MeJA-induced ROS production in guard cells of wild-type plants.

Epidermal leaf tissues were pre-treated with PNBC, IDM and EA at 10  $\mu$ M for 30 min were treated with or without 1  $\mu$ M ABA or MeJA. The vertical scale represents the percentages of DCF fluorescent levels when the fluorescent intensities of ABA- and MeJA-treated cells were normalized to the control value taken as 100% for each experiment. Each data was obtained from at least 120 guard cells ( $n = 3$ ). Error bars represent standard deviation. The different letters indicate the statistically significant difference at 5% level.

#### 4.4.5. Effects of GSH depletion on ABA- and MeJA-induced cytosolic alkalization in guard cells

Depletion of GSH by PNBC, by IDM and by EA did not significantly change  $\text{pH}_{\text{cyt}}$  in the absence of ABA and MeJA (Fig. 4.4 A). ABA and MeJA at 1  $\mu$ M significantly induced cytosolic alkalization in guard cells untreated with PNBC, IDM, or EA (Fig. 4.4 B and C). Depletion of GSH by PNBC, IDM and EA did not significantly affect ABA- or MeJA-induced cytosolic alkalization in guard cells (Fig. 4.4 B and C), suggesting that intracellular GSH does not modulate ABA- or MeJA-induced cytosolic alkalization in guard cells.



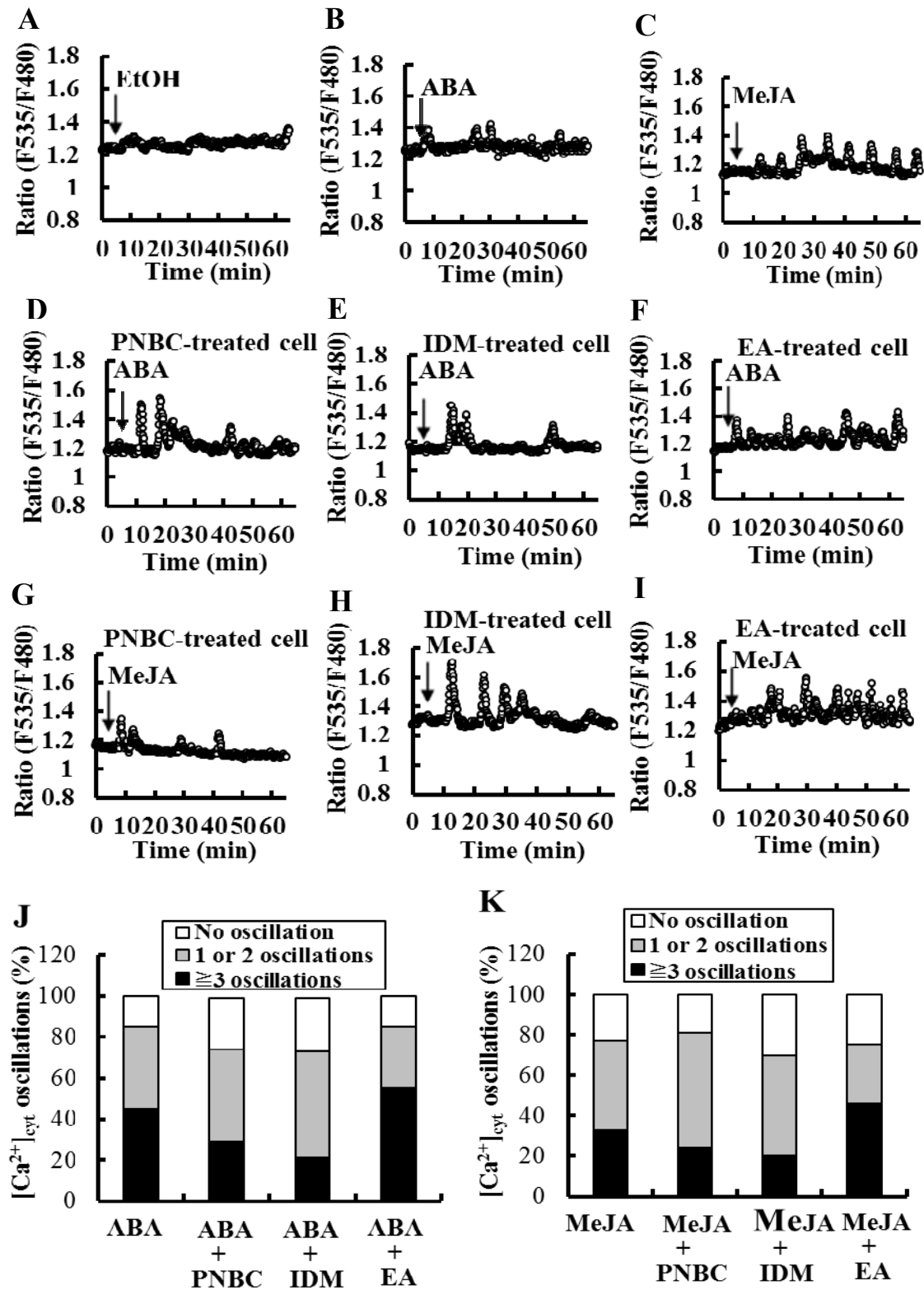


**Fig. 4.5.** Cytosolic alkalization in PNBC-, IDM-, and EA-treated guard cells. A, Effect of PNBC, IDM, and EA on cytosolic pH ( $\text{pH}_{\text{cyt}}$ ) in wild-type plants. B, C, Effects of PNBC, IDM, and EA on ABA- and MeJA-induced cytosolic alkalization in guard cells of wild-type plants. Epidermal leaf tissues were pre-treated with PNBC, IDM and EA at  $10 \mu\text{M}$  for 30 min were treated with or without  $1 \mu\text{M}$  ABA or MeJA. The vertical scale represents the percentages of BCECF fluorescent levels when the fluorescent intensities of ABA- and MeJA-treated cells were normalized to the control value taken as 100% for each experiment. Each data set was obtained from at least 180 guard cells ( $n = 3$ ). Error bars represent standard deviations. The different letters indicate the statistically significant difference at 5% level.

#### 4.4.6. Effects of GSH depletion on ABA- and MeJA-induced cytosolic $\text{Ca}^{2+}$ oscillation in guard cells

The guard cells untreated with ABA or MeJA did not show  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillation (Fig. 4.6 A). The application of  $1 \mu\text{M}$  ABA (85%,  $n = 17$  of 20

cells) and 1  $\mu\text{M}$  MeJA (77%,  $n = 14$  of 18 guard cells) induced  $[\text{Ca}^{2+}]_{\text{cyt}}$



**Fig. 4.6.**  $[Ca^{2+}]_{cyt}$  oscillations in wild-type guard cells. A, B, C,  $[Ca^{2+}]_{cyt}$  oscillations (changes in fluorescence emission ratio, 535/480 nm) in YC3.6 expressing guard cells untreated or treated with 1  $\mu$ M ABA or MeJA in wild-type guard cells. D, E, and F,  $[Ca^{2+}]_{cyt}$  oscillations in guard cells treated with PNBC, IDM or EA at 10  $\mu$ M in the presence of 1  $\mu$ M ABA. G, H, and I,  $[Ca^{2+}]_{cyt}$  oscillations in guard cells treated with PNBC, IDM or EA in the presence of 1  $\mu$ M MeJA.  $[Ca^{2+}]_{cyt}$  oscillations were counted when changes in  $[Ca^{2+}]_{cyt}$  ratios were more than or equal to 0.1 units from the baseline. J, K, Stack column representation of ABA- and MeJA-elicited  $[Ca^{2+}]_{cyt}$  oscillations (%) with PNBC, IDM or EA-untreated (n = 20 for ABA, n = 18 for MeJA) or treated (n = 24 for PNBC, n = 19 for IDM, n = 20 for EA, ABA; n = 21 for PNBC, n = 20 for IDM, n = 24 for EA, MeJA) guard cells.

oscillation in wild-type guard cells untreated with PNBC, IDM, or EA (Fig. 4.6 B, C, J, and K). In the presence of ABA or MeJA, pretreatment of guard cells with PNBC (74%, n = 18 of 24 cells for ABA; 81%, n = 17 of 21 cells for MeJA), IDM (73%, n = 14 of 19 cells for ABA; 70%, n = 14 of 20 cells for MeJA) and EA (85%, n = 17 of 20 cells for ABA; 75%, n = 18 of 24 cells for MeJA) induced  $[Ca^{2+}]_{cyt}$  oscillation in guard cells (Fig. 4.6 D-K). Taken together, there were no significant difference in  $[Ca^{2+}]_{cyt}$  oscillation between the PNBC, IDM, and EA-untreated cells and the PNBC, IDM, and EA-treated cells ( $p = 0.41$  for PNBC,  $p = 0.38$  for IDM,  $p = 1$  for EA, ABA;  $p = 0.80$  for PNBC,  $p = 0.58$  for IDM,  $p = 0.83$  for EA, MeJA), suggesting that depletion of GSH does not affect ABA- and MeJA-induced  $[Ca^{2+}]_{cyt}$  oscillation in guard cells.

#### 4.5. Discussion

GSH has many functions in growth, development, cell defense, and regulation of gene expression (May et al., 1998; Noctor & Foyer 1998). ABA- and MeJA-induced stomatal closure is accompanied by decreasing GSH levels in guard cells and depletion of GSH in guard cells enhances ABA- and MeJA-induced stomatal closure (Okuma et al., 2011; Akter et al., 2010). This suggests that GSH is a key factor in stomatal closure, but evidence for this has been limited.

ABA- and MeJA-induced stomatal closure are accompanied by ROS production and cytosolic alkalization, and  $[Ca^{2+}]_{\text{cyt}}$  oscillation in guard cells (Islam et al., 2010b). In the present study, depletion of GSH in guard cells did not affect either ABA- or MeJA-induced ROS production (Fig. 4.3), in agreement with our previous results (Okuma et al., 2011; Akter et al., 2010). An increment of intracellular GSH due to GSHmex does not decrease ROS accumulation in guard cells (Okuma et al., 2011). Moreover, unlike glutathione peroxidases (GPX) in animals (Arthur et al., 2000), Arabidopsis GPX use thioredoxin as electron donor rather than GSH to scavenge  $H_2O_2$  and organic hydroperoxides (Iqbal et al., 2006). Together, these results suggest that intracellular GSH does not involved in ABA- and MeJA-induced ROS production in guard cells.

Vacuolar-type (V-type) ATPase contributes to  $pH_{\text{cyt}}$  homeostasis (Sze et al., 1992). ABA and MeJA induces alkalization in guard cells, which is closely related to  $[Ca^{2+}]_{\text{cyt}}$  oscillation (Islam et al., 2010b). In the present study, GSH depletion in guard cells also did not affect either ABA- or MeJA-induced cytosolic alkalization or  $[Ca^{2+}]_{\text{cyt}}$  oscillation (Figs. 4.5 and 4.6), in agreement with the previous result that V-type ATPase activity is not modulated by GSH in leaf vacuoles (Dietz et al., 1998). On the other

hand, Tavakoli *et al.* reported that H<sub>2</sub>O<sub>2</sub> at higher than 0.5 mM deactivated V-type ATPase, which was reactivated by GSH *in vitro* (Tavakoli *et al.*, 2001). However, levels of ROS production elicited by ABA and MeJA were less than 100  $\mu$ M (Tossi *et al.*, 2009; Hung and Kao 2007). Therefore, ROS levels under physiological conditions are not so high as to inhibit V-type ATPase, resulting in no effects of GSH on the alkalization in guard cells.

In this study, GST substrates, PNBC and IDM depleted GSH in guard cells and enhanced ABA- and MeJA-induced stomatal closure (Figs. 4.1 and 4.2), in agreement with our previous results for GSH depletion by CDNB (Okuma *et al.*, 2011). These results support mechanism that GSH negatively regulates ABA- and MeJA-induced stomatal closure. However, EA treatment did not enhance ABA- or MeJA-induced stomatal closure (Fig. 4.2 C) although EA decreased intracellular GSH levels. There was a difference in GSH depletion between the EA and the PNBC or IDM-treated guard cells (Fig. 4.1 A). EA and its conjugate play also as an inhibitor of alpha-, mu-, and pi-class GSTs in animals and humans (Ploemen *et al.*, 1990), but, to our knowledge, neither PNBC nor IDM inhibits any class of GSTs. Hence this difference might result in differences in effects of the depletion of GSH on ABA- and MeJA-induced stomatal closure, suggesting that certain GST is involved in ABA- and MeJA-induced stomata closure.

PNBC and IDM slightly enhanced ABA inhibition of light-induced stomatal opening (Fig. 4.3A and B), suggesting that GSH depletion is involved in ABA inhibition of light-induced stomatal opening. In addition, EA did not enhance the inhibition of light-induced stomatal opening (Fig. 4.3 C). These results are in agreement with results of ABA-induced stomatal closure (Fig. 4.2 C).

In conclusion, depletion of GSH enhances ABA- and MeJA-induced stomatal closure without affecting early signal components, ROS production and cytosolic alkalization in guard cells.

## Summary

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Absciscic acid (ABA) and Methyl jasmonate (MeJA) induce stomatal closure *via* signal cascade which involves in redox regulation of protein and low-molecular-weight compound. Glutathione (GSH), one of the most abundant low molecular-weight thiol compounds that maintain redox homeostasis under normal and stressful conditions in plants. To clarify whether GSH is involved in redox regulation of stomatal closure in *Arabidopsis thaliana*, we employed ABA and MeJA induced stomatal closure using the GSH deficient mutants, the *chlorinal-1* (*chl-1*), which is defective in light harvesting protein of photosystem-II and *cad2-1*, which is deficient in the first GSH biosynthesis enzyme  $\gamma$ -glutamylcysteine synthetase, the NAD(P)H oxidase mutants, *atrbohD*, *atrbohF*, and *atrbohD atrbohF*, protein phosphatases 2C (PP2Cs) mutants, *abi1-1* and *abi2-1*, and the GSH decreasing chemicals, *p*-nitrobenzyl chloride (PNBC), iodomethane (IDM), and ethacrynic acid (EA).

Genetically depletion of GSH mutants i.e. *chl-1* and *cad2-1* plants accumulated significantly lower GSH than that of wild types did (chapter 2 and 3), and showed higher stomatal closure to MeJA than wild-type plants (chapter 2 and 3). In *chl-1* and *cad2-1* plants, glutathione monoethylester (GSHmee) restored GSH level (chapter 2 and 3) and reduced MeJA sensitivity to guard cells. Depletion or increment of GSH did not significantly increase or decrease the production of ROS accumulation in guard cells, cytosolic alkalization or cytosolic  $\text{Ca}^{2+}$   $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillation in guard cells. Application of 10  $\mu\text{M}$  MeJA and 10  $\mu\text{M}$  ABA induced stomatal closure and GSH depletion in the *atrbohD* and *atrbohF* single mutants but not in the *atrbohD atrbohF* double mutant, suggesting that AtRBOHD and

AtRBOHF redundantly regulate GSH depletion induced by MeJA and ABA in guard cells. In addition, MeJA-induced stomatal closure and GSH depletion was impaired in the *abi1-1* and *abi2-1* mutants. Based on these observations it has been proposed that ABI1 and ABI2 redundantly regulate stomatal closure and GSH depletion induced by MeJA in guard cells. Moreover, we examined whether exogenous ROS-induced stomatal closure is accompanied by GSH depletion in wild-type guard cells. In the wild-type plants, H<sub>2</sub>O<sub>2</sub> induces stomatal closure but did not deplete GSH in guard cells (chapter 3). These results suggest that intracellular GSH is not involved in ROS scavenging during stomatal closure in Arabidopsis.

On the other hand, in the presence of PNBC, IDM or EA, MCB dependent fluorescence was decreased in a larger amount in guard cells than PNBC, IDM or EA-untreated guard cells, indicating that MCB specificity to cytosolic GSH in guard cells not to other thiol or protein in guard cells. Our results showed that GSH decreases in guard cells are accompanied by MeJA and ABA induced stomatal closure (chapter 4). In wild-type guard cells, PNBC, IDM or EA decrease GSH in guard cells, and depletion of GSH by PNBC and IDM enhances sensitivity to MeJA- and ABA-induced stomatal closure (chapter 4), whereas EA did not show sensitivity to MeJA- and ABA-induced stomatal closure.

Stomatal opening provides access to inner leaf tissues for many pathogens, so narrowing stomatal apertures may be advantageous for plant defense. Treatment with ABA at 1  $\mu$ M significantly inhibited light-induced stomatal opening in the wild type. PNBC and IDM enhanced inhibition of light-induced stomatal opening by ABA in a dose dependent manner in Arabidopsis wild-type plants, suggesting that GSH depletion is involved in ABA inhibition of light-induced stomatal opening. However, EA did not

enhance the inhibition of light-induced stomatal opening. These results are in agreement with results of ABA-induced stomatal closure (chapter 4).

Environmental stresses induced oxidative damage *via* ROS production in plants. Under oxidative stresses, GSH acts as an antioxidant in rice plants and tobacco BY-2 cells. Both ABA and MeJA increased ROS and lead to a decreased in GSH in rice and wheat plants. However, the mechanisms of GSH depletion in guard cells by ABA and MeJA are different from other cells. In Arabidopsis, depletion of GSH by conjugation to MCB occurs in the cytosol but not in chloroplasts; suggest that GSH depletion in guard cells is related to cytosolic ROS accumulation but not chloroplastic ROS accumulation. Therefore, GSH regulate ROS-related and redox-sensitive signal transduction factors.

In conclusion, GSH negatively regulates ABA- and MeJA-induced stomatal closure *via* modulation of common signal components other than early signal components, ROS production,  $[Ca^{2+}]_{\text{cyt}}$  elevation, and cytosolic alkalization.

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## **Acknowledgements**

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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.

It is with a deep sense of respect and heartfelt gratitude that I extend my unreserved indebtedness to my supervisor, Yoshiyuki Murata, PhD, Professor, Faculty of Agriculture, Okayama University, Japan. His expert guidance, advice and genuine assistance in all aspects of my studies have been very helpful to my course of study.

I would like to express my sincere thanks to Dr. Yoshimasa Nakamura for his cordial suggestions, constructive criticism and guidance during my study. I am thankful to Dr. Yoshinobu Kimura to critically assess the thesis. My enormous thanks go to Dr. Shintaro Munemasa and Dr. Misugi Uraji for their help and valuable advice at the beginning of my research period. I also express my sincere appreciation and thanks to all members of the lab.

I am grateful to the JASSO foundation for giving me the JASSO scholarship during my research period.

I would like to dedicate this thesis to my beloved parents.

I am grateful to my beloved husband Dr. Muhammad Abdus Sobahan and son Md. Farhan Sadiq (Ornab) for sharing my feelings, and for their special sacrifices, continuous cooperation and encouragement during the study period.

I would like to express my heartfelt appreciation to my brother, sisters and all relatives for their prayers and wishes.

## Journal Articles

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- I Akter N, Sobahan MA, Hossain MA, Uraji M, Nakamura Y, Mori IC, Murata Y (2010)** The involvement of intracellular glutathione in methyl jasmonate signaling in *Arabidopsis* guard cells. *Biosci. Biotechnol. Biochem.* **74 (12)**, 2504-2506.
- II Akter N, Okuma E, Sobahan MA, Uraji M, Munemasa S, Nakamura Y, Mori IC, Murata Y (2012a)** Negative regulation of methyl jasmonate-induced stomatal closure by glutathione in *Arabidopsis*. *J Plant Growth Regul.* DOI 10.1007/s00344-012-9291-7. In Press.
- III Akter N, Sobahan MA, Uraji M, Wenxiu Ye, Hossain MA, Nakamura Y, Mori IC, Murata Y (2012b)** Effects of glutathione depletion on abscisic acid- and methyl jasmonate-induced stomatal closure in *Arabidopsis thaliana*. *Biosci. Biotechnol. Biochem.* BBB-120384. In press.