Journal of Experimental Botany, Vol. 71, No. 22 pp. 6921–6931, 2020
doi:10.1093/jxb/eraa420 Advance Access Publication 10 September 2020

RESEARCH PAPER

Stomatal response to isothiocyanates in *Arabidopsis thaliana*

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Received 10 June 2020; Editorial decision 2 September 2020; Accepted 8 September 2020

Editor: Christine Foyer, University of Birmingham, UK

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), were investigated in *A. thaliana*. 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 ($[Ca^{2+}]_{cyt}$) spikes (transient elevation), while PEITC and BITC showed the highest frequency. There was a strong positive correlation between the number of $[Ca^{2+}]_{cyt}$ spikes per guard cell and the decrease in stomatal aperture. Both cytosolic alkalization and GSH content have a positive correlation with the decrease in stomatal aperture, but ROS production did not have a significant correlation with the decrease in stomatal apertures. These results indicate that the molecules with a functional ITC group induce stomatal closure that is accompanied by GSH depletion, cytosolic alkalization, $[Ca^{2+}]_{cyt}$ spikes, and ROS production, and that the former three cellular events, rather than ROS production, are highly correlated with the decrease in stomatal aperture.

Keywords: Cytosolic alkalization, cytosolic calcium spike, glutathione, isothiocyanate, reactive oxygen species, stomatal closure.

Introduction

Isothiocyanates (ITCs) are produced by myrosinase-dependent hydrolysis of glucosinolates in cruciferous plants (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 the *Brassicaceae* family of plants (Ishida *et al.*, 2014).

Degradation products of glucosinolates via the glucosinolate-myrosinase system have a repelling effect on many herbivores (Halkier and Gershenzon, 2006). Arabidopsis can biosynthesize a variety of glucosinolates, including 2-propenylglucosinolate, 2-phenylethylglucosinolate,and4-(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 inhibiting animal cell growth (Miyoshi et al., 2008).

Isothiocyanates can readily react with nucleophiles in the cells because the carbon atom of the 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 a 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., 2015a). Spraying of high doses of AITC or PEITC (≥10 mM) inhibited plant growth and caused severe chlorophyll degradation, whereas, a lower dose (1 mM PEITC) enhanced glutathione *S*-transferase expression in *A. thaliana* (Hara et al., 2010). Moreover, treatment with AITC led to a substantial decrease in 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, ITC signalling mechanisms in plant cells remain to be elucidated.

Stomata are tiny pores surrounded by a pair of guard cells, and are located mainly in the lower epidermis of plant leaves. Several recent reports confirmed that AITC induced stomatal closure in the glucosinolate-producing *A. thaliana* (Khokon et al., 2011a; Hossain et al., 2013), as well as in the glucosinolate-non-producing *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), although no information was provided about signalling events.

The content of ITCs in ground leaves of *A. thaliana* was estimated to be approximately 10 μmol g^{-1} fresh weight (FW; Khokon et al., 2011a) and the content of ITCs in ground cabbage sprouts was about 2 mg g^{-1} FW (Wang et al., 2015), which indicate that ITC content can reach around 1 mM in ground leaves, and up to 10 μM in certain damaged parts of leaves. 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+} ($[Ca^{2+}]_{cyt}$) spikes (transient elevation of $[Ca^{2+}]_{cyt}$) are important signalling events in AITC-induced stomatal closure (Khokon et al., 2011a; Sobahan et al., 2015). However, to the best of our knowledge, there is no report on details about the stomatal closure mechanism induced by other ITCs, besides AITC.

Glutathione (GSH) content 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 derivatives of GSH, GSH monoethyl ester (GSHmee), suppressed MeJA-induced stomatal closure (Akter et al., 2013), and phenocopied the stomatal response of a GSH-deficient mutant in response to ABA (Jahan et al., 2008; Okuma et al., 2011) and MeJA (Akter et al., 2013), where GSHmee was hydrolysed 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., 2011a) 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.

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 content 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 a degree of decrease in stomatal aperture induced by ITCs. In addition, how the signalling components downstream of ROS, such as cytosolic alkalization, or $[Ca^{2+}]_{cyt}$ spikes, regulate the degree of decrease in stomatal aperture induced by ITCs has not yet been demonstrated.

The ITC group in the ITCs can conjugate with the thiol group of GSH, resulting in a decrease in GSH content when animal cells (Zhang, 2000, 2001) and plant cells (Øverby et al., 2015b) are treated with ITCs. It was also demonstrated that accumulation of four ITCs − SFN, AITC, BITC, and PEITC in human cells were proportional to the GSH conjugation reaction with ITCs (Zhang, 2001). Likewise, GSH content in guard cells was shown to decrease during AITC-induced stomatal closure in *A. thaliana* and *V. faba* (Khokon et al., 2011a; Sobahan et al., 2015), but it still remains unclear whether GSH content regulates the degree of decrease in stomatal aperture induced by ITCs.

In this study, we investigated stomatal responses to four ITCs, namely SFN, AITC, BITC, and PEITC. Furthermore, we determined ROS accumulation, cytosolic alkalization, GSH depletion, and $[Ca^{2+}]_{cyt}$ spikes in guard cells of *A. thaliana*, in order to clarify how these signalling events correlate to the extent of stomatal closure induced by ITCs.

Materials and Methods

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 four to five week-old plants were employed for all experiments.

Measurement of stomatal aperture

Stomatal aperture measurements were performed as described previously (Munemasa et al., 2019). Briefly, rosette leaves were floated with the abaxial side down and incubated on a stomatal bioassay solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris

(pH 5.6) under light (80 μmol m^{-2} s^{-1}) for 2 h to open stomata. After that, dimethyl sulfoxide (DMSO) or SFN, BITC, AITC, or PEITC (dissolved in DMSO) were added to the stomatal bioassay solution and incubated for another 2 h. The final concentrations of each ITC and DMSO in stomatal bioassay solution were 10 μM and 0.1%, respectively. A physiological concentration of ITC at 10 μM was used to induce stomatal closure, because ITC concentrations can reach 10 μmol g^{-1} FW in ground leaves (Khokon *et al.*, 2011a). The incubated leaves were shredded in water using a commercial blender (700BUJ, Waring Commercial, Torrington, CT, USA), and epidermal tissues were collected using a nylon mesh. At least 20 stomatal apertures from two leaves collected from two independent plants were measured in each distinct experiment, using WinRoof 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). Three independent experiments were performed.

The decrease in stomatal aperture was calculated as follows: "decrease in stomatal aperture (μm)"="stomatal aperture of DMSO-treated leaves (μm)" - "stomatal aperture of ITC-treated leaves (μm)".

Measurement of ROS production in guard cells

Production of ROS in guard cells was examined using the fluorescent dye 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 for 2 h and then incubated in the bioassay solution supplemented with 50 μM H_2DCF-DA at 25±1 °C under dark conditions for 30 min. Following this, 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 bioassay solution and treated with 0.1% DMSO, or 10 μM SFN, BITC, AITC, or PEITC at 25±1 °C, under dark conditions for another 30 min. Subsequently, fluorescence of guard cells was observed under a fluorescence microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with an OP-66 835 BZ filter (excitation wavelength 480/30 nm, absorption wavelength 510 nm, and dichroic mirror wavelength 505 nm). The captured fluorescence images were analysed using ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA). Three independent experiments were performed. In each experiment, four or five leaves were collected from four or five independent plants and the fluorescence images from at least 20 guard cells were analysed.

Measurement of cytosolic alkalization in guard cells

The cytosolic pH of guard cells was monitored using a fluorescent 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 four or five leaves with the commercial blender. The collected tissues were incubated for 2 h in the stomatal bioassay solution under light conditions. After this incubation, the tissues were incubated in the bioassay solution supplemented with 20 μM BCECF-AM for 30 min in the dark at 25± 1°C to load the dye. The tissues were then washed several times with bioassay buffer in order to remove excess dye. The dye-loaded epidermal fragments were treated with 0.1% DMSO, or 10 μM 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 analysed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, four or five leaves were collected from four or five independent plants, and the fluorescence images from at least 20 guard cells were analysed.

Measurement of GSH content in guard cells

Glutathione in guard cells was fluorometrically quantified using monochlorobimane (MCB; Okuma *et al.*, 2011). Briefly, the leaves were shredded and the resulting epidermal fragments were soaked in stomatal bioassay solution under light for 2 h. The fragments were treated with 0.1% DMSO, or 10 μM SFN, BITC, AITC, or PEITC, and 50 μM of MCB for 2 h at 25±1 °C. Monochlorobimane reacts with GSH to form fluorescent glutathione *S*-bimane in guard cells. After washing the fragments to remove excess chemicals, the fluorescence of guard cells was imaged using a fluorescent microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan) with an OP-66 834 BZ filter (excitation wavelength 360/40 nm, absorption wavelength 460/50 nm, and dichroic mirror wavelength 400 nm). The captured fluorescence images were analysed using ImageJ 1.52a. Three independent experiments were performed. In each experiment, four or five leaves were collected from four or five independent plants, and the fluorescence images from at least 20 guard cells were analysed.

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

Changes in $[Ca^{2+}]_{cyt}$ in guard cells were observed using transgenic Arabidopsis plants expressing a Ca^{2+} sensor protein, Yellow Cameleon 3.6 (YC3.6), 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 a growth chamber for 2 h to promote stomatal opening. Following this, 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 analysed using AQUA COSMOS software (Hamamatsu Photonics). Note that we used the same exposure time for both CFP and YFP. A leaf was collected from a plant and the fluorescence intensities of at least two or three guard cells on the leaf were recorded. For each treatment, at least six independent leaves were employed, and fluorescence intensities of a total of 16–18 guard cells were analysed.

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

Determination of the partition-coefficient values and electron density of the carbon atom of the ITC group in 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).

Statistical analysis

The significant differences between mean values of stomatal apertures, ROS accumulation, cytosolic pH, and GSH content were assessed by Tukey's multiple comparison test, and the significant differences between frequency of $[Ca^{2+}]_{cyt}$ spikes was assessed by a 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). We considered differences at the level of $P<0.05$ as being significant for Tukey's multiple comparison test, and for the 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.

Results

ITCs induce stomatal closure

Compared with DMSO controls, both AITC and SFN significantly ($P<0.05$, Tukey's test) reduced stomatal apertures (Fig. 1A–D), in agreement with previous results (Khokon

et al., 2010; Montillet *et al.*, 2013; Sobahan *et al.*, 2015). Similar to AITC and SFN, both BITC and PEITC significantly ($P<0.05$, Tukey's test) reduced stomatal apertures, with PEITC and BITC being most effective (Fig. 1). There were no significant ($P>0.05$, Tukey's test) differences in stomatal apertures between other treatment groups (SFN, BITC, and AITC) (Fig. 1A, C-E).

ITCs induce ROS accumulation in guard cells

Compared with DMSO controls, allyl isothiocyanate (AITC) induced ROS accumulation in guard cells, as observed via DCF fluorescence (Fig. 2A-C), and in agreement with previous results (Khokon *et al.*, 2011a; Hossain *et al.*, 2013; Sobahan *et al.*, 2015). Similar to AITC, the three ITCs, BITC, PEITC, and SFN, were found to induce ROS accumulation in guard cells 30 min after treatment (Fig. 2). There were no significant ($P>0.05$, Tukey's test) differences in ROS accumulation among all four ITCs (Fig. 2A, C-F).

Effect of ITCs on cytosolic pH and GSH content in guard cells

Allyl isothiocyanate (AITC) significantly ($P<0.05$, Tukey's test) increased fluorescence of cytosolic pH indicator BCECF in *A. thaliana* guard cells (Fig. 3A, C), similar to that found in *V. faba* guard cells (Sobahan *et al.*, 2015). The other three ITCs, SFN, BITC, and PEITC significantly ($P<0.05$, Tukey's test) increased BCECF fluorescence intensity in *A. thaliana* guard cells (Fig. 3A, D-F). The strongest BCECF fluorescence was emitted from PEITC-treated guard cells (Fig. 3A, F) and there were no significant ($P>0.05$, Tukey's test) differences in BCECF fluorescence among other treatment groups (SFN, BITC, and AITC; Fig. 3A, C-E).

Monochlorobimane reacts with intracellular GSH to form fluorescent GSH *S*-bimane in guard cells. Allyl isothiocyanate (AITC) significantly ($P<0.05$, Tukey's test) induced GSH depletion in *A. thaliana* guard cells (Fig. 4A, C), as previously seen in *V. faba* guard cells (Sobahan *et al.*, 2015). The GSH depletion in guard cells was significantly ($P<0.05$, Tukey's test) induced by SFN, BITC, and PEITC (Fig. 4A, D-F), and PEITC induced the most prominent effect on GSH depletion, followed by BITC (Fig. 4A, E, F).

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

When guard cells were treated with DMSO, only a few cells (18.75% of guard cells out of 16 cells measured) showed $[Ca^{2+}]_{cyt}$ spikes (Fig. 5A). AITC triggered $[Ca^{2+}]_{cyt}$ spikes in guard cells (62.5% of guard cells out of 16 cells measured) (Fig. 5B), in agreement with previous results (Khokon *et al.*, 2011a; Hossain *et al.*, 2013; Sobahan *et al.*, 2015; Ye *et al.*, 2020).

The $[Ca^{2+}]_{cyt}$ spikes were also observed in guard cells treated with SFN (77.7% of guard cells out of 18 cells measured), BITC (93.75% of guard cells out of 16 cells measured), and PEITC (93.75% of guard cells out of 16 cells measured), and were more frequent than those in the DMSO-treated guard cells (Fig. 5C, D, E). The highest percentage of $[Ca^{2+}]_{cyt}$ spikes was observed in PEITC- and BITC-treated guard cells, which

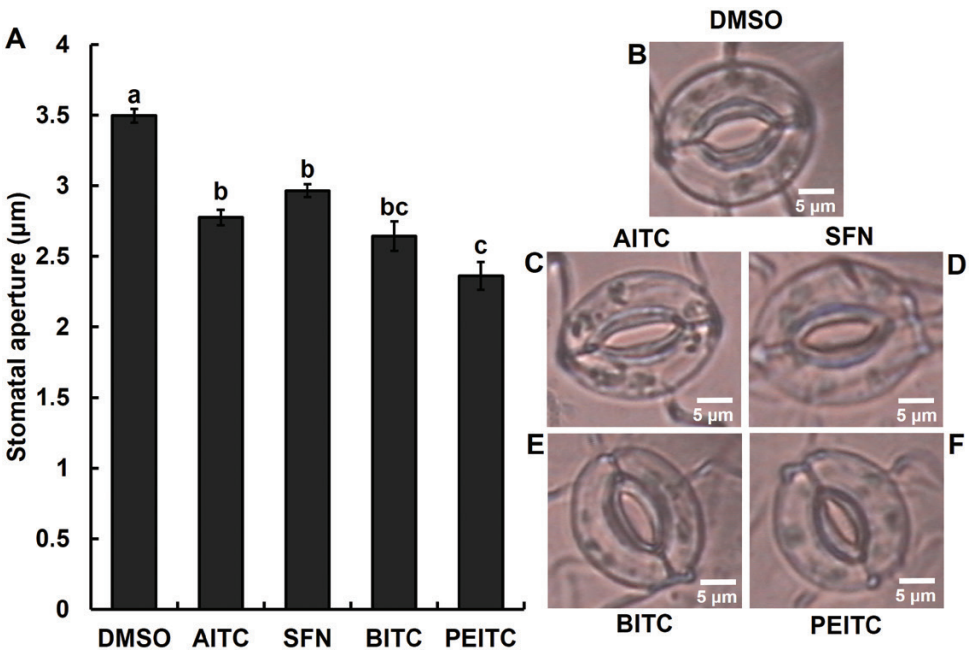

Fig. 1. Stomatal response to ITCs in *A. thaliana*. (A) Effects of AITC, SFN, BITC, and PEITC on stomatal movement. The rosette leaves were incubated with 10 μM of AITC, SFN, BITC, or PEITC for 2 h. There are no effects of 0.1% DMSO as solvent on stomatal movement. Data are the mean ±SE (n=three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P<0.05$ based on Tukey's test. (B, C, D, E, F) Representative images of the guard cells of DMSO, AITC, SFN, BITC, and PEITC treatments, respectively. Scale bar=5 μm. (This figure is available in colour at *JXB* online.)

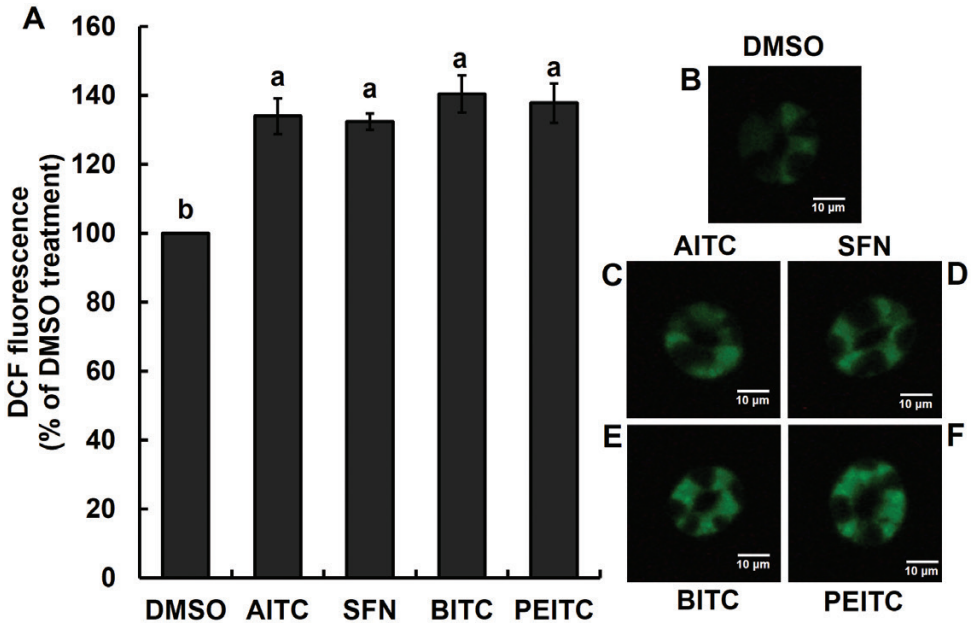

Fig. 2. ITC-induced ROS production in guard cells. (A) Effects of AITC, SFN, BITC, and PEITC on ROS production. The leaf epidermal tissues were treated with 10 μM of AITC, SFN, BITC, or PEITC for 30 minutes after treatment with H_2DCF-DA. There are no effects of 0.1% DMSO as solvent on ROS accumulation in guard cells. Data are the mean ±SE (n=three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P<0.05$ based on Tukey's test. (B, C, D, E, F) Representative images of the guard cells of DMSO, AITC, SFN, BITC, and PEITC treatments, respectively. Scale bar=10 μm. (This figure is available in colour at *JXB* online.)

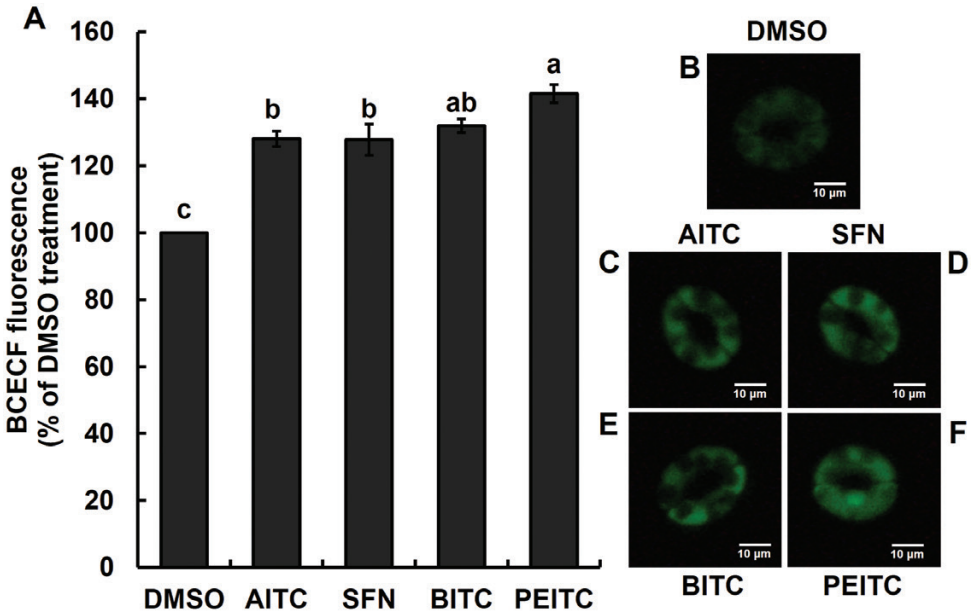

Fig. 3. ITC-induced cytosolic alkalization in guard cells. (A) Effects of AITC, SFN, BITC, and PEITC on cytosolic alkalization. The leaf epidermal tissues were treated with 10 μM of AITC, SFN, BITC, or PEITC for 30 minutes after treatment of BCECF-AM. There are no effects of 0.1% DMSO as solvent on cytosolic alkalization in guard cells. Data are the mean ±SE (n=three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P<0.05$ based on Tukey's test. (B, C, D, E, F) Representative images of the guard cells of DMSO, AITC, SFN, BITC, and PEITC treatments, respectively. Scale bar=10 μm. (This figure is available in colour at *JXB* online.)

was significantly ($P<0.05$, $\chi 2$ test) higher than that in the SFN- or AITC-treated guard cells (Fig. 5F).

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

There was a negative and significant correlation between GSH content (GSH *S*-bimane fluorescence) in guard cells and decrease in stomatal aperture; the correlation coefficient (r) is -1.0 ($R^2=0.9999$, $P=0.00004$; Fig. 6A; Supplementary Fig. S1 available at JXB online). However, there was no significant correlation between ROS accumulation (DCF fluorescence) and decrease in stomatal aperture (r=0.71, $R^2=0.4981$, $P=0.294$; Fig. 6B; Supplementary Fig. S1), or between ROS accumulation and GSH depletion (r=-0.71, $R^2=0.5032$, $P=0.290$; Fig. 6C; Supp. Fig. S1) in guard cells. Furthermore, the levels of cytosolic alkalization (BCECF fluorescence) and $[Ca^{2+}]_{cyt}$ spikes per guard cell had a positive and significant correlation with

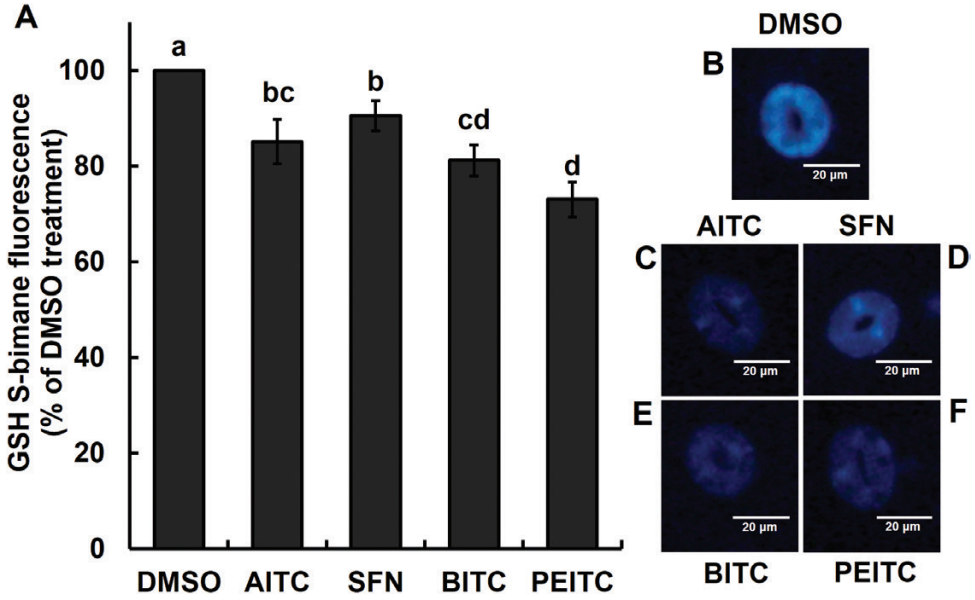

Fig. 4. Effects of ITCs on GSH content in guard cells. (A) Effects of AITC, SFN, BITC, and PEITC on GSH content. The leaf epidermal tissues were treated with 10 µM of AITC, SFN, BITC, or PEITC for 2 h in presence of the dye monochlorobimane. There are no effects of 0.1% DMSO as solvent on GSH content in guard cells. Data are the mean ±SE (n=three independent experiments, >60 stomata for each data point). Bars with the same letters are not significantly different at $P<0.05$ based on Tukey's test. (B, C, D, E, F) Representative images of the guard cells of DMSO, AITC, SFN, BITC, and PEITC treatments, respectively. Scale bar=20 µm. (This figure is available in colour at *JXB* online.)

a decrease in stomatal aperture (r=0.95, R^2=0.8955, P=0.050 and r=0.96, R^2=0.9167, P=0.042, for cytosolic alkalinisation and calcium spikes, respectively; Fig. 6D, E; Supp. Fig. S1).

In order to elucidate ITC signalling 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 (Fig. 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 the 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 can reduce its electrophilicity due to the electron-releasing substituent/group. In this study, the correlation coefficient is -0.95 (R^2=0.898, P=0.049) for log *P* of ITCs and -0.06 (R^2=0.004, P=0.936) for electron density of the carbon atom of the ITC group, with a decrease in stomatal aperture (Fig. 6F, G; Supp. Fig. S1). These results indicate that lipophilicity rather than electrophilicity of ITCs is closely related to the stomatal response. Moreover, the log *P* of ITCs had a positive and significant correlation (r=0.95, R^2=0.8978, P=0.049) with GSH content in guard cells (Fig. 6H; Supp. Fig. S1).

Discussion

Reactive oxygen species function as second messengers during 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.*, 2011b), 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), indicating that ITC-induced stomatal closure is accompanied by ROS production, in agreement with 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 differences in the degree of decrease in stomatal aperture (Figs. 1, 2). This is further supported by the non-significant correlation between ITC-induced ROS accumulation and decrease in stomatal aperture (Figs 6B; Supp. Fig. S1). These results suggest that ROS production is one of the necessary conditions, but not a determinant of the degree of decrease in stomatal aperture, following ITC treatment.

It has been established that 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 induces cytosolic alkalization in *V. faba* (Sobahan *et al.*, 2015). In this study, SFN, BITC, and PEITC, as well as AITC prompted cytosolic alkalization in *A. thaliana* guard cells (Fig. 3). In guard cells, 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). In this study, there was a positive correlation between ITC-induced cytosolic alkalization and decrease in stomatal aperture (Figs 6D; Supp. Fig. S1). These results suggest that outward rectifying potassium channels could be activated by cytosolic alkalization during ITC-induced stomatal closure.

In plant cells, $[Ca^{2+}]_{cyt}$ spikes occur in response to many stimuli, and this 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). Here, AITC-induced stomatal closure was accompanied by $[Ca^{2+}]_{cyt}$ spikes in guard cells (Fig. 5B), in agreement

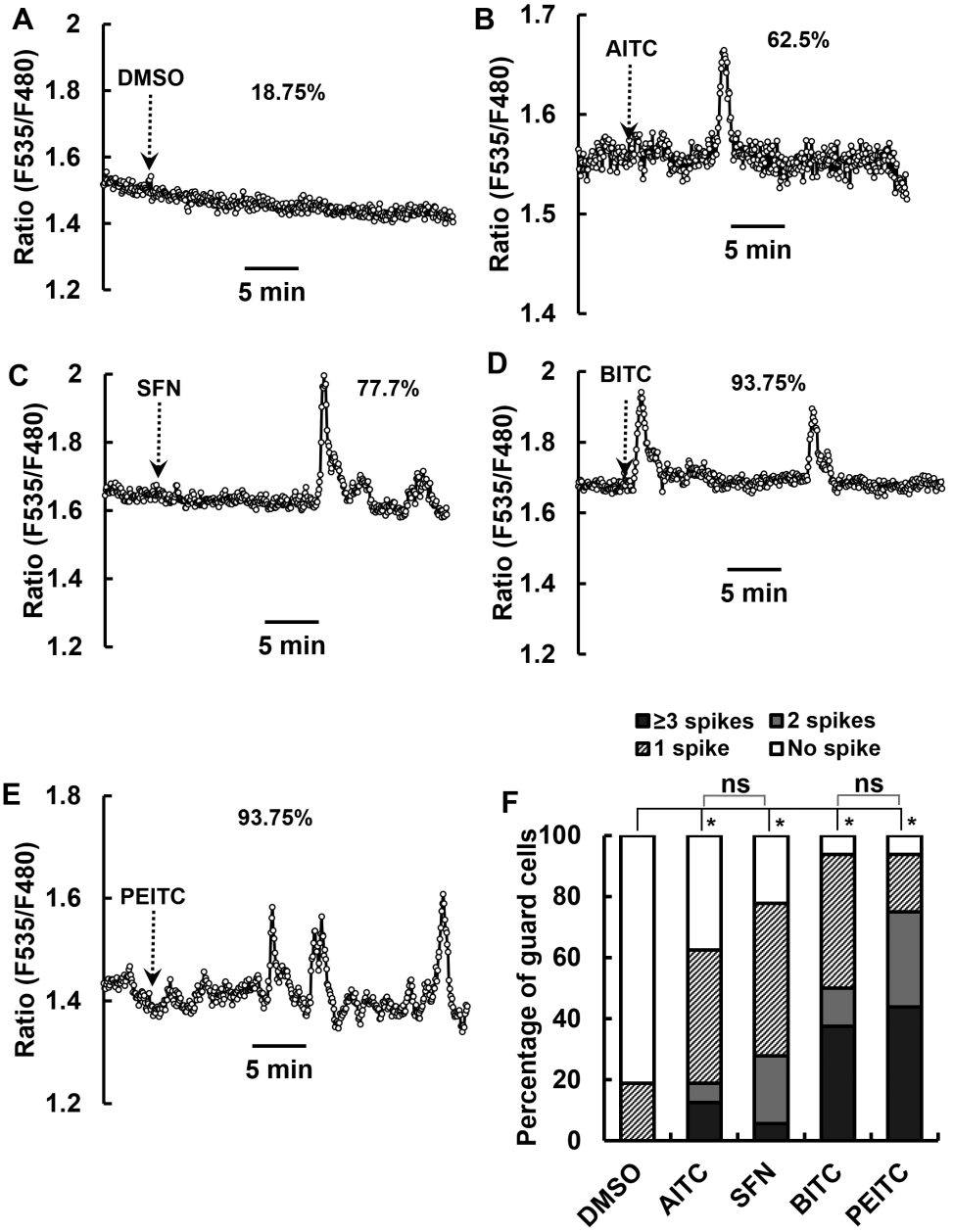

Fig. 5. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) in *A. thaliana* guard cells during stomatal closure induced by different ITCs. The $[Ca^{2+}]_{cyt}$ in guard cells expressing Yellow Cameleon 3.6 was monitored. (A) Treatment with 0.01% dimethyl sulfoxide (DMSO), 18.75% (3 out of 16) guard cells showed $[Ca^{2+}]_{cyt}$ spike(s) (transient $[Ca^{2+}]_{cyt}$ elevation). Treatment with 10 µM (B) AITC, (C) SFN, (D) BITC, and (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) of guard cells with $[Ca^{2+}]_{cyt}$ spikes, respectively. (F) Percentage bar chart showing the number of $[Ca^{2+}]_{cyt}$ spikes 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 ≥0.1 from the baseline. The significance of differences between frequency of $[Ca^{2+}]_{cyt}$ spikes was by chi-squared ($\chi 2$) test. * indicates statistical significance compared with DMSO treatment ($P<0.05$) and 'ns' indicates no significant difference. Scale bars indicate 5 min.

with previous studies (Khokon *et al.*, 2011a; Hossain *et al.*, 2013; Ye *et al.*, 2020). Moreover, the stomatal closure induced by SFN, BITC, and PEITC was also accompanied by $[Ca^{2+}]_{cyt}$ spikes in guard cells (Fig. 5C, D, E). The stomatal apertures decreased more with a rise in the number of $[Ca^{2+}]_{cyt}$ spikes per guard cell (Fig. 6E). It is already known that the frequency of $[Ca^{2+}]_{cyt}$ oscillations in guard cells controls a reduction in stomatal apertures (Allen *et al.*, 2001; Yang *et al.*, 2003). These results suggest that stomatal apertures regulated by ITC treatment are controlled by $[Ca^{2+}]_{cyt}$ signatures.

In this study, there was a significant negative correlation between GSH content in guard cells and decrease in stomatal aperture (Figs 6A; Supp. Fig. S1), suggesting that GSH is consumed during stomatal closure, for example, to form glutathione conjugates with ITCs or signalling molecules such as reactive carbonyl species (RCS; Øverby *et al.*, 2015b; Yin *et al.*, 2017; Mano *et al.*, 2017). Previous studies demonstrated that AITC-induced stomatal closure is accompanied by GSH depletion in *A. thaliana* and *V. faba* guard cells, and is suppressed by a supplement of GSH with GSHmee

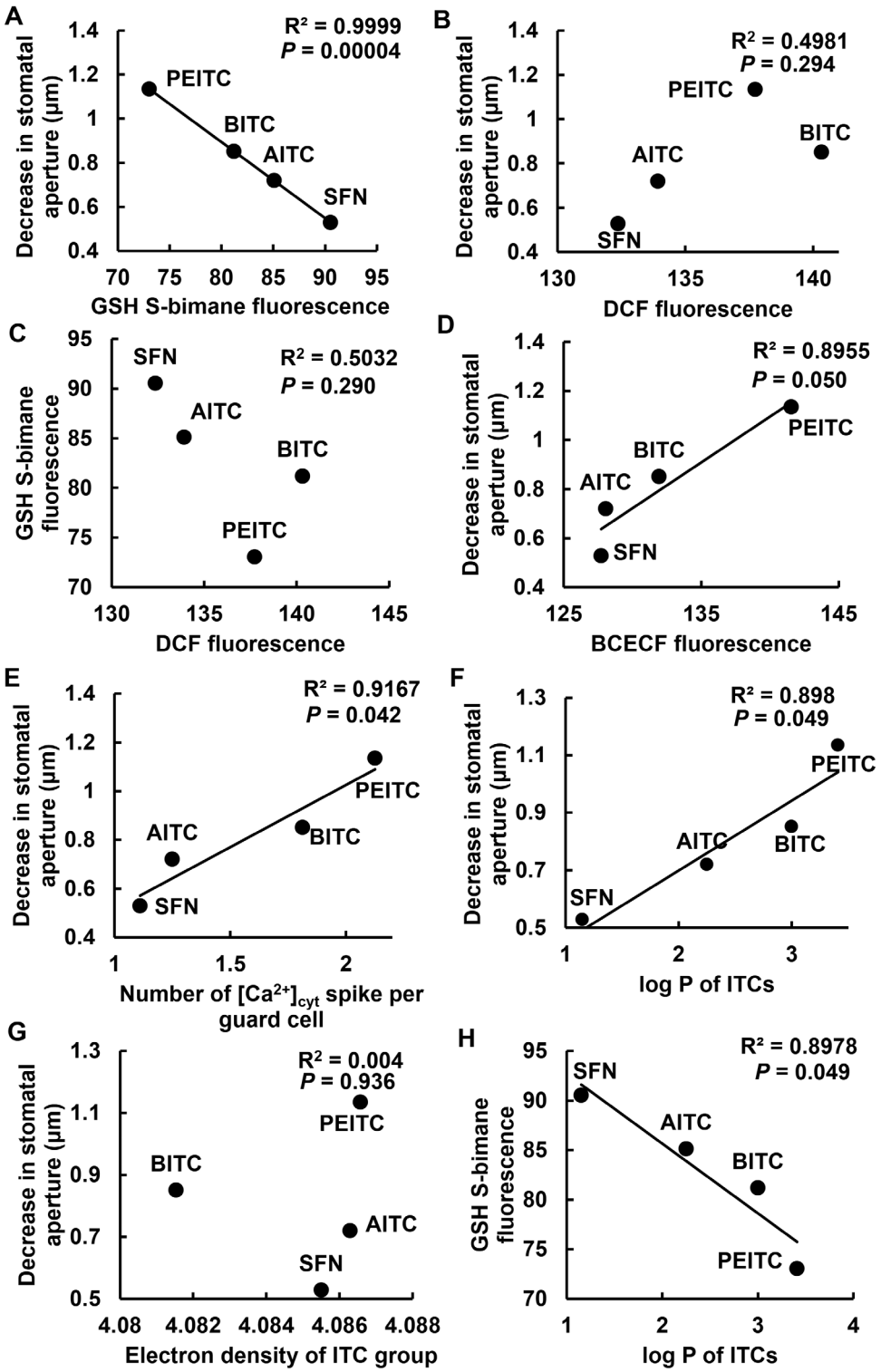

Fig. 6. Plots showing the correlation between two parameters. Plots of (A) decrease in stomatal aperture (μm) versus GSH content in guard cells, (B) decrease in stomatal aperture (μm) versus DCF fluorescence in guard cells, (C) GSH content versus DCF fluorescence in guard cells, (D) decrease in stomatal aperture (μm) versus BCECF fluorescence in guard cells, (E) decrease in stomatal aperture (μm) versus number of $[Ca^{2+}]_{cyt}$ spikes per guard cell, (F) decrease in stomatal aperture (μm) versus log *P* of ITCs, (G) decrease in stomatal aperture (μm) versus electron density of ITC groups, and (H) GSH content versus log *P* of ITCs, which were treated with SFN, BITC, AITC, or PEITC. The number of $[Ca^{2+}]_{cyt}$ spikes 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 being statistically significant.

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 GSH deficient mutants, with the phenotypes being complemented with GSH treatment (Jahan *et al*., 2008; Okuma *et al*., 2011). Taken together, certain signalling

reactions regulated by GSH may be critical to regulate stomatal closure induced by ITCs.

Analysis of the electron density of the C atoms in the ITC groups revealed that the membrane permeability of ITCs is favourable to ITC-induced stomatal closure, and that ITCs interact with certain intracellular signal components to trigger stomatal closure. The most likely potential candidate for these 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. 4; Sobahan *et al.*, 2015). This argument can be further strengthened by the strong negative correlation between log *P* values of ITCs and GSH content in guard cells (Figs 6H; Supp. Fig. S1).

Both ABA (Okuma *et al.*, 2011; Akter *et al.*, 2012) and MeJA (Akter *et al.*, 2013) also decrease GSH content 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 reactive carbonyl species (RCS) production. ITC-induced ROS may not directly regulate GSH in guard cells (Fig. 6C), but might have certain indirect effects. RCS 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 may also be accompanied by RCS production following ROS synthesis, resulting in GSH depletion in guard cells.

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

We conclude that ITCs induce stomatal closure accompanied by ROS accumulation, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ spikes in *A. thaliana* guard cells. In signalling events examined in this study, the decrease in GSH content induced by ITCs was most tightly correlated with the decrease in stomatal aperture. Moreover, the ITC-induced increase in mean number of $[Ca^{2+}]_{cyt}$ spikes in guard cells enhanced stomatal closure. Finally, we conclude that ITC-induced ROS accumulation in guard cells may create a necessary environment for signalling events leading to stomatal closure in guard cells.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Y.M. conceived the research plans; S.A., E.O., M.T.-U.-A., and M.S.J. performed the experiments; Y.M. supervised the experiments; T.N., Y.N. and S.M. provided suggestions; S.A., E.O. and M.T.-U.-A. wrote the manuscript; all authors approved the final manuscript.

Data availability

The data supporting the findings of this study are available from the corresponding author, Yoshiyuki Murata, upon request.

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