Stomatal Response to Isothiocyanates in *Arabidopsis thaliana*

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Stomatal Response to Isothiocyanates in *Arabidopsis thaliana*

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Chapter 1

General Introduction

1.1 Stomata and guard cells

Stomata (singular stoma) are the microscopic pores (openings or apertures) formed by pairs of specialized parenchyma cells known as guard cells. Stomata are located in the epidermis of leaves and stems including other aerial parts of most of the land plants. Usually, more stomata are present in the lower epidermis of leaves of the dicotyledon plants, whereas a same number of stomata are present on the both epidermis (upper and lower) of monocotyledons. In general, aquatic plants have stomata on the upper epidermis of the floating leaves but entirely lacks in the submerged leaves. Guard cells respond to a variety of abiotic and biotic stimuli such as light, drought, carbon dioxide, relative humidity, ozone, external calcium, phytohormones and elicitors, and regulates stomatal movements (Shimazaki et al., 2007; Melotto et al., 2008; Murata et al., 2015). Guard cells have become one of the well-developed model systems for elucidating dynamic signal transduction within a network in a single cell. The model plant Arabidopsis (*Arabidopsis thaliana*) is frequently used to study the mechanisms of stomatal movement, because of its short life cycle, small size, completely sequenced genome, and the availability of the mutants (The Arabidopsis Genome Initiative, 2000).

1.2 Second messengers in guard cell signaling

Second messengers are molecules that relay signals from receptors to the target molecules inside cells and serve to amplify the strength of signals. They can integrate information from multiple upstream inputs and can disintegrate to several

downstream targets, and thereby expanding the scope of signal transduction. Different types of second messengers such as ROS, Ca^{2+}, NO, cytosolic pH (pH_{cyt}), phosphatidic acid, cyclic GMP and cyclic ADP ribose are involved in guard cells signaling. Among them, ROS and Ca^{2+} are involved in many signaling pathways in guard cells and are well-studied (Khokon et al, 2011, Ye et al., 2013a, 2013b; Hossain et al., 2014). Here, I briefly described the role of ROS, cytosolic pH, and Ca^{2+} in guard cell signaling.

ROS are chemical species that are formed due to the partial reduction of oxygen, which includes H_2O_2, superoxide anion (O^{2-}), singlet oxygen and hydroxyl radical. Environmental and endogenous stresses elicit ROS production that is related to guard cell signaling. Mainly two types of enzymes such as plasma membrane NAD(P)H oxidases and cell wall peroxidase (class III peroxidases) catalyzed ROS generation. Plasma membrane NAD(P)H oxidases are responsible for ROS production in guard cell ABA signaling (Kwak et al., 2003). ROS production is crucial for signal integration between ABA signaling and other signaling in guard cells (Mori et al., 2009; Murata et al., 2015). AtrbohD and AtrbohF, two NADPH oxidases, catalyze superoxide production in the apoplasts in ABA and MeJA signaling (Kwak et al., 2003; Jannat et al., 2012), while apoplastic peroxidases are responsible for superoxide production in signaling in guard cells induced by isothiocyanates (ITCs) (Khokon et al., 2011a). Then H_2O_2 is generated through dismutation by unknown superoxide dismutase, which is accumulated in guard cells through diffusion and water channels (Henzler and Steudle, 2000; Bienert et al, 2007).

Cytosolic Ca^{2+} $[Ca^{2+}]_{cyt}$ functions as a key second messenger in signaling in guard cells (Pei et al., 2000; Roelfsema et al., 2010). Our understanding of Ca^{2+} signaling in guard cells has greatly advanced after the discovery of live guard cell

imaging based on a Ca^{2+}-sensing fluorescent protein, yellow cameleon (Nagai et al., 2004). Stomatal closure responds to a specific pattern of elevation of free $[Ca^{2+}]_{cyt}$ to close (Allen et al., 2000). Ca^{2+} influx is essential for the elevation of free $[Ca^{2+}]_{cyt}$ in guard cells induced by stimuli (Klusener et al., 2002; Siegel et al., 2009). In Arabidopsis, many proteins (about 250 proteins) possibly having the Ca^{2+}-binding EF-hand motif (Day et al., 2002), which transduce Ca^{2+} signals to downstream targets.

Increment of cytosolic pH (alkalization) in guard cells is a common phenomenon in both ABA- and MeJA-induced stomatal closure in Arabidopsis, *Pisum* and *Paphiopedilum* (Irving et al., 1992; Gehring et al., 1997; Suhita et al., 2004; Gonugunta et al., 2008; Islam et al., 2010). It has been reported that ABA elevates $[Ca^{2+}]_{cyt}$ in accordance with increment of pH_{cyt} in *Paphiopedilum* guard cells and Arabidopsis guard cells (Irving et al., 1992; Islam et al., 2010). Cytosolic alkalization activates outward K^+ currents and inactivates inward K^+ currents to promote net efflux of K^+ in *Vicia* guard cells (Blatt and Armstrong, 1993). Grabov and Blatt (1997) showed that cytosolic acidification activates inward K^+ currents but does not significantly change $[Ca^{2+}]_{cyt}$. These results indicate that changes in pH_{cyt} are closely involved in the modulation of ion mobilization to lead stomatal movement.

1.3 Isothiocyanates

Isothiocyanates (ITCs) are biodegradation products found in several plants belonging to the *Brassicaceae* family, and are produced upon disruption of the tissue by herbivores or pathogens. Members of the *Brassicaceae* family include several vegetables commonly consumed by humans such as broccoli, brussels sprouts, watercress, wasabi, cauliflower and mustard. ITCs are stored as their biologically inert precursor glucosinolates that is physically separated from the hydrolyzing

enzyme myrosinase. Upon damage to the cells both substrate and enzyme is released and the subsequent hydrolysis of glucosinolate yields several products including ITCs (Halkier and Gershenzon, 2006; Kissen and Bones, 2009). Being volatile and exerting a strong and pungent taste, ITCs are meant to repel herbivores (Lambrix et al., 2001; Yan and Chen, 2007). In addition, ITCs are highly reactive, making them an efficient countermeasure against pathogens at the site of the wound (Olivier et al., 1999). ITCs are composed of an –N=C=S reactive group linked to an R moiety dictating other physiochemical properties. Previously studied examples include the aliphatic allyl ITC (AITC) and sulforaphane (SFN), and the aromatic benzyl ITC (BITC), and phenethyl ITC (PEITC).

1.4 Physiological roles of ITCs

ITCs are able to antagonize multiple targets due to an electrophilic central C-atom in the reactive part, which can conjugate with any accessible thiol group. In addition to cysteine containing proteins, the abundantly present GSH presents an inevitable target for ITCs as demonstrated with AITC (Zhang et al., 1995). Activity of ITCs in plants has also been reported. Previous reports of ITCs or ITC-deriving extracts in weed control have shown promising results (Haramoto and Gallandt, 2005; Norsworthy et al., 2006). ITC-induced growth inhibition has been reported in wheat, velvet leaf, palmer amaranth, lettuce and the model plant *A. thaliana* (Norsworthy and Meehan, 2005; Hara et al., 2010). More recently, a physiological role for ITCs in defense has been proposed. A recent report showed that exposure of *A. thaliana* to AITC led to a stomatal closure, possibly to prevent water loss and intrusion of microorganisms trough stomata (Khokon et al., 2011). Moreover, a role in protection against heat stress has also been reported (Hara et al., 2013). To fully understand

the presumable physiological purpose of ITCs, more knowledge about the molecular events initiated in plant cells by ITCs is required. In *A. thaliana*, ITCs results in disintegration of microtubules and upregulation of stress-related genes (Øverby et al., 2015). Furthermore, exposure of *A. thaliana* to AITC results in reduction of actin-mediated intracellular transport (Sporsheim et al., 2015). Moreover, AITC was able to induce a shift in the cell cycle distribution in *A. thaliana* (Åsberg et al., 2015).

1.5 AITC-induced stomatal closure

AITC induced stomatal closure in Arabidopsis accompanied by ROS and nitric oxide (NO) production as second messengers. At higher than 1 mM, AITC shows cytotoxicity on Arabidopsis (Hara et al., 2010), whereas even 10 μM AITC significantly induced stomatal closure (Khokon et al., 2011). Both ROS and NO are second messengers which function in ABA and MeJA signaling to lead stomatal closure (Bright et al., 2006; Munemasa et al., 2007). AITC also elicited ROS and NO production (Khokon et al., 2011), suggesting that AITC employs parts of ABA- and MeJA-signal pathway to induce stomatal closure.

Calcium mobilization is one of key signal components in ABA and MeJA signaling in guard cells. It is well known that ABA and MeJA elicit $[Ca^{2+}]_{cyt}$ elevations following ROS production, resulting in stomatal closure (Munemasa et al., 2007; Hossain et al., 2011). AITC elicits $[Ca^{2+}]_{cyt}$ oscillations in guard cells like ABA and MeJA (Khokon et al., 2011). These results indicate that AITC induces stomatal closure via $[Ca^{2+}]_{cyt}$ elevations, that is, AITC induces stomatal closure via Ca^{2+}-dependent pathway along with ROS production.

GSH is closely concerned with ROS homeostasis and redox status (Noctor & Foyer 1998), and GSH can enzymatically and non-enzymatically react with ITCs

(Zhang et al., 1995). The pretreatment with GSHmee completely inhibited AITC-induced stomatal closure (Khokon et al., 2011), and treatment with GSHmee increases intracellular GSH contents in Arabidopsis guard cells (Jahan et al., 2008). Moreover, AITC decreased intracellular GSH level (Khokon et al., 2011). Recently, we have reported that ABA and MeJA decreased intracellular GSH contents in guard cells and that depletion of intracellular GSH enhanced ABA- and MeJA-induced stomatal closure (Jahan et al. 2008; Akter et al., 2010). These results suggest that AITC react with certain intracellular components to trigger AITC signaling and affect stomatal aperture via AITC-induced GSH depletion.

AITC induces stomatal closure accompanied by cytosolic Ca^{2+} elevation and the AITC-induced stomatal closure requires MeJA priming. AITC and MeJA cooperatively induce stomatal closure, for example, when plants are wounded. Moreover, when attacked by insects and herbivores, crucifer plants can biosynthesize ITCs as degradation products of glucosinolates. AITC induces stomatal closure along with MeJA priming and that ITCs do not only repel insects and herbivores but also induce stomatal closure to suppress water loss caused by their bite.

Overall, AITC induces stomatal closure in Arabidopsis via production of ROS and NO and elevation of cytosolic Ca^{2+}. Moreover, AITC-induced stomatal closure requires MeJA priming but not ABA priming, and AITC signaling shares some signal components with ABA signal and MeJA signaling.

1.6 Purpose of the study

The objectives of this study are as follows:

- To understand the cellular effects of different ITCs (SFN, AITC, BITC, and PEITC.) in guard cells and shed light on the role of these ITCs in guard cell signaling.
- To clarify the roles of different second messengers such as ROS accumulation, reactive carbonyl species (RCS) accumulation, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ spike in ITC-induced guard cell signaling in *A. thaliana*.
- To understand the possible role of GSH in ITC-induced stomatal closure and the underlying mechanism of ITC-induced GSH depletion.
- To identify the determinant of the extent of sotmatal closure in response to ITCs.

CHAPTER 2

Role of different isothiocyanates on stomatal signaling in *Arabidopsis thaliana*

2.1 Abstract

Allyl isothiocyanate (AITC) induces stomatal closure accompanied by reactive oxygen species (ROS) production and glutathione (GSH) depletion in *Arabidopsis thaliana*. In this study, stomatal responses to three other isothiocyanates (ITCs), benzyl isothiocyanate (BITC), sulforaphane (SFN), and phenethyl isothiocyanate (PEITC), in *A. thaliana* were investigated. All these ITCs significantly induced stomatal closure, where PEITC and BITC were most effective. The selected ITCs also induced ROS accumulation, cytosolic alkalization, and GSH depletion in guard cells. Moreover, all ITCs increased the frequency of cytosolic free calcium ($[Ca^{2+}]_{cyt}$) spike (transient elevation) while the PEITC and BITC showed the highest frequency. There was a strong positive correlation between the number of $[Ca^{2+}]_{cyt}$ spike per guard cell and the decrease in stomatal aperture. The cytosolic alkalization and GSH contents have a positive correlation with the decrease in stomatal aperture but the ROS production did not have a significant correlation with the decrease in stomatal apertures. These results indicate that the molecules functionalized with an ITC group induce stomatal closure that is accompanied by GSH depletion, cytosolic alkalization, $[Ca^{2+}]_{cyt}$ spike, and ROS production and that the GSH contents, cytosolic alkalization, and the number of $[Ca^{2+}]_{cyt}$ spike rather than ROS production are highly correlated with the decrease in stomatal aperture.

2.2 Introduction

Isothiocyanates (ITCs) are produced by the hydrolysis of glucosinolates in crucifer plants, which is catalyzed by myrosinases (Narbad and Rossiter, 2018). In intact leaves, a very small amount of ITCs is detected because glucosinolates and myrosinases are stored separately in the plant cells (Wittstock *et al.*, 2016). However, a large amount of ITCs is produced rapidly when the leaves are damaged by insects or herbivores as glucosinolates come in contact with myrosinases (Sugiyama and Hirai, 2019; Parchem *et al.*, 2020). This glucosinolate-myrosinase system has been found mainly in *Brassicaceae* family plants (Ishida *et al.*, 2014).

Degradation products of glucosinolates via glucosinolate-myrosinase system have repellent effects on many herbivores (Halkier and Gershenzon, 2006). Arabidopsis can biosynthesize a variety of glucosinolates including 2-propenylglucosinolate, 2-phenylethylglucosinolate, and 4-(methylsulfiny) butylglucosinolate (Fahey *et al.*, 2001), which are degraded to allyl ITC (AITC), phenethyl ITC (PEITC), and sulforaphene (SFN), respectively. In addition, benzylglucosinolate is converted to benzyl ITC (BITC), which has been found as a reactive compound to inhibit animal cell growth (Miyoshi *et al.*, 2008).

Isothiocyanates can readily react with nucleophiles in the cells because the carbon atom of isothiocyanate group is highly electrophilic (Kaschula and Hunter, 2016). The electrophilicity of ITCs is dictated by the side groups of the ITCs (Von Weymarn *et al.,* 2007). Isothiocyanates exhibit various effects on the physiological processes of plants (Urbancsok *et al.*, 2017). For instance, SFN induced cell death as an herbicide in *Abutilon theophrasti* (Brinker and Spencer, 1993) and *Arabidopsis thaliana* (Andersson *et al.*, 2015), and gaseous ITCs including AITC inhibited growth through disruption of microtubules in *A. thaliana* (Øverby *et al.*, 2015*a*). Spraying of

high doses of AITC or PEITC (≥10 mM) inhibited plant growth and caused severe chlorophyll degradation, whereas, lower dose (1 mM PEITC) enhanced glutathione *S*-transferase expression of *A. thaliana* (Hara *et al.*, 2010). Moreover, treatment with AITC led to a substantial decrease of plant growth in a dose-dependent manner (Urbancsok *et al.*, 2017). Furthermore, application of several ITCs (up to 5 mM) enhanced the heat tolerance of *A. thaliana* (Hara *et al.*, 2013). However, the ITC signaling in plant cells remains to be elucidated.

Tiny pores surrounded by a pair of guard cells are located mainly in the lower epidermis of plant leaves and are known as stomata. Several recent reports confirmed that AITC induced stomatal closure in the glucosinolate-producing *A. thaliana* (Khokon *et al.*, 2011*a*; Hossain *et al.*, 2013) as well as in the glucosinolate-nonproducing *Vicia faba* (Sobahan *et al.*, 2015) at physiological concentrations. Moreover, one recent study reported that SFN also induced stomatal closure in A. thaliana (Montillet *et al.* 2013) though no information was provided about signaling events. The content of ITCs in the ground leaves of *A. thaliana* was approximately 10 μmol g^{-1} fresh weight (FW) (Khokon *et al.*, 2011*a*) and the content of ITCs in the ground cabbage sprouts was about 2 mg g^{-1} FW (Wang *et al.*, 2015), which indicate that ITCs contents can reach around 1 mM in ground leaves and up to 10 μM in certain damaged parts of leaves. The AITC-induced stomatal closure is accompanied by reactive oxygen species (ROS) production, which is mediated by activation of salicylhydroxamic acid (SHAM)-sensitive peroxidases in *A. thaliana* (Hossain *et al.*, 2013) and *V. faba* (Sobahan *et al.*, 2015). Moreover, in guard cells, both cytosolic alkalization and cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) spike (transient elevation of $[Ca^{2+}]_{cyt}$) are important signaling events in AITC-induced stomatal closure (Khokon *et al.*, 2011*a*; Sobahan *et al.*, 2015). However, to the best of my knowledge, there is no report on

details about stomatal closure mechanism induced by other ITCs besides AITC.

Glutathione (GSH) contents in *A. thaliana* guard cells decreased with stomatal closure induced by abscisic acid, ABA (Okuma *et al.*, 2011; Akter *et al.*, 2012) and methyl jasmonate, MeJA (Akter *et al.*, 2012).Glutathione-depletion chemicals enhanced ABA- (Okuma *et al.*, 2011; Akter *et al.*, 2012) and MeJA- (Akter *et al.*, 2012) induced stomatal closure. The application of cell permeable derivative of GSH, GSH monoethyl ester (GSHmee), suppressed MeJA-induced stomatal closure (Akter *et al.*, 2013) and phenocopied the stomatal response of GSH-deficient mutant in response to ABA (Jahan *et al.*, 2008; Okuma *et al.*, 2011) and MeJA (Akter *et al.*, 2013), where GSHmee is hydrolyzed by cytosolic esterases to release free intracellular GSH (Puri and Meister, 1983). Likewise, AITC induced GSH depletion during stomatal closure in *A. thaliana* (Khokon *et al.*, 2011*a*) and *V. faba* (Sobahan *et al.*, 2015) and GSHmee induced stomatal reopening in *V. faba* (Sobahan *et al.*, 2015). These results suggest that intracellular GSH functions as a negative regulator of AITC-induced stomatal closure as well as that of ABA- and MeJA-induced stomatal closure.

The GSH depletion did not affect ABA-induced ROS accumulation in *A. thaliana* guard cells (Okuma *et al.*, 2011) and exogenous application of H_2O_2 did not change GSH contents in *A. thaliana* guard cells (Akter *et al.*, 2013). These results suggest that both ROS accumulation and GSH depletion play crucial roles in ABA-induced stomatal closure even though there is no significant correlation between ROS accumulation and GSH depletion. However, it remains unclear whether ROS levels modulate degree of decrease in stomatal aperture induced by ITCs. In addition, how the downstream signaling components of ROS such as cytosolic alkalization or $[Ca^{2+}]_{cyt}$ spike regulate the degree of decrease in stomatal aperture induced by ITCs has not been demonstrated yet.

The ITC group of the ITCs can conjugate with thiol group of GSH, resulting in decrease of GSH contents when animal cells (Zhang, 2000, 2001) and plant cells (Øverby *et al.*, 2015*b*) were treated with ITCs. It was also demonstrated that accumulations of four ITCs − SFN, AITC, BITC, and PEITC in human cells were proportional to GSH conjugation reaction with the ITCs (Zhang, 2001). Likewise, GSH contents in guard cells proved to decrease during AITC-induced stomatal closure in plants (Khokon *et al.*, 2011*a*; Sobahan *et al.*, 2015) but it still remains unclear whether GSH contents regulate degree of decrease in stomatal aperture induced by ITCs. In this study, I investigated stomatal responses to four ITCs namely SFN, AITC, BITC, and PEITC. Furthermore, I determined ROS accumulation, cytosolic alkalization, GSH depletion, and $[Ca^{2+}]_{cyt}$ spike in the guard cells in order to clarify how these signaling events control the extents of ITCs-induced stomatal closure in *A. thaliana*.

2.3 Materials and Methods

2.3.1 *Plant materials and growth conditions*

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was grown on a soil mixture [vermiculite:soil, 1:1 (v/v)] in the growth chamber at 21 ± 2°C and 80 μmol m^{-2} s^{-1} light intensity under a 16-h-light/8-h-dark regime. The plants were watered with deionized water containing 0.1% (v/v) HYPONeX (Hyponex Japan, Osaka, Japan) once a week. Rosette leaves from 4- to 5-week-old plants were employed for all experiments.

2.3.2 *Measurement of stomatal aperture*

Stomatal aperture measurements were performed as described previously (Munemasa *et al.*, 2019). Briefly, the rosette leaves were floated with the abaxial

side down and incubated on stomatal bioassay solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 5.6) under light condition (80 μmol m^{-2} s^{-1}) for 2 h to open stomata. After that, dimethyl sulfoxide (DMSO) or SFN, BITC, AITC, or PEITC were added to the stomatal bioassay solution and incubated for another 2 h. The final concentrations of each ITC and DMSO on stomatal bioassay solution were 10 μM and 0.1%, respectively. The incubated leaves were shredded by a commercial blender (700BUJ, Waring Commercial, Torrington, Connecticut) and epidermal tissues were collected using a nylon mesh. At least 20 stomatal apertures were measured on each distinct experiment using WinRoof 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). Three independent experiments were performed. In each experiment, two leaves were collected from two independent plants and at least 20 stomatal apertures were measured.

2.3.3 Measurement of ROS production in guard cells

Production of ROS in guard cells was examined using a fluorescence dye of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Sigma, St Louis, MO) as described previously (Khokon et al., 2011a; Salam et al., 2013). Briefly, the detached leaves were shredded in the commercial blender and the resulting fragments were collected by a 100-μm pore nylon mesh. The epidermal fragments were soaked in the stomatal bioassay solution under light condition for 2 h and then were incubated in the bioassay solution supplemented with 50 μM H_2DCF-DA at room temperature under dark condition for 30 min. After that, the epidermal fragments were collected again using 100-μm pore nylon mesh and washed with the stomatal bioassay solution. The samples were resuspended in the assay solution and treated with 0.1% DMSO or 10 μM of SFN, BITC, AITC, or PEITC at room temperature under dark condition for

another 30 min. Then, fluorescence of guard cells was observed under a fluorescence microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with filter: OP-66835 BZ filter (excitation wavelength, 480/30 nm, absorption wavelength, 510 nm, and dichroic mirror wavelength, 505 nm). The captured fluorescence images were analyzed using ImageJ 1.52a (National Institutes of Health, Bethesda, MD, USA). Three independent experiments were performed. In each experiment, 4 or 5 leaves were collected from 4 or 5 independent plants and the fluorescence images from at least 20 guard cells were analyzed

2.3.4 Measurement of cytosolic alkalization in guard cells

Cytosolic pH of guard cells were monitored using a fluorescence dye BCECF-AM (3'-*O*-acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester; Dojindo, Kumamoto, Japan) as described previously (Islam et al., 2010). Epidermal tissues were isolated from 4-5 leaves with the commercial blender. The collected tissues were incubated for 2 h in the stomatal bioassay solution under light condition. After this incubation, the tissues were incubated in the bioassay solution supplemented with 20 μM of BCECF-AM for 30 min in the dark at room temperature to load BCECF-AM. Then the tissues were washed several times with assay buffer in order to remove excess dye. The dye-loaded epidermal fragments were treated with 0.1% DMSO or 10 μM of SFN, BITC, AITC, or PEITC for 20 min. Fluorescence images of guard cells were captured using a fluorescence microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with the following settings: excitation 480 nm and emission 535 nm. The captured fluorescence images were analyzed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, 4 or 5 leaves were collected from 4 or 5 independent plants and the fluorescence images

from at least 20 guard cells were analyzed.

2.3.5 Measurement of GSH contents in guard cells

Glutathione in guard cells was fluorometrically quantitated using monochlorobimane (MCB) (Okuma *et al.*, 2011). Briefly, the leaves were shredded and the resulting fragments were soaked into stomatal bioassay solution under light for 2 h. Then the fragments were treated with 0.1% DMSO or 10 μM of SFN, BITC, AITC, or PEITC, and 50 μM of MCB for 2 h at room temperature. Monochlorobimane reacts with GSH to form fluorescent glutathione *S*-bimane in guard cells. After washing the fragments to remove excess chemicals, fluorescence of guard cells was imaged using the fluorescent microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with filter: OP-66834 BZ filter (excitation wavelength 360/40 nm, absorption wavelength 460/50 nm, and dichroic mirror wavelength 400 nm). The captured fluorescence images were analyzed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, 4 or 5 leaves were collected from 4 or 5 independent plants and the fluorescence images from at least 20 guard cells were analyzed.

2.3.6 Measurement of guard cell $[Ca^{2+}]_{cyt}$

Changes in $[Ca^{2+}]_{cyt}$ in guard cells were observed using a Ca^{2+} sensor Yellow Cameleon 3.6 (YC3.6)-expressing wild-type plants as described previously (Hossain et al., 2011; Munemasa et al., 2011). The abaxial epidermal peels attached on a glass slide were incubated in the stomatal bioassay solution in growth chamber for 2 h to promote stomatal opening. After that, the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fluorescence intensities (F_{535} and F_{480}) of guard cells were measured under a fluorescence microscope (IX71, Olympus, Tokyo, Japan)

equipped with a dual-emission imaging system [W-View system; 440AF21 excitation filter, 445DRLP dichroic mirror and two emission filters, 480DF30 for CFP and 535DF25 for YFP; Hamamatsu Photonics, Hamamatsu, Japan] and a CCD camera (Hamamatsu ORCA-ER digital camera; Hamamatsu Photonics). The fluorescence intensities were ratiometrically analyzed using AQUA COSMOS software (Hamamatsu Photonics). Note that I used the same exposure time for both CFP and YFP. A leaf was collected from a plant and the fluorescence intensities of at least 2-3 guard cells on the leaf were recorded. For each treatment, at least 6 independent leaves were employed and fluorescence intensities of a total of 16-18 guard cells were analyzed.

For the analysis of $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{cyt}$ spikes were counted when changes in fluorescence ratios (F_{535}/F_{480}) were more than or equal to 0.1 from the baseline.

2.3.7 Determination of the partition-coefficient values and electron density of the carbon atom of the ITC group of ITCs

Partition-coefficient (log *P*) values were calculated using ‘molinspiration’ online services (http://www.molinspiration.com/cgi-bin/properties). The electron density of carbon atom of ITC group was calculated based on a software, Winmostar V6.004 (https://winmostar.com/en/index.php).

2.3.8 Statistical analysis

The significance of differences between mean values of stomatal apertures, ROS accumulation, cytosolic pH, and GSH contents were assessed by Tukey’s multiple comparison test and the significance of differences between frequency of $[Ca^{2+}]_{cyt}$ spike was by chi-squared (χ^2) test. The correlation analysis and visualization of

correlation matrix by plot were performed by R 3.6.1 using 'corrplot' package (Wei and Simko, 2017). I considered differences at the level of $P < 0.05$ as significant for Tukey's multiple comparison test and chi-squared test, at the level of coefficient of determination (R^2) ≥ 0.85, and at the level of correlation coefficient (r) ≥ 0.90 as significant.

2.4 Results

2.4.1 ITCs induce stomatal closure

Both AITC and SFN significantly induced stomatal closure (Fig. 2.1), in agreement with the previous results (Khokon et al., 2010; Montillet et al., 2013; Sobahan et al., 2015). Both BITC and PEITC like AITC and SFN significantly induced stomatal closure, where PEITC and BITC were most effective (Fig. 2.1). There were no significant differences in stomatal apertures among other treatment groups (SFN, BITC, and AITC) (Fig. 2.1).

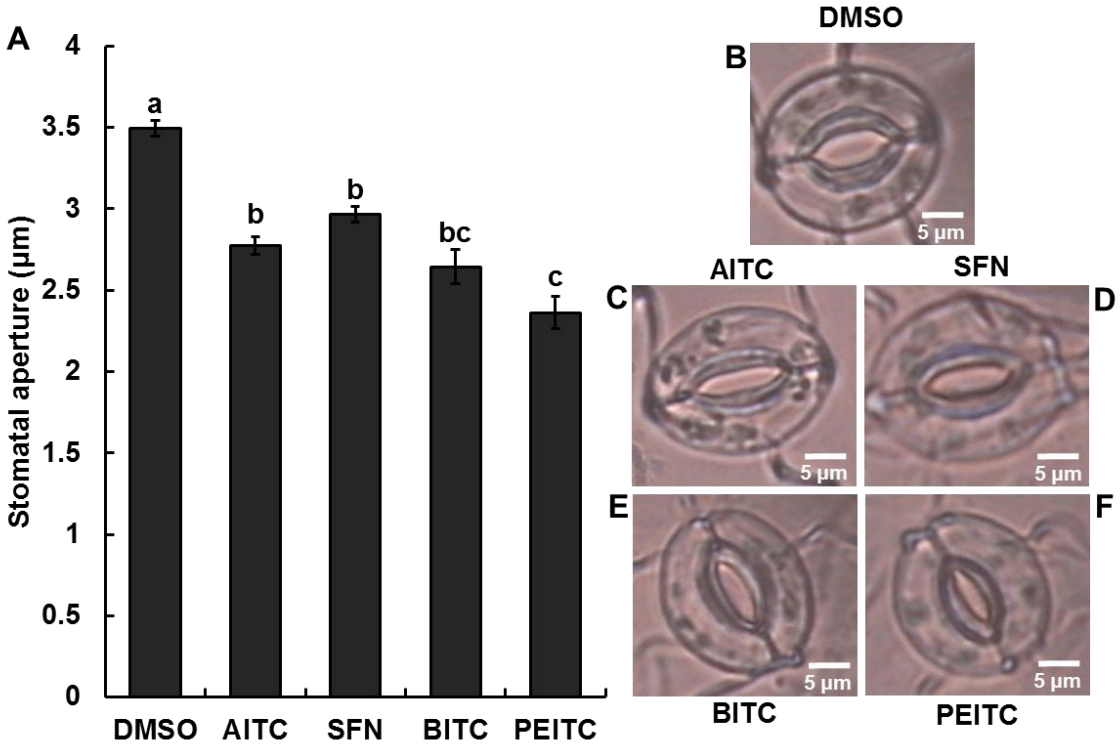

Fig. 2.1. Stomatal response to ITCs in *A. thaliana*.

The rosette leaves were incubated with 10 μM of AITC, SFN, BITC, or PEITC for 2 h. There are no effects of 0.1% DMSO as solvent on stomatal movement. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

2.4.2 ITCs induce ROS accumulation in guard cells

Allyl isothiocyanate (AITC) induced ROS accumulation in guard cells (Fig. 2.2), in agreement with the previous results (Khokon et al., 2011a; Hossain et al., 2013; Sobahan et al., 2015). The three ITCs, BITC, PEITC, and SFN, like AITC were found to induce ROS accumulation in guard cells (Fig. 2.2). There were no significant differences in ROS accumulation among all four ITCs (Fig. 2.2).

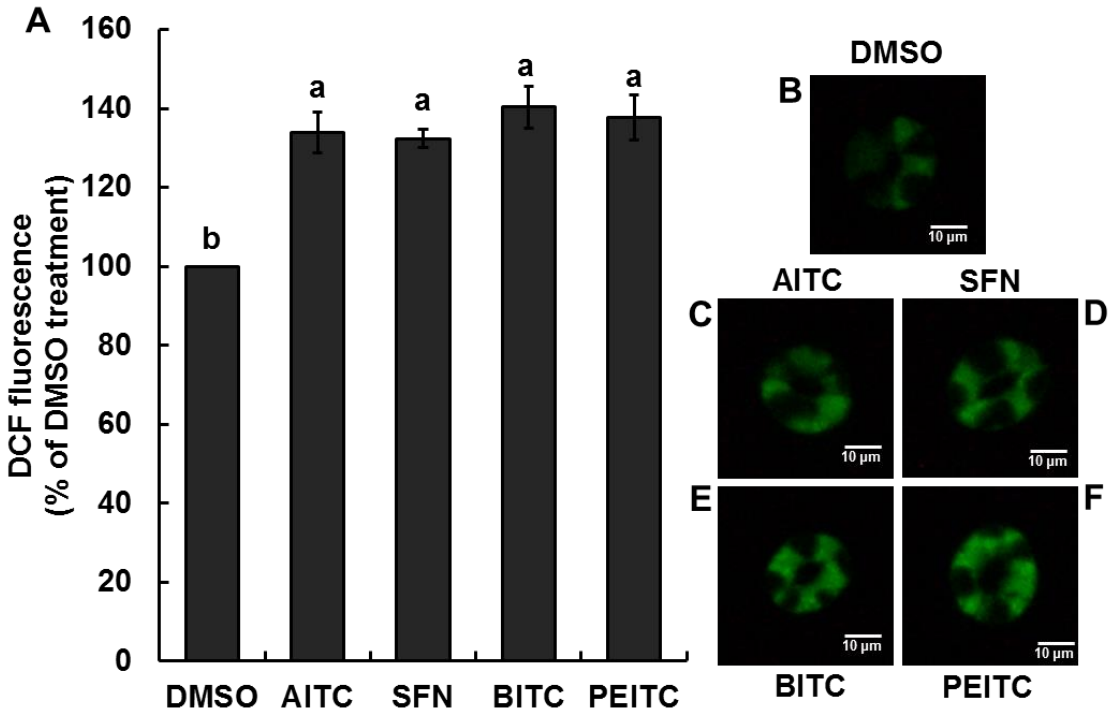

Fig. 2.2. ITC-induced ROS production in guard cells.

The leaf epidermal tissues were treated with 10 μM of AITC, SFN, BITC, or PEITC for 30 minutes after treatment of H_2DCF-DA. There are no effects of 0.1% DMSO as

solvent on ROS accumulation in guard cells. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

2.4.3 Effects of ITCs on cytosolic pH and GSH contents in guard cells

Allyl isothiocyanate (AITC) significantly increased BCECF fluorescence in *A. thaliana* guard cells (Fig. 2.3) as well as in *V. faba* guard cells (Sobahan et al., 2015). The other three ITCs, SFN, BITC, and PEITC significantly increased BCECF fluorescence intensity in the *A. thaliana* guard cells. The strongest BCECF fluorescence was emitted from the PEITC-treated guard cells (Fig. 2.3) and there were no significant differences in BCECF fluorescence among other treatment groups (SFN, BITC, and AITC) (Fig. 2.3).

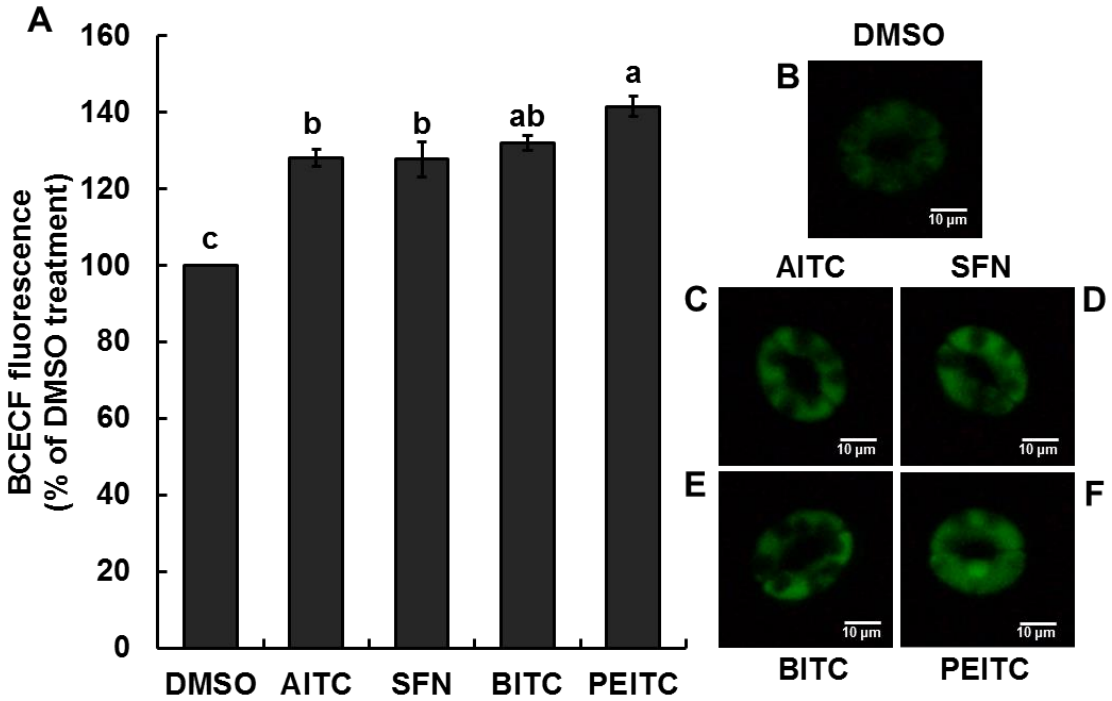

Fig. 2.3. ITC-induced cytosolic alkalization in guard cells.

The leaf epidermal tissues were treated with 10 µM of AITC, SFN, BITC, or PEITC for 30 minutes after treatment of BCECF-AM. There are no effects of 0.1% DMSO as

solvent on cytosolic alkalization in guard cells. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

Allyl isothiocyanate (AITC) significantly induced GSH depletion in *A. thaliana* guard cells (Fig. 2.4) as well as in *V. faba* guard cells (Sobahan et al., 2015). The GSH depletion in guard cells was significantly induced by SFN, BITC, and PEITC (Fig. 2.4) and PEITC exhibited prominent effect on GSH depletion, followed by BITC (Fig. 2.4).

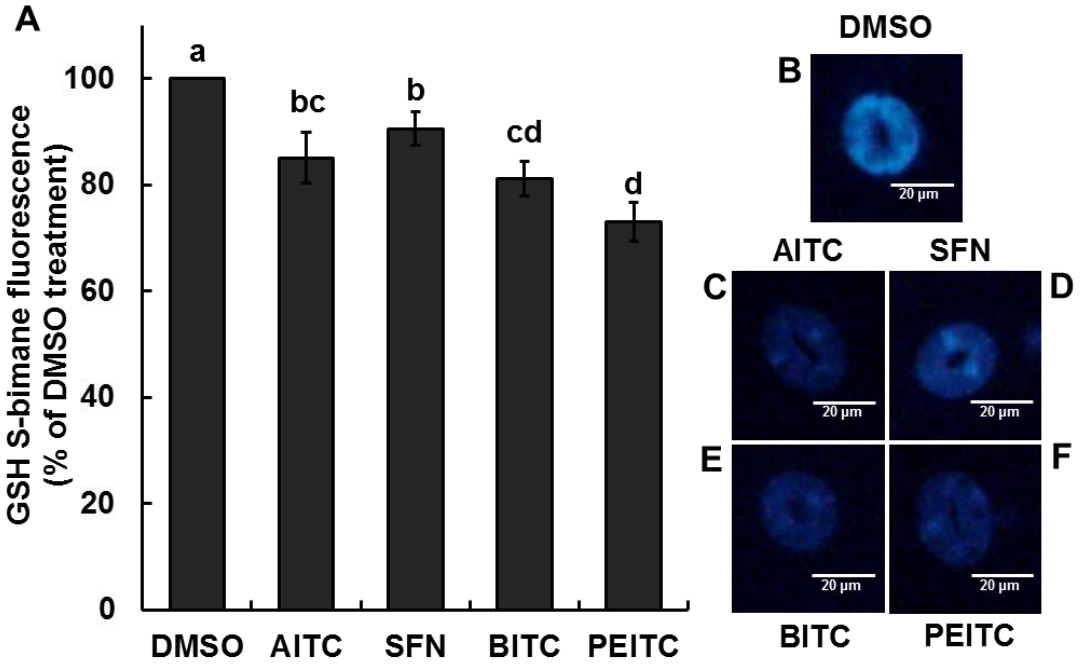

Fig. 2.4. Effects of ITCs on GSH contents in guard cells.

The leaf epidermal tissues were treated with 10 µM of AITC, SFN, BITC, or PEITC for 2 h in presence of a dye monochlorobimane. There are no effects of 0.1% DMSO as solvent on GSH contents in guard cells. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

2.4.4 Effects of ITCs on $[Ca^{2+}]_{cyt}$ in guard cells

When guard cells were treated with DMSO, only few cells (18.75%, n = 3 from 16 cells) showed $[Ca^{2+}]_{cyt}$ spikes (Fig. 2.5A). The AITC triggered $[Ca^{2+}]_{cyt}$ spikes in guard cells (62.5%, n = 10 from 16 cells) (Fig. 2.5B), in agreement with the previous results (Khokon et al., 2011a; Hossain et al., 2013; Sobahan et al., 2015; Ye et al., 2020). The $[Ca^{2+}]_{cyt}$ spikes were observed in the guard cells treated with SFN (77.7%, n = 14 from 18 cells), BITC (93.75%, n = 15 from 16 cells), and PEITC (93.75%, n = 15 from 16 cells) was more frequent than that in the DMSO-treated guard cells (Fig. 2.5C, D, E). The highest frequency of $[Ca^{2+}]_{cyt}$ spike was observed in the PEITC and BITC-treated guard cells, which was significantly higher than that in the SFN or AITC-treated guard cells (Fig. 2.5F).

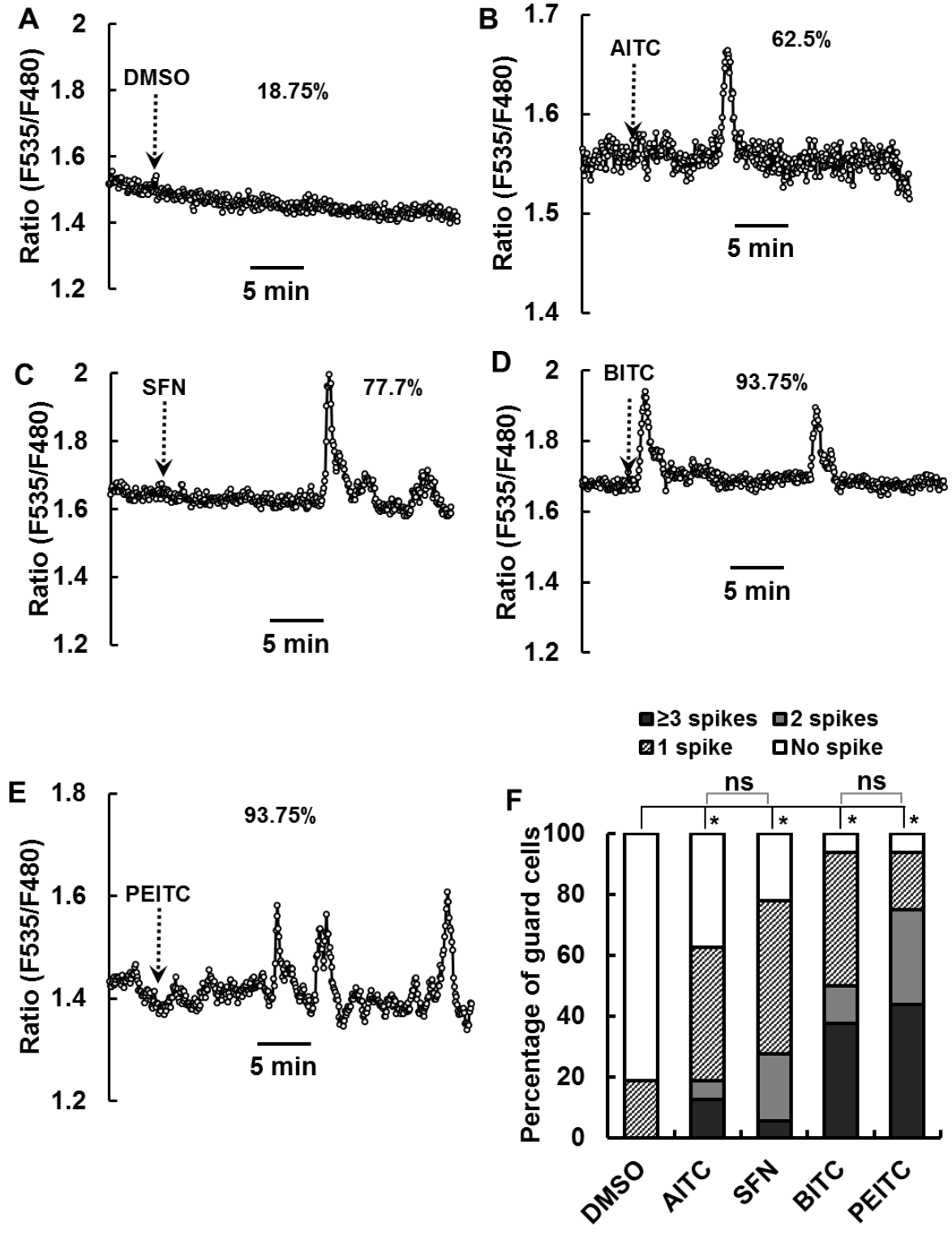

Fig. 2.5. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) in *A. thaliana* guard cells during different ITCs-induced stomatal closure.

The $[Ca^{2+}]_{cyt}$ in guard cells expressing Yellow Cameleon 3.6 was monitored. (A)

Treatment with 0.01% dimethyl sulfoxide, 3 of 16 (18.75%) guard cells showed $[Ca^{2+}]_{cyt}$ spike(s) (transient $[Ca^{2+}]_{cyt}$ elevation). Treatment with 10 μM (B) AITC, (C) SFN, (D) BITC, or (E) PEITC showed 62.65% (10 out of 16 guard cells), 77.7% (14 out of 18 guard cells), 93.75% (15 out of 16 guard cells) and 93.75% (15 out of 16 guard cells) $[Ca^{2+}]_{cyt}$ spike(s), respectively. (F) Percentage bar chart showing the number of $[Ca^{2+}]_{cyt}$ spike in wild-type guard cells treated with AITC, SFN, BITC, and PEITC. $[Ca^{2+}]_{cyt}$ spikes were counted when changes in fluorescence ratios (F_{535}/F_{480}) were more than or equal to 0.1 from the baseline. The significance of differences between frequency of $[Ca^{2+}]_{cyt}$ spike was by chi-squared ($\chi 2$) test. * indicates statistical significant compared with DMSO treatment ($P < 0.05$) and ns indicates no significant difference. Scale bars indicate 5 min.

2.4.5 Correlation analysis between signal events induced by ITCs and decrease in stomatal aperture

There was a negative and significant correlation between GSH contents in guard cells and decrease in stomatal aperture; the correlation coefficient (r) is -1.0 (P = 0.00004) (Figs 2.6A, 2.7). There was no significant correlation between ROS accumulation and decrease in stomatal aperture (r = 0.71, P = 0.294) (Figs 2.6B, 2.7) or between ROS accumulation and GSH depletion (r = -0.71, P = 0.290) (Figs 2.6C, 2.7) in guard cells. Furthermore, the levels of cytosolic alkalization and $[Ca^{2+}]_{cyt}$ spike per guard cell had a positive and significant correlation with decrease in stomatal aperture (r = 0.95, P = 0.050 and 0.96, P = 0.049, respectively) (Figs 2.6D, E; 2.7).

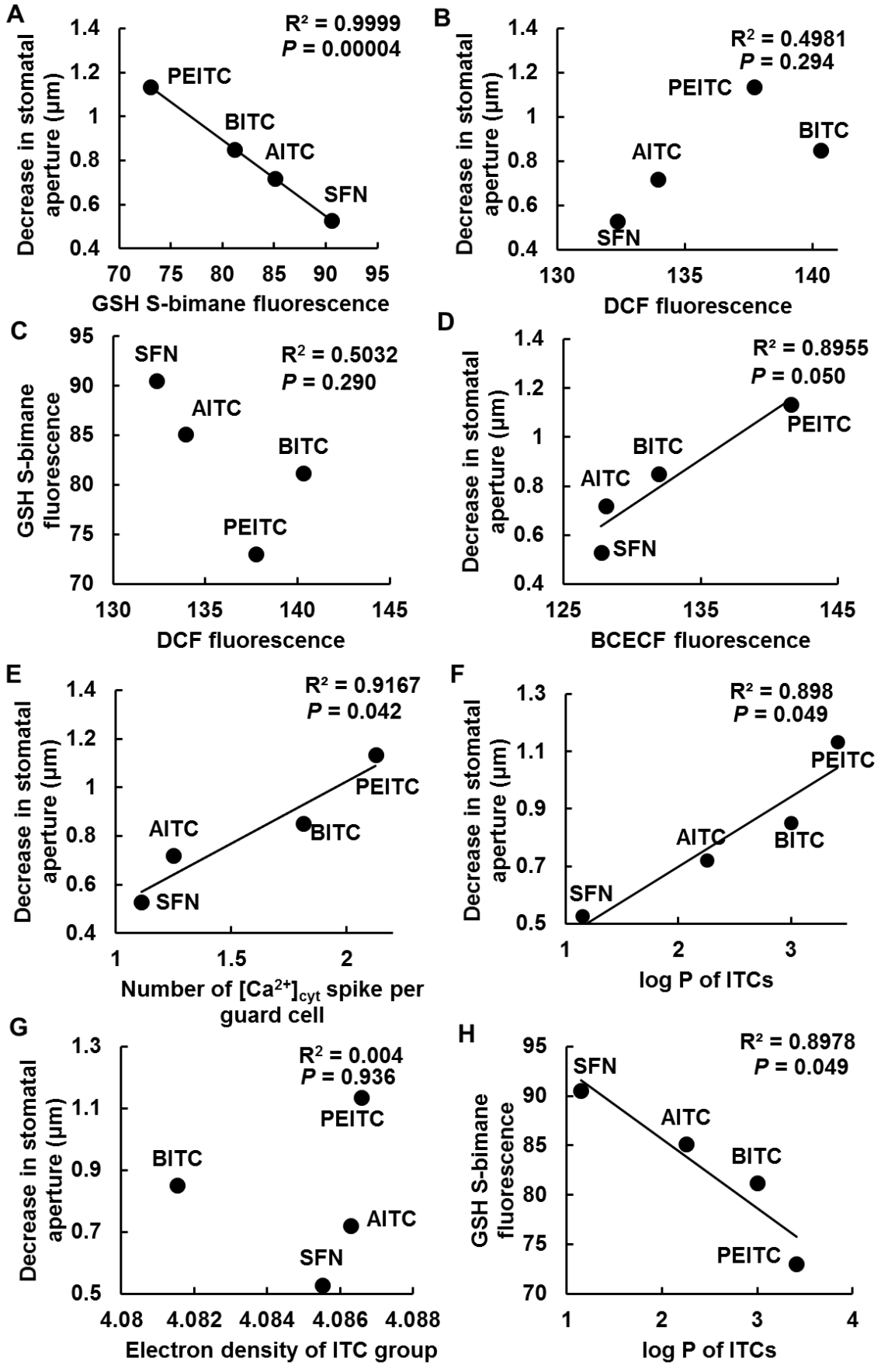
A
R² = 0.9999
P = 0.00004
PEITC
BITC
AITC
SFN
Decrease in stomatal aperture (μm)
GSH S-bimane fluorescence
B
R² = 0.4981
P = 0.294
PEITC
BITC
AITC
SFN
Decrease in stomatal aperture (μm)
DCF fluorescence
C
R² = 0.5032
P = 0.290
SFN
AITC
BITC
PEITC
GSH S-bimane fluorescence
DCF fluorescence
D
R² = 0.8955
P = 0.050
PEITC
BITC
AITC
SFN
Decrease in stomatal aperture (μm)
BCECF fluorescence
E
R² = 0.9167
P = 0.042
PEITC
AITC
BITC
SFN
Decrease in stomatal aperture (μm)
Number of [Ca2+]cyt spike per guard cell
F
R² = 0.898
P = 0.049
PEITC
AITC
BITC
SFN
Decrease in stomatal aperture (μm)
log P of ITCs
G
R² = 0.004
P = 0.936
PEITC
BITC
AITC
SFN
Decrease in stomatal aperture (μm)
Electron density of ITC group
H
R² = 0.8978
P = 0.049
SFN
AITC
BITC
PEITC
GSH S-bimane fluorescence
log P of ITCs

Fig. 2.6. Plots showing the relationship between two parameters.

Plots of (A) decrease in stomatal aperture (μm) *vs* GSH content in guard cells, (B) decrease in stomatal aperture (μm) *vs* DCF fluorescence in guard cells, (C) GSH content *vs* DCF fluorescence in guard cells, (D) decrease in stomatal aperture (μm) *vs* BCECF fluorescence in guard cells, (E) decrease in stomatal aperture (μm) *vs* number of $[Ca^{2+}]_{cyt}$ spike per guard cell, (F) decrease in stomatal aperture (μm) *vs* log *P* of ITCs, (G) decrease in stomatal aperture (μm) *vs* electron density of ITC group, and (H) GSH content *vs* log *P* of ITCs, which were treated with SFN, BITC, AITC, or PEITC. The degree of stomatal closure was calculated as follows: “decrease in stomatal aperture (μm)” = “stomatal aperture of DMSO-treated leaves (μm)” - "stomatal aperture of ITCs-treated leaves (μm)”. The number of $[Ca^{2+}]_{cyt}$ spike per guard cell was estimated by dividing the total number of $[Ca^{2+}]_{cyt}$ spikes of a treatment by the total number of guard cells of that treatment. Coefficient of determination (R^2) ≥ 0.85 was considered as statistically significant.

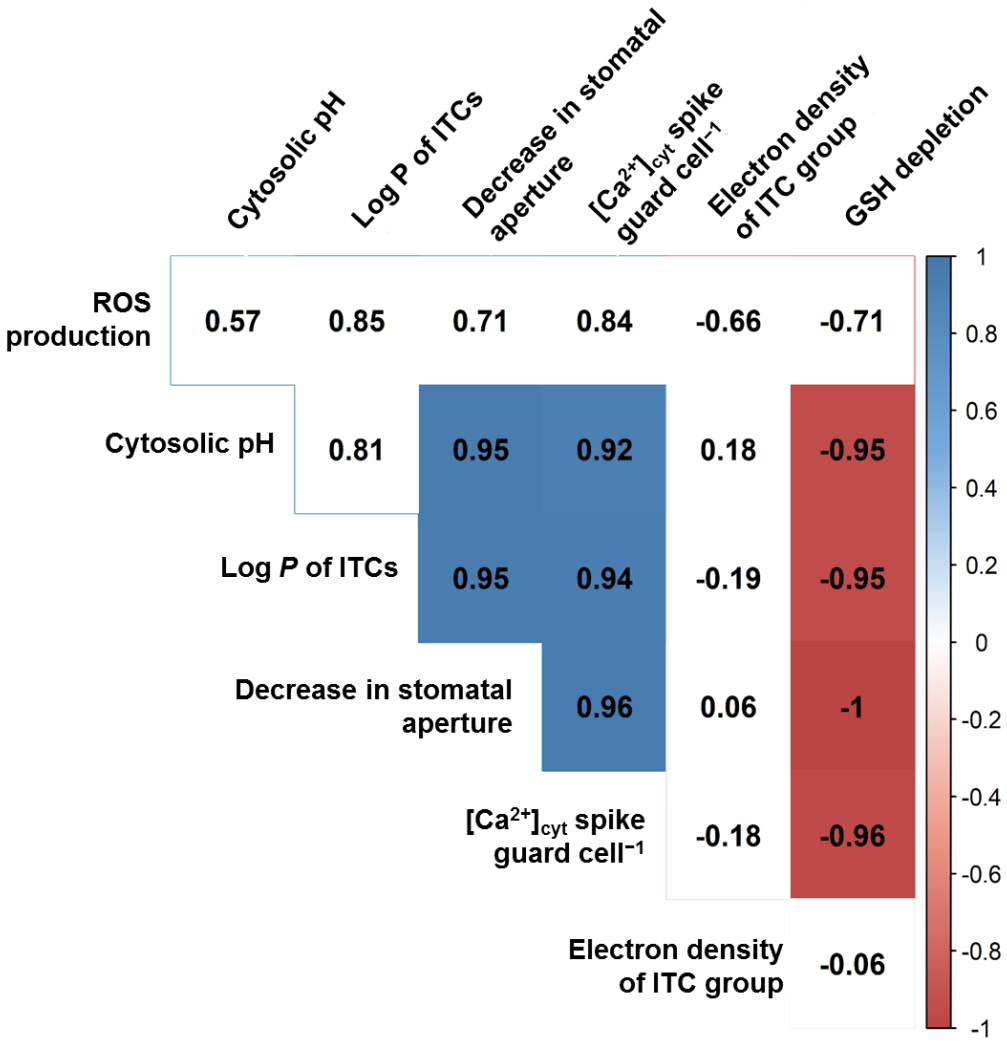

Fig. 2.7. The correlation plot showing the Pearson correlation coefficient matrix among all the selected parameters.

The color scale indicates the extent of positive or negative correlation. The color filled boxes indicates a significant correlation between two parameters at $P < 0.05$. pH_{cyt}, cytosolic pH; ROS, reactive oxygen species; GSH, glutathione; $[Ca^{2+}]_{cyt}$, cytosolic free calcium.

2.5 Discussion

Reactive oxygen species function as second messengers in stomatal closure induced by various stimuli such as ABA (Pei et al., 2000), MeJA (Munemasa et al., 2007), salicylic acid (Mori et al., 2001; Khokon et al., 2011*b*), and yeast elicitor (Khokon et al., 2010; Ye et al., 2013). In this study, SFN, BITC, AITC, and PEITC induced ROS accumulation in guard cells (Fig. 2.2), indicating that ITC-induced stomatal closure is accompanied by ROS production in agreement with the previous studies (Khokon et al., 2011a; Hossain et al., 2013; Sobahan et al., 2015). There are no significant differences in ROS accumulation among all four ITCs despite of the differences in degree of decrease in stomatal aperture (Figs 2.1, 2,2), which is further supported by the non-significant correlation (r = 0.71, P = 0.294) between ROS accumulation and decrease in stomatal aperture (Figs 2.6B, 2.7). These results suggest that ROS production is one of the necessary conditions but not a determinant of degree of decrease in stomatal aperture. Moreover, an increase of guard cell cytosolic pH is indispensable for stomatal closure induced by ABA (Irving et al., 1992; Gonugunta et al., 2009) and MeJA (Islam et al., 2010). Previously, it has been shown that AITC induced cytosolic alkalization in *V. faba* (Sobahan et al., 2015). In this study, SFN, BITC, and PEITC as well as AITC prompted the cytosolic alkalization in *A. thaliana* guard cells (Fig. 2.3). The outward rectifying potassium channels that are closely related to ABA-induced stomatal closure are activated by cytosolic alkalization (Blatt and Armstrong, 1993; Gonugunta et al., 2009) and there was a positive correlation between the cytosolic alkalization and decrease in stomatal aperture (r = 0.95, P = 0.050) (Figs 2.6D, 2.7). These results suggest that the outward rectifying potassium channels are activated by cytosolic alkalization on the ITC-induced stomatal closure.

In plant cells, $[Ca^{2+}]_{cyt}$ spikes occur in response to many stimuli and that dynamic $[Ca^{2+}]_{cyt}$ fluctuation regulates many physiological responses including stomatal closure (McAinsh et al., 1990; Allen et al., 2000, 2001; Pei et al., 2000; Siegel et al., 2009). The AITC-induced stomatal closure was accompanied by $[Ca^{2+}]_{cyt}$ spike in guard cells (Fig. 2.5B), in agreement with the previous studies (Khokon et al., 2011a; Hossain et al., 2013; Ye et al., 2020) and the stomatal closure induced by SFN, BITC, and PEITC was also accompanied by $[Ca^{2+}]_{cyt}$ spike in guard cells (Fig. 2.5C, D, E). The stomatal apertures decreased with a rise in the number of $[Ca^{2+}]_{cyt}$ spike per guard cell (Fig. 2.6E). In addition, the frequency of $[Ca^{2+}]_{cyt}$ oscillation in guard cells controls reduction of stomatal apertures (Allen et al., 2001; Yang et al., 2003). These results suggest that stomatal apertures are sophisticatedly controlled by $[Ca^{2+}]_{cyt}$ signature.

In this study, there was a significant negative correlation ($r = -1$, $P = 0.00004$) between GSH contents in guard cells and decrease in stomatal aperture (Figs 2.6A, 2.7), suggesting that GSH is consumed during stomatal closure, for example, to form glutathione conjugation with ITCs and signaling molecules such as reactive carbonyl species (RCS) (Øverby et al., 2015b; Yin et al., 2017; Mano et al., 2017). The previous studies demonstrated that AITC-induced stomatal closure is accompanied by GSH depletion in *A. thaliana* and *V. faba* and is suppressed by supplement of GSH with GSHmee treatment (Khokon et al., 2011a; Sobahan et al., 2015). In addition, ABA-induced stomatal closure and MeJA-induced stomatal closure are accompanied by GSH depletion and are enhanced in the GSH deficient mutants and the phenotypes are complemented with GSH treatment (Jahan et al., 2008; Okuma et al., 2011). Taken together, certain signaling reaction regulated by GSH may be a critical step to regulate stomatal apertures on stomatal closure.

In order to elucidate ITCs signaling in guard cells, I assessed the correlation between decrease in stomatal aperture in response to ITCs and log *P* values of the ITCs or electron densities of the carbon atom of the ITC group (Fig. 2.6F, G). The log *P* is the lipophilicity or hydrophobicity parameter of a molecule and is a crucial factor governing passive membrane partitioning. An increase in log *P* value of a particular compound enhances its permeability to plasma membrane and vice versa (Bennion et al., 2017). The central carbon atom of ITC group is highly electrophilic and can react with N-, O-, or S-based nucleophiles (Zhang et al., 1995). The higher electron densities of the carbon atom of the ITC group due to electron-releasing substituent/group can reduce its electrophilicity. In this study, the correlation coefficient is -0.95 (*P* = 0.049) for log *P* of ITCs and -0.06 (*P* = 0.936) for electron density of the carbon atom of the ITC group with the decrease in stomatal aperture (Figs 2.6F, G; 2.7). These results indicate that lipophilicity rather than electrophilicity of ITCs is closely related to the stomatal response. It implies that the membrane permeability of ITCs is favorable to ITC-induced stomatal closure and that ITCs interact with certain intracellular signal component to trigger stomatal closure. The highest-potential candidate of intracellular components is GSH because ITCs can directly conjugate with GSH (Zhang, 2000) and because ITC-induced stomatal closure is accompanied by intracellular GSH depletion (Fig. 2.4) (Sobahan et al., 2015). This argument can be further strengthened by the strong negative correlation (r = -0.95, *P* = 0.049) between log *P* values of ITCs and GSH contents in guard cells (Figs 2.6H, 2.7).

The stomatal closure requires activation/deactivation of ion transporters and channels such as potassium channels and anion channels (Schroeder et al., 1987; Pandey et al., 2007; Jezek and Blatt, 2017) and protein kinase such as CDPK (Li et

al., 1998; Yip Delormel and Boudsocq, 2019). In animal cells, ITCs can form a covalent binding with thiol group of protein (Mi et al., 2008). Hence, in plants, ITCs may react with the transporters, channels, and kinases and modulate their activities, resulting in stomatal closure.

It is concluded that ITCs induce stomata closure accompanied by ROS accumulation, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ spike in *A. thaliana*, that in signal events studies in this study, the decrease in GSH content is most tightly related to the decrease in stomatal aperture, that the increasing number of $[Ca^{2+}]_{cyt}$ spike in guard cells can enhance stomatal closure, and that ROS accumulation in guard cells may create a necessary environment for signaling to lead to stomatal closure.

Chapter 3

Involvement of RCS in the regulation of isothiocyanate signaling in Arabidopsis guard cells

3.1. Abstract

In this present study, I investigated the involvement of reactive carbonyl species (RCS) in ITC signaling in *A. thaliana* guard cells. I found that ITCs significantly increased RCS production in the *A. thaliana* guard cells. Application of PEITC induced stomatal closure and RCS scavenger slightly impaired PEITC-induced stomatal closure. Application of PEITC also induced cytosolic alkalization and GSH depletion in guard cells and RCS scavenger slightly reduced PEITC-induced cytosolic alkalization and GSH depletion. Together, these results suggests RCS is involved in ITC signaling in guard cells and that ITC-induced RCS can affect the cytosolic alkalization and GSH depletion in *A. thaliana* guard cells.

3.2. Introduction

Guard cells, which form stomatal pores in leaf epidermis of higher plants, respond to numerous biotic and abiotic signaling stimuli (Melotto et al., 2008). Many studies have shown that ABA, MeJA, and ITCs stimulate stomatal closure in many plant species (Irving et al., 1992, Blatt and Armstrong, 1993, Gehring et al., 1997, Suhita et al., 2004, Islam et al. 2010; Khokon et al., 2011).

Allyl isothiocyanate induced stomatal closure in *A. thaliana* (Khokon et al., 2011a; Hossain et al., 2013) and *Vicia faba* (Sobahan et al., 2015). The AITC-induced stomatal closure is accompanied by ROS production (Hossain et al., 2013; Sobahan

et al., 2015). Moreover, in guard cells, both cytosolic alkalization and cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) spike (transient elevation of $[Ca^{2+}]_{cyt}$) are important signaling events in AITC-induced stomatal closure (Khokon et al., 2011a; Sobahan et al., 2015). Moreover, AITC induced GSH depletion during stomatal closure in guard cells (Khokon et al., 2011a; Sobahan et al., 2015). The GSH depletion did not affect ABA-induced ROS accumulation in *A. thaliana* guard cells (Okuma et al., 2011) and exogenous application of H_2O_2 did not change GSH contents in *A. thaliana* guard cells (Akter *et al.*, 2013). These results suggest that both ROS accumulation and GSH depletion play crucial roles in ABA-induced stomatal closure even though there is no significant correlation between ROS accumulation and GSH depletion. However, it remains unclear how GSH depletion occurs during ITC-induced stomatal closure.

ROS are constitutively formed in cells and can oxidize lipids to their peroxides (Mène-Saffrané et al. 2007). Lipid peroxides decompose to form various aldehydes and ketones in the presence of redox catalysts such as transition metal ions or free radicals (i.e. carbonyls; Farmer and Mueller 2013). Among the lipid peroxide-derived carbonyls, the α,β-unsaturated carbonyls, such as acrolein and 4-hydroxy-(E)-2-nonenal (HNE), are termed reactive carbonyl species (RCS) because of their high reactivity (Mano 2012). There is a close relationship between the RCS level and damage in plants under stress conditions (Mano et al. 2010, Yin et al. 2010). However, it is not clear whether the RCS production is involved in ITC-induced stomatal closure. In this present study, I investigated the involvement of RCS in ITC signaling in *A. thaliana* guard cells.

3.3. Materials and methods

3.3.1. *Plant materials and growth conditions*

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was grown on a soil mixture [vermiculite:soil, 1:1 (v/v)] in the growth chamber at 21 ± 2°C and 80 μmol m^{-2} s^{-1} light intensity under a 16-h-light/8-h-dark regime. The plants were watered with deionized water containing 0.1% (v/v) HYPONeX (Hyponex Japan, Osaka, Japan) once a week. Rosette leaves from 4- to 5-week-old plants were employed for all experiments.

3.3.2. Measurement of ITC-induced RCS production in guard cells

RCS accumulation in guard cells in response to ITCs was monitored using a fluorescence dye AcroleinRED. Epidermal tissues were isolated from 4-5 leaves with the commercial blender. The collected tissues were incubated for 2 h in the stomatal bioassay solution under light condition. After that the epidermal fragments were treated with 0.1% DMSO or 10 μM of PEITC for 1.5 h. After this incubation, the tissues were incubated in the bioassay solution supplemented with 20 μM of AcroleinRED for 30 min in the dark at room temperature to load dye. Then the tissues were washed several times with assay buffer in order to remove excess dye. Fluorescence images of guard cells were captured using a fluorescence microscope with the following settings: excitation 560 nm and emission 585 nm. The captured fluorescence images were analyzed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, 4 or 5 leaves were collected from 4 or 5 independent plants and the fluorescence images from at least 20 guard cells were analyzed.

3.3.3. *Measurement of stomatal aperture*

Stomatal aperture measurements were performed as described previously (Munemasa et al., 2019). Briefly, the rosette leaves were floated with the abaxial side down and incubated on stomatal bioassay solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 5.6) under light condition (80 μmol m^{-2} s^{-1}) for 2 h to open stomata. After that, dimethyl sulfoxide (DMSO) or PEITC were added to the stomatal bioassay solution and incubated for another 2 h. The final concentrations of each ITC and DMSO on stomatal bioassay solution were 10 μM and 0.1%, respectively. The RCS scavenger, 1 mM carnosine was added 30 min prior to the PEITC application. The incubated leaves were shredded by a commercial blender (700BUJ, Waring Commercial, Torrington, Connecticut) and epidermal tissues were collected using a nylon mesh. At least 20 stomatal apertures were measured on each distinct experiment using WinRoof 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). Three independent experiments were performed. In each experiment, two leaves were collected from two independent plants and at least 20 stomatal apertures were measured.

3.3.4. Measurement of cytosolic alkalization in guard cells

Cytosolic pH of guard cells were monitored using a fluorescence dye BCECF-AM (3'-*O*-acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester; Dojindo, Kumamoto, Japan) as described previously (Islam et al., 2010). Epidermal tissues were isolated from 4-5 leaves with the commercial blender. The collected tissues were incubated for 2 h in the stomatal bioassay solution under light condition. After this incubation, the tissues were incubated in the bioassay solution supplemented with 20 μM of BCECF-AM for 30 min in the dark at room temperature

to load BCECF-AM. Then the tissues were washed several times with assay buffer in order to remove excess dye. The dye-loaded epidermal fragments were treated with 0.1% DMSO or 10 μM of PEITC for 30 min. The RCS scavenger, 1 mM carnosine was added 15 min prior to the PEITC application. Fluorescence images of guard cells were captured using a fluorescence microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with the following settings: excitation 480 nm and emission 535 nm. The captured fluorescence images were analyzed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, 4 or 5 leaves were collected from 4 or 5 independent plants and the fluorescence images from at least 20 guard cells were analyzed.

3.3.5. Measurement of GSH contents in guard cells

Glutathione in guard cells was fluorometrically quantitated using monochlorobimane (MCB) (Okuma *et al.*, 2011). Briefly, the leaves were shredded and the resulting fragments were soaked into stomatal bioassay solution under light for 2 h. Then the fragments were treated with 0.1% DMSO or 10 μM of PEITC, and 50 μM of MCB for 2 h at room temperature. The RCS scavenger, 1 mM carnosine was added 15 min prior to the PEITC application. After washing the fragments to remove excess chemicals, fluorescence of guard cells was imaged using the fluorescent microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with filter: OP-66834 BZ filter (excitation wavelength 360/40 nm, absorption wavelength 460/50 nm, and dichroic mirror wavelength 400 nm). The captured fluorescence images were analyzed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, 4 or 5 leaves were collected from 4 or 5 independent plants and the fluorescence images from at least 20 guard cells were analysed.

3.3.6. Statistical analysis

The significance of differences between mean values of stomatal apertures, RCS accumulation, cytosolic pH, and GSH contents were assessed by Tukey's multiple comparison test.

3.4. Results

3.4.1. Effects ITCs in RCS production in guard cells

The four ITCs, AITC, SFN, BITC, and PEITC significantly increased AcroleinRED fluorescence intensity in the *A. thaliana* guard cells. The strongest AcroleinRED fluorescence was emitted from the PEITC and BITC-treated guard cells (Fig. 3.1) and there were no significant differences in AcroleinRED fluorescence between AITC and SFN treatment groups (Fig. 3.1).

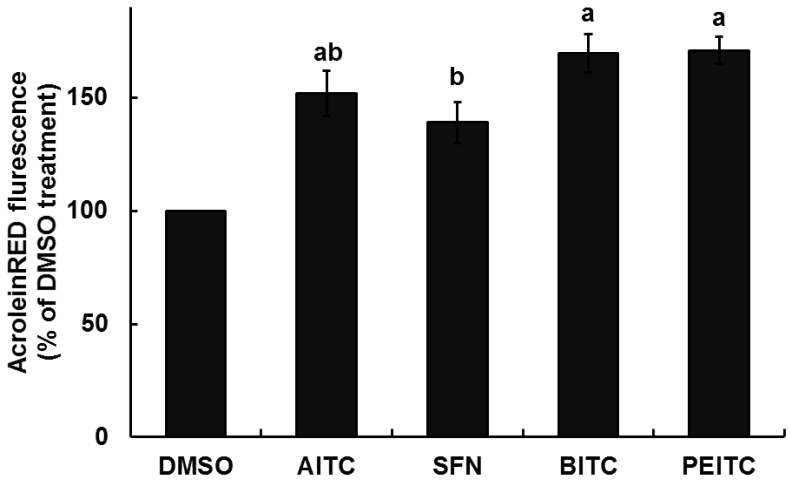

Fig. 3.1. ITC-induced RCS production in guard cells.

The leaf epidermal tissues were treated with 10 μM of AITC, SFN, BITC, or PEITC for 2 h after treatment of AcroleinRED. There are no effects of 0.1% DMSO as solvent on RCS accumulation in guard cells. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

3.4.2. Effects of RCS scavenger on PEITC-induced stomatal closure

Application of 10 μM PEITC induced stomatal closure in the *A. thaliana* (Fig. 3.2). The PEITC-induced stomatal closure was slightly reduced by 1 mM carnosine (Fig. 3.2). This results suggest that guard cells in *A. thaliana* responded to ITC-induced RCS production and exerted stomatal closure.

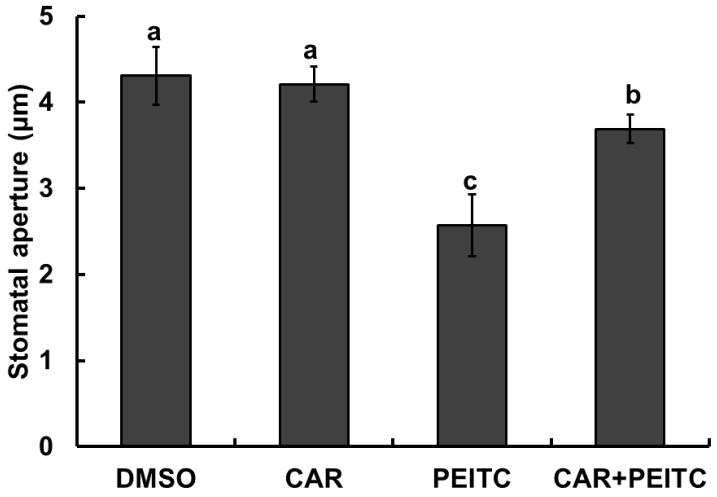

Fig. 3.2. Effects of RCS scavengers on PEITC-induced stomatal closure in *A. thaliana*.

The rosette leaves were incubated with 10 μM of PEITC for 2 h. The RCS scavenger, 1 mM carnosine (CAR) was added 30 min prior to the PEITC application. There are no effects of 0.1% DMSO as solvent on stomatal movement. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

3.4.3. Effects of RCS scavenger on PEITC-induced cytosolic alkalization

Application of 10 μM PEITC induced cytosolic alkalization in the *A. thaliana* guard cells (Fig. 3.3). The PEITC-induced cytosolic alkalization was slightly reduced by 1 mM carnosine (Fig. 3.3). This results suggest that ITC-induced RCS accumulation involve in cytosolic alkalization of *A. thaliana* guard cells.

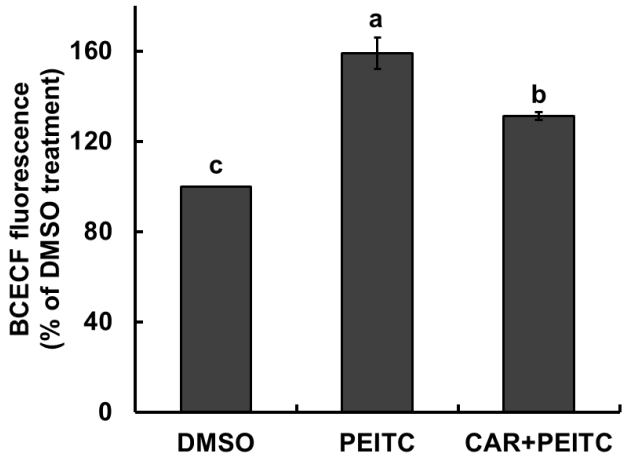

Fig. 3.3. Effects of RCS scavengers on PEITC-induced cytosolic alkalization in guard cells

The leaf epidermal tissues were treated with 10 μM of AITC, SFN, BITC, or PEITC for 30 minutes after treatment of BCECF-AM. The RCS scavenger, 1 mM carnosine (CAR) was added 15 min prior to the PEITC application. There are no effects of 0.1% DMSO as solvent on cytosolic alkalization in guard cells. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

3.4.4. Effects of RCS scavenger on PEITC-induced GSH depletion

Application of 10 μM PEITC reduced GSH content in the *A. thaliana* guard cells (Fig. 3.4). The PEITC-induced GSH depletion was slightly reduced by 1 mM carnosine treatment (Fig. 3.4). This results suggest that ITC-induced RCS accumulation involve in GSH depletion of *A. thaliana* guard cells.

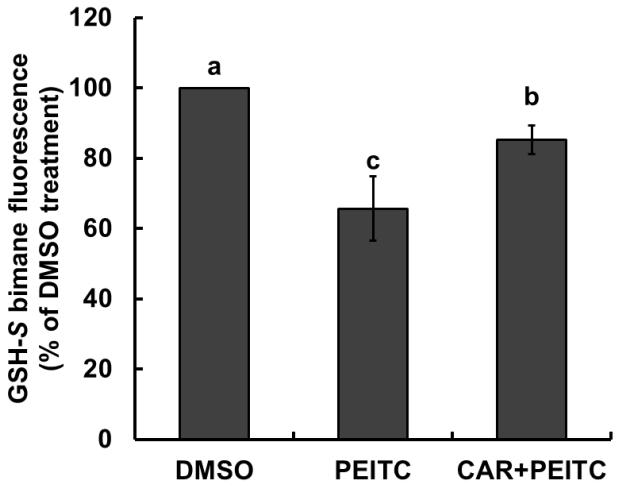

Fig. 3.4. Effects of RCS scavengers on PEITC-induced GSH depletion in guard cells

The leaf epidermal tissues were treated with 10 μM of PEITC for 2 h in presence of a dye monochlorobimane. The RCS scavenger, 1 mM carnosine (CAR) was added 15 min prior to the PEITC application. There are no effects of 0.1% DMSO as solvent on GSH contents in guard cells. Data are the mean ± standard error (n = three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P < 0.05$.

3.5. Discussion

Guard cells treated with ABA and MeJA accumulated a significant amount of RCS (Islam et al., 2016; 2020). RCS function as signal mediators downstream of H_2O_2 production in the ABA and MeJA signaling pathway in guard cells (Islam et al., 2016; 2020). In this study, I found that a significant amount of RCS was accumulated in guard cells treated with different ITCs, such as AITC, SFN, BITC, PEITC (Fig. 3.1). This result indicates that as like as ABA and MeJA signaling, ITC signaling in guard cells also involved with RCS production in *A. thaliana*. Furthermore, the PEITC-induced stomatal closure was delayed when guard cells were treated with RCS

scavenger (Fig. 3.2), suggesting that RCS is a signal mediator of ITC signaling in guard cells that regulates the ITC-stomatal closure to a certain extent.

Both ABA (Okuma et al., 2011; Akter et al., 2012) and MeJA (Akter et al., 2013) also decrease GSH contents in guard cells but it is unlikely that ABA or MeJA conjugates with GSH because ABA and MeJA are not as electrophilic as ITCs. Thus, ABA- and MeJA-induced GSH depletion might be triggered by other mechanism(s) such as ROS production and/or RCS production. Reactive oxygen species have certain indirect effect on GSH depletion by producing RCS in guard cells. Reactive carbonyl species can easily react with GSH in animal and plant cells (Ishikawa et al., 1986; Spitz et al., 1990; Grune et al., 1994; Ullrich et al., 1994; Islam et al., 2016; Yin et al., 2017) and ABA induces production of RCS following ROS production in guard cells (Islam et al., 2016, 2019). Therefore, ITC-induced stomatal closure is also be accompanied by RCS production following ROS production, resulting in GSH depletion in guard cells. Findings of this study support this mechanism because I found that guard cells treated with RCS scavenger and PEITC accumulate more GSH than that of only PEITC-treated guard cells (Fig 3.4). Furthermore, ITC-induced RCS accumulation in guard cells resulted in cytosolic alkalization in guard cells (Fig. 3.3), implying that ITC-induced RCS in guard cells not only reduces GSH contents but also regulates other secondary messengers/ signaling mediators that can increase the extent of stomatal closure.

In conclusion, the presented results suggest that RCS functions as a signal mediator in ITC signaling and regulates cytosolic alkalization and GSH depletion in the ITC signal pathway in Arabidopsis guard cells.

Summary

A pair of guard cells forms a tiny pore called “stoma”, which is present mainly on the leaf surface of higher plants. In response to a variety of abiotic (e.g., light, drought, and temperature) and biotic (e.g., pathogen, and elicitors) factors, guard cells regulate stomatal movements. The opening and closing of stomatal pores can modulate the exchange between CO_2 and O_2 to promote photosynthesis, transpiration of water to support nutrient uptake from soil and entry of pathogens. Thus, stomata play pivotal roles during plant adaptation to stress conditions.

Isothiocyanates are produced by the hydrolysis of glucosinolates in crucifer plants, which is catalyzed by myrosinases. A large amount of ITCs is produced rapidly when the leaves are damaged by insects or herbivores as glucosinolates come in contact with myrosinases. Isothiocyanates can readily react with nucleophiles in the cells because the carbon atom of ITC group is highly electrophilic. Isothiocyanates exhibit various effects on the physiological processes of plants. However, the ITC signaling in plant cells remains to be elucidated. In this study, I investigated stomatal responses to four ITCs namely SFN, AITC, BITC, and PEITC. Furthermore, I determined ROS accumulation, cytosolic alkalization, GSH depletion, and $[Ca^{2+}]_{cyt}$ spike in the guard cells in order to clarify how these signaling events control the extents of ITCs-induced stomatal closure in *A. thaliana*.

The AITC-induced stomatal closure is accompanied by ROS production, which is mediated by activation of salicylhydroxamic acid (SHAM)-sensitive peroxidases in *A. thaliana* and *V. faba*. Moreover, in guard cells, both cytosolic alkalization and $[Ca^{2+}]_{cyt}$ spike (transient elevation of $[Ca^{2+}]_{cyt}$) are important signaling events in AITC-induced stomatal closure. However, in this study, I have tried to reveal the details about stomatal closure mechanism induced by other ITCs besides AITC.

We found that there are no significant differences in ROS accumulation among all four ITCs despite of the differences in degree of decrease in stomatal aperture. These results suggest that ROS production is one of the necessary conditions but not a determinant of degree of decrease in stomatal aperture. In this study, I also found that the outward rectifying potassium channels are activated by cytosolic alkalization on the ITC-induced stomatal closure. The stomatal apertures decreased with a rise in the number of $[Ca^{2+}]_{cyt}$ spike per guard cell. In addition, the frequency of $[Ca^{2+}]_{cyt}$ oscillation in guard cells controls reduction of stomatal apertures. These results suggest that stomatal apertures are sophisticatedly controlled by $[Ca^{2+}]_{cyt}$ signature.

Intracellular GSH functions as a negative regulator of AITC-induced stomatal closure as well as that of ABA- and MeJA-induced stomatal closure. Glutathione contents in A. thaliana guard cells decreased with stomatal closure induced by ABA and MeJA. I also found that GSH depletion in response to SFN, AITC, BITC, and PEITC in guard cells. In order to elucidate ITCs signaling in guard cells, we assessed the correlation between decrease in stomatal aperture in response to ITCs and log *P* values of the ITCs or electron densities of the carbon atom of the ITC group. The log *P* is the lipophilicity or hydrophobicity parameter of a molecule and is a crucial factor governing passive membrane partitioning. It implies that the membrane permeability of ITCs is favorable to ITC-induced stomatal closure and that ITCs interact with certain intracellular signal component to trigger stomatal closure.

RCS functions as a signal mediator in ITC signaling and regulates cytosolic alkalization and GSH depletion in the ITC signal pathway in Arabidopsis guard cells.

Conclusions

- ITCs induce stomata closure accompanied by ROS accumulation, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ spike in *A. thaliana*
- ROS accumulation in guard cells may create a necessary environment for signaling to lead to stomatal closure in guard cells
- Decrease in GSH content is most tightly related to the decrease in stomatal aperture.
- Levels of cytosolic alkalization and the increasing number of $[Ca^{2+}]_{cyt}$ spike in guard cells can enhance stomatal closure.

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