Roles of glucosinolate-myrosinase system in stomatal closure in Arabidopsis

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Mohammad Shakhawat Hossain

Graduate School of

Natural Science and Technology

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Roles of glucosinolate-myrosinase system in stomatal closure in Arabidopsis

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Submitted by
Mohammad Shakhawat Hossain

Department of Biofunctional Chemistry
Graduate School of Natural Science and Technology
Okayama University, Japan
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List of contents

List of contents i-iii

Abbreviations used iv

List of figures v

Chapter 1 General Introduction

1.1 Plant physiology 1

1.2 Stress and stress responses in plants 1

1.3 Stomata and guard cells 2

1.4 Guard cell signaling 2

1.5 Glucosinolate 3

1.6 Myrosinase 4

1.7 Isothiocyanate 5

1.8 Nitrile 5

1.9 Thiocyanate 6

1.10 Second messengers in stress signaling

1.10.1 Reactive oxygen species (ROS) 7

1.10.2 Nitric Oxide (NO) 8

1.10.3 Calcium 8

1.10.4 Cytosolic pH 9

1.11 Arabidopsis mutant 10

1.11.1 *tgg* mutants 10

1.11.2 *atrbohD atrbohF* mutant 11

1.12 Purpose of the study 12

Chapter 2 Glucosinolate degradation products, isothiocyanates, nitriles, and thiocyanates, induce stomatal closure accompanied by peroxidase-mediated reactive oxygen species production in *Arabidopsis thaliana*

2.1 Abstract 13

2.2 Introduction 14-15

2.3 Materials and Methods 15

2.3.1 Plant materials and growth conditions 16

2.3.2 Measurement of stomatal aperture 16

2.3.3 Measurement of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in whole leaves 16

2.3.4 Measurement of ROS and NO production in guard cells 17

2.3.5 Measurement of guard cell $[Ca^{2+}]_{cyt}$ oscillations 18

2.3.6 Statistical analysis 18

2.3.7 Accession numbers 19

2.4. Results

2.4. 1 Glucosinolate degradation product-induced stomatal closure 19-21

2.4.2 Glucosinolate degradation product-induced extracellular ROS production in whole leaves 21

2.4.3 Glucosinolate degradation product-induced intracellular ROS accumulation in guard cells 21-23

2.4.4 Glucosinolate degradation product-induced NO production in guard cells 23

2.4.5 Glucosinolate degradation product-induced cytosolic Ca^{2+} oscillations in guard cells 23-26

2.5 Discussion

2.5.1 Glucosinolate degradation products, isothiocynates, nitriles, and thiocyanates, induce stomatal closure 26-29

2.5.2 Sharing signal components with other signaling 29-30

2.5.3 Physiological significance of degradation products of glucosinolate 30

2.5.4 Conclusion 31

Chapter 3 Allyl isothiocyanate-induced stomatal closure impaired by *tgg1 tgg2* mutation in Arabidopsis

3.1 Abstract 32

3.2 Introduction 33-34

3.3 Materials and Methods 34

3.3.1 Plant materials and growth conditions 34

3.3.2 Measurement of stomatal aperture 35

3.3.3 Measurement of ROS and NO production 35

3.3.4 Measurement of cytosolic pH 35

3.3.5 Measurement of $[Ca^{2+}]_{cyt}$ oscillation 35

3.3.6 Statistical analysis 36

3.3.7 Accession numbers 36

3.4 Results

3.4.1 Stomatal response of *tgg1 tgg2* mutant 36-38

3.4.2 Allyl isothiocyanate (AITC)-induced ROS and NO accumulation in guard cells 39

3.4.3 AITC-induced cytosolic alkalization in guard cells 39-41

3.4.4 AITC-induced $[Ca^{2+}]_{cyt}$ oscillations in *tgg1 tgg2* guard cells 41-42

3.5 Discussion 42-47

General summary 48-51

Acknowledgements 52

References 53-65

Abbreviations used

ABA	abscisic acid
AITC	allyl isothiocyanate
BCECF-AM	2',7'-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester
CAT	Catalase
$[Ca^{2+}]_{cyt}$	cytosolic free calcium concentration
DAB	3,3′-diaminobenzidine tetrahydrochloride hydrate
DAF-2DA	4,5-diaminofluorescein-2 diacetate
DPI	diphenyleneiodonium chloride
EGTA	(*0,0′*-Bis (2-aminoethyl)ethyleneglycol-*N,N,N′*-*N′*-tetraacetic acid
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
ITC	isothiocyanate
MeJA	methyl jasmonate
MES	2-(N-morpholino)ethanesulfonic acid
NBT	nitro blue tetrazolium
pH_{cyt}	cytosolic alkalization
ROS	reactive oxygen species
SA	salicylic acid
SHAM	salicylhydroxamic acid
TGG	thioglucoside glucohydrolase
YEL	yeast elicitor
YC3.6	yellow cameleon3.6
YFP	yellow fluorescent protein

List of figures

Fig. No.		Page No.
1.1	A model showing closing and opening of stomata under various conditions	3
2.1	Glucosinolate degradation product-induced stomatal closure in Arabidopsis	20
2.2	ROS production in whole leaves by degradation products of glucosinolate	22
2.3	Glucosinolate degradation product-induced ROS accumulation in guard cells	24
2.4	Glucosinolate degradation product-induced NO production in guard cells	25
2.5	AITC-, 3-butenenitrile (3BN)-, and ethyl thiocyanate (ESCN)-induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells of wild-type plants	27
3.1	Stomatal movement in Arabidopsis in response to AITC	37
3.2	AITC-induced ROS and NO accumulation in guard cells of wild type and *tgg* mutants	40
3.3	AITC-induced cytosolic alkalization in guard cells of wild type and *tgg* mutants	41
3.4	$[Ca^{2+}]_{cyt}$ oscillations elicited by AITC in Arabidopsis wild-type and *tgg1 tgg2* guard cell.	43

Chapter 1

General Introduction

1.1 Plant physiology

Physiology is the science of the function of living systems. This includes how organ, organ systems, organisms, cells, and bio-molecules carry out the physical or chemical functions that exist in a living system. Plant physiology is the sub discipline of botany concerned with the functioning or physiology of plants. It is also the base of development of methods for the control of plant physiological and biochemical processes. It is divided into three major parts: a) the physiology of nutrition and metabolism, which deals with the uptake, transformations, and release of materials, and also their movement within and between the cells and organs of the plant, b) the physiology of growth, development and reproduction, which is concerned with these aspects of plant function, and c) environmental physiology, which seeks to understand the manifold responses of plants to the environment. Closely related fields include plant morphology, plant ecology, phytochemistry, cell biology and molecular biology. Plant physiology includes the study of all the internal activities of plants and the physical and chemical processes associated with life as they occur in plants such as photosynthesis, respiration, plant nutrition, plant hormone functions, circadian rhythms, environmental stress physiology, seed germination, dormancy and stomata function and transpiration etc.

1.2 Stress and stress responses in plants

Stress can be defined as the external conditions that adversely affect plant growth, development or productivity. Stresses trigger a wide range of plant responses including changes in growth rate and crop yield, cellular metabolism, altered gene expression etc. Stress can be biotic or abiotic. Biotic stress can be imposed by other

organisms, while abiotic stress can be arising from an excess or deficit in the physical or chemical environment. A stress response is initiated when plant recognizes stress at the cellular level. Stress recognition activates signal transduction pathways that transmit information within the individual cells and through the plant. Changes in gene expression may modify growth and development and even influence the reproductive capabilities. To withstand with these challenges, plant defense responses rely on signaling mechanisms of hormones and plants specific substances (Bostock 2005; Zhang et al. 2008).

1.3 Stomata and guard cells

Stomata are natural microscopic pores on the leaf epidermis of higher plants that regulate gas exchange and water loss *via* transpiration (Hetherington and Woodward 2003). The stomatal pores are surrounded by a pair of specialized parenchyma cells known as guard cells which act as a turgor-driven valve that open and close the stomatal pores in response to given environmental condition. The most important function of stomata is to allow entry of sufficient CO_2 for optimal photosynthesis while conserving water as required by the plant. In addition, these specialized structures also play critical roles in the control of leaf temperature by modulating rates of transpirational water loss, and restrict pathogen invasion *via* stomatal closure. Multiple environmental factors such as drought, CO_2 concentration, light, humidity, biotic stresses and different plant hormones module stomatal apertures. Stomatal movement is facilitated by the activity of ion channels and ion transporters found in the plasma membrane and vacuolar membrane of guard cells.

1.4 Guard cell signaling

Guard cells have become a highly developed model system for characterizing

early signal transduction mechanisms in plants and for elucidating how individual signaling mechanisms can interact within a network in a single cell (Schroeder et al. 2001). Stomata generally open in response to light, low CO_2 concentrations, and high atmospheric humidity and close in response to darkness, high CO_2 concentrations, low humidity, H_2O_2, and plant hormones like abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA), and elicitors (Schroeder et al. 2001; Zhang et al. 2001; Hetherington and Woodward 2003; Suhita et al. 2004; Young et al. 2006; Khokon et al. 2010a, 2011b). During stomatal opening, an increase in guard cell volume is driven by uptake and intracellular generation of solutes, which decreases guard cell water potential and creates a driving force for water uptake into the guard cell. Due to the radial reinforcement of the guard cell wall, the resultant increase in turgor causes the two guard cells of the stomata to separate, widening the stomatal pore. During stomatal closure, there is a reduction in guard cell solute concentration and volume, which results in guard cell deflation and narrowing of the stomatal aperture (Panday et al. 2007).

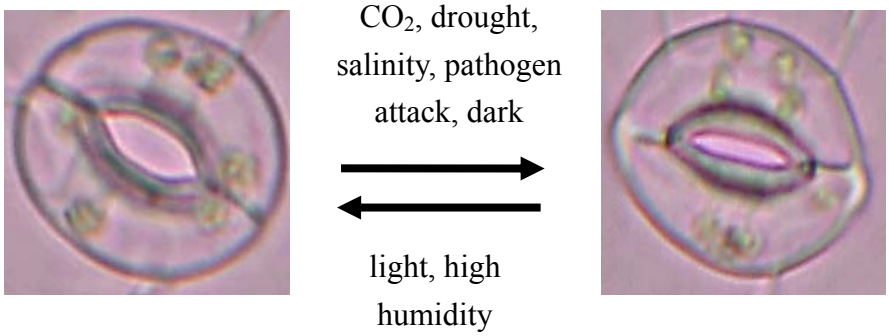

Fig. 1.1 A model showing closing and opening of stoma under various conditions.

1.5 Glucosinolate

Glucosinolates (GSLs) are amino acid-derived plant secondary metabolites that contain sulfur, nitrogen, and a group derived from glucose. They are naturally

occurring *β*-thioglucoside *N*-hydroxysulfates found in sixteen families of dicotyledonous plants including Brassicaceae such as the model plant Arabidopsis. GSLs are grouped into three categories such as aliphatic, aromatic, and indolic. Aliphatic GSLs are derived from alanine, leucine, isoleucine, valine, and methionine. Tyrosine or phenylalanine gives aromatic GSLs, and tryptophan-derived GSLs are called indolic GSLs. Although more than 120 different GSLs have been identified and thirty-four GSLs have been found in Arabidopsis. Intact GSLs are mostly considered to be biologically inactive. The enzymatic degradation of GSLs, which occur massively upon tissue damage, are catalyzed by plant thioglucosides called myrosinase. Depending on several factors (e.g. GSLs structure, pH, proteins, metal ions, and other cofactors) myrosinase-catalyzed hydrolysis of GSLs can lead to a variety of products such as isothiocyanates, nitriles, and thiocyanates. Moreover, most degradation products play important roles in plant defense systems against pest, pathogen, fungi, and bacterial infections (Vaughn et al. 2006).

1.6 Myrosinase

Myrosinase (*β*-thioglucoside glucohydrolase, TGG; EC 3.2.1.147) is an enzyme. Myrosinases and glucosinolates are localized in separate plant cells, myrosin cells and S-cells, respectively. Under stress condition, *β*-thioglucoside linkage is cleaved by myrosinase. Isothiocyanates are products of the spontaneous rearrangement of the glucosinolate aglycones released by myrosinase and the formation of nitriles and thiocyanates are depend on both myrosinase and specifier proteins or pH (Halkier and Gershenzon 2006). Six myrosinase, *TGG*, genes are identified and two functional myrosinase genes, *TGG1* (At5g26000) and *TGG2* (At5g25980), have been found in the Arabidopsis genome (Xue et al. 1995; Zhang et al. 2002). *TGG4*

(At1g47600) and *TGG5* (At1g51470) are expressed in root tissues. *TGG3* (At5g48375) is a pseudogene that is expressed in stamens and petals (Zhang et al. 2002). The remaining member *TGG6* (At1g51490) is expressed in flowers, specifically in the stamens (Toufighi et al. 2005). Moreover, myrosinases, TGG1 and TGG2, have redundant function in glucosinolates breakdown (Barth and Jander 2006) and the glucosinolate-myrosinase system serves as a major chemical defense mechanism against insects, bacteria, and fungi (Bones and Rossiter 1996). Furthermore, glucosinolate-myrosinase system enhances ABA-inhibition of guard cell inward K^+ channels and of stomatal closure (Zhao et al. 2008). However, it is still unknown whether myrosinases function during stomatal closure response to isothiocyanates, nitriles, and thiocyanates.

1.7 Isothiocyanate

Isothiocyanates (ITCs) are sulfur-containing electrophiles produced by Brassicaceae plants. The most intensively studied hydrolysis products, isothiocyanates, are formed through a spontaneous rearrangement of the glucosinolate aglucone in the absence of supplementary proteins. Allyl isothiocyanate (AITC) is one of the degradation products during glucosinolate-myrosinase interaction in Arabidopsis and has repellent effect on plant enemies (Lambrix et al. 2001). AITC also shows antimicrobial activity against a wide spectrum of pathogens (Olivier et al. 1999). Moreover, in animal cells, ITCs inhibits carcinogenesis and tumourigenesis, and induce apoptosis (Hayes et al. 2008). However, the effects of ITCs in animals have been well studied but the effects of ITCs in planta still remain to be elucidated.

1.8 Nitrile

The most common glucosinolate hydrolysis product is nitrile. The formation of nitriles is favored at a pH of less than three or in the presence of Fe^{2+} ions. Burow et al. (2009) have reported that protein factors are involved in nitrile formation, such as the epithiospecifier protein (ESP). The recent isolation of an Arabidopsis gene encoding an ESP showed that this protein not only promotes the formation of epithionitriles, but also the formation of simple nitriles from a large variety of glucosinolates (Kissen and Bones 2009). When the glucosinolate side chain has a terminal double bond, ESP promotes the reaction of the sulfur atom of the thioglucoside linkage with the double bond to form a thirane ring, giving an epithionitrile and simple nitrile (Lambrix et al. 2001). On the other hand, formation of simple nitriles are also produced in the absence of specifier proteins in vitro when the myrosinase reaction takes place at low pH values (< 5) or high ferrous ion concentrations (> 0.01 mM) (Wittstock and Burow 2007). Moreover, nitrile-specifier proteins (NSPs) promote simple nitrile formation at physiological pH values, but do not catalyze epithionitrile or thiocyanate formation (Burow et al. 2009). Furthermore, ITCs are known to be toxic to a wide variety of organism (Wittstock et al. 2003), while nitriles appear to have lower direct toxicity but may instead serve to deter attract plant enemies (Lambrix et al. 2001; Burow et al. 2006).

1.9 Thiocyanate

Thiocyanates are formed from only three glucosinolates: allyl-, benzyl-, and 4-methylsulfinylbutyl-glucosinolate (Burow et al. 2009). Thiocyanates are produced from a very limited number of glucosinolates, and their formation necessitates the presence of a thiocyanate-forming factor in addition to myrosinase. A thiocyanate-forming protein (TFP) has recently been identified in *Lepidium sativum* (Burow et al. 2007). On the other hand, thiocyanate formation requires at least two

enzymes, a myrosinase and a thiocyanate-forming factor.

1.10 Second messengers in stress signaling

1.10.1 Reactive oxygen species

Reactive oxygen species (ROS) are continuously produced in plants as by-products of several aerobic metabolic processes like photosynthesis and respiration. Electrons that have a high-energy state are transferred to molecular oxygen (O_2) to form ROS (Mittler 2002). ROS comprise of singlet oxygen ($^1O_2$), super oxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). Under optimal growth conditions, ROS are mainly produced at a low level in organelles such as chloroplast, mitochondria, apoplast and peroxisomes. However, during stress, their rate of production is dramatically increased. ROS accumulation depends on the balance between ROS production and ROS scavenging (Mittler et al. 2004). Antioxidants such as ascorbic acid (AsA), glutathione (GSH), α-tocopherol and ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), and peroxiredoxin (PrxR) are essential for ROS detoxification during normal metabolism, and particularly during stress (Takahashi and Asada 1998; Apel and Hirt 2004; Mittler et al. 2004).

There are several sources of ROS like mitochondria, chloroplasts, plasma membrane bound NADPH oxidases, cell wall peroxidases, peroxisomes and glyoxysomes (Allan and Fluhr 1997; Bolwell et al. 1998; Pei et al. 2000; Zhang et al. 2001; Torres et al. 2002; Kwak et al. 2003; Bindschedler et al. 2006; Asada 2006; del Rio et al. 2006). ROS function as signaling molecules in plants involved in regulating various processes including pathogen defense, programmed cell death, stress responses and stomatal behavior (Apel and Hirt 2004). ROS act as a second messenger in hormonal signaling. ROS production mediated by plasma membrane NADPH oxidases

is required by ABA- and MeJA-induced stomatal closure (Kwak et al. 2003; Suhita et al. 2004; Munemasa et al. 2007) while extracellular ROS production mediated by cell wall peroxidases is required by salicylic acid (SA)-, yeast elicitor (YEL)-, and chitosan-induced stomatal closure (Bolwell et al. 1998; Khokon et al. 2010a, b, 2011b). However, it is still unknown whether glucosinolate degradation products induce ROS production in guard cells.

1.10.2 Nitric oxide

Nitric oxide (NO) has been shown to serve as an important signal molecule involved in many aspects of developmental processes, including floral transition, seed germination, root growth, fruit maturation, and orientation of pollen tube growth (Beligni and Lamattina 2000). NO is generated by nitrate reductase (NR), NO synthase (NOS) and non-enzymatic system in plant cells (Yamasaki 2000). Both NR and NOS are involved in NO production during stomatal closure induced by ABA and MeJA (Desikan et al. 2002; Munemasa et al. 2011a). NR reduces nitrite to NO, and the *nia1*, *nia2* NR deficient mutant in Arabidopsis showed reduced ABA induction to stomatal closure (Desikan et al. 2002; Bright et al. 2006). In contrast, NOS-like enzyme is required for jasmonate signaling in guard cells but NR is not required (Munemasa et al. 2011a). However, the involvement of NO to signal transduction leading to stomatal closure is still controversial since Lozano-Juste and Leon (2010) have reported that the Arabidopsis *nia1 nia2 noa1-2* triple mutant, which is deficient endogenous NO production, is hypersensitive to ABA.

1.10.3 Calcium

The calcium ion (Ca^{2+}) plays a role in the regulation of cellular function as a

major intracellular messenger. Changes in cytosolic Ca^{2+} concentration take place in response to a wide variety of abiotic and biotic signals (Sanders et al. 1999; Sanders et al. 2002). Ca^{2+} is stored both in extra- and intracellular stores. Inside the cell, the vacuole is the most prominent Ca^{2+} storage place, but its concentration is higher than in the cytosol also in the endoplasmic reticulum, mitochondria and chloroplasts. Different effectors can induce an elevation in cytosolic Ca^{2+} in guard cells. Cytosolic Ca^{2+} elevation can be achieved by Ca^{2+} influx from the apoplast into the cytosol *via* hyperpolarization activated Ca^{2+} channels in the plasma membrane of guard cells and by its release from intracellular stores, primarily from the vacuole. Both ABA and MeJA activate Ca^{2+}-permeable cation channels and elicit $[Ca^{2+}]_{cyt}$ oscillation of various frequencies and amplitudes (Allen et al. 2002; Kwak et al. 2003; Munemasa et al. 2007, 2011b, Islam et al. 2010a, b). Moreover, an Arabidopsis calcium dependent protein kinases, (CDPKs), function as important $[Ca^{2+}]_{cyt}$ sensors in many aspects of plant physiological processes (Klimecka and Muszynska 2007). CDPK, *CPK6*, is a positive regulator in guard cell ABA and MeJA signaling (Mori et al. 2006; Munemasa et al. 2011b). In guard cell, ABA and MeJA signaling are dependent on $[Ca^{2+}]_{cyt}$ and a $[Ca^{2+}]_{cyt}$ sensor. CPK6 is a central regulator of the signaling (Mori et al. 2006; Munemasa et al. 2011b).

1.10.4 Cytosolic pH

Increment of guard cell cytosolic pH (pH_{cyt}) is favorable for stomatal closure. Changes of pH_{cyt} in guard cells are considered as an important step during ABA- and MeJA-induced stomatal closure (Gehring et al. 1997; Suhita et al. 2004). Both ABA and MeJA increase in guard cell pH_{cyt} during stomatal closure in Arabidopsis, Pisum, Paphiopedilum (Irving et al. 1992; Gehring et al. 1997; Suhita et al. 2004; Gonugunta et

al. 2008). Moreover, ABA inhibits plasma membrane H^+-ATPase activity and activates vacuolar H^+-ATPase (V-ATPase) activity, resulting in cytosolic alkalization (Goh et al. 1996; Barklay et al. 1999; Zhang et al. 2004). Blatt and Armstrong (1993) reported that acidification of the cytoplasm by external sodium butyrate inhibited the outward K^+ channel, prevented the ABA-induced alkalization of the cytoplasm, and blocked the ABA-induced activation of the outward K^+ channel, arguing for regulation by cytoplasmic pH, and positioning pH in the signaling chain leading from ABA to this channel. However, no study has elucidated whether the degradation products regulate pH_{cyt} in Arabidopsis guard cells.

1.11 Arabidopsis mutant

Arabidopsis thaliana is a model plant having a large collection of mutants with defect in different defense and stress related signaling pathways and use of these mutants makes it possible to determine which pathways are controlling an observed response (Glazebrook 1997). Pharmacological, cell biological, genetical and electrophysiolocal studies have elucidated the multiple regulatory protein components, enzymes and second messengers involved in ABA and MeJA signaling, and biosynthesis (Schroeder et al. 2001; Munemasa et al. 2007). In my experiments, *atrbohD atrbohF* and *tgg* mutants were used to clarify the involvement of ROS-catalyzing enzymes in the glucosinolates degradation products, isothiocyanate, nitrile, and thiocyanate signaling.

1.11 .1 *tgg* mutants

Myrosinases are cytosolic and vacuolar enzymes (Luthy and Matile 1984). Glucosinolates are hydrolyzed by myrosinases to form toxin products, isothiocyanates,

nitriles, and thiocyanates that are active against insects, pathogens and herbivores (Lambrix et al., 2001). In Arabidopsis, six myrosinase (thioglucoside glucohydrolases, *TGGs*) genes are identified (Xue et al. 1995; Zhang et al. 2002). Two functional myrosinase genes, *TGG1* (thioglucoside glucohydrolase 1) and *TGG2* (thioglucoside glucohydrolase 2), have been found in the Arabidopsis genome (Xue et al. 1995; Husebye et al. 2002). Barth and Jander (2006) have demonstrated that TGG1 and TGG2 have redundant function in glucosinolates breakdown. The *tgg1* mutation impairs inhibition of light-induced stomatal opening by ABA (Zhao et al. 2008) and the *tgg1 tgg2* mutation impairs ABA- and MeJA-induced stomatal closure (Islam et al. 2009). To clarify whether myrosinases function during stomatal response, *tgg* mutants were used. Arabidopsis genome initiative numbers for the genes are *TGG1* (At5g26000) and *TGG2* (At5g25980).

1.11.2 *atrbohD atrbohF* mutant

NADPH oxidase, known as the respiratory burst oxidase homolog (RBOH), Arabidopsis has 10 *Atrboh* (Arabidopsis RBOH) genes homologus to $gp91^{phox}$ (Torres et al. 2002). NADPH oxidases are plasma membrane protein (Keller et al. 1998) that may produce ROS in the vicinity of plasma membrane ion channels. Guard cell expressed NADPH oxidase catalytic subunit genes, $gp91^{phox}$ homologous sequence (Torres et al. 1998), were identified using degenerate oligomers. A guard cell expressed $gp91^{phox}$ homologous gene, *AtrbohD* was identified from guard cell cDNA libraries. Genechip experiments followed to identify another $gp91^{phox}$ homologous gene *AtrbohF*. The *atrbohD atrbohF* double mutant was obtained from crosses between homozygous *atrbohD* and *atrbohF* mutants. ABA-induced stomatal closure, ROS production and cytosolic calcium increases were strongly impaired in *atrbohD atrbohF* mutant (Kwak

et al. 2003). Exogenously applied H_2O_2 can rescue wild type stomatal responses in the *atrbohD atrbohF* mutant (Kwak et al. 2003). In this study, I used *atrbohD atrbohF* mutant to clarify the involvement of ROS-catalyzing enzyme NADPH oxidase in isothiocyanate, nitrile, and thiocyanate signaling. Arabidopsis genome initiative numbers for *AtRBOH* genes are *AtRBOHD* (At5g47910) and *AtRBOHF* (At1g64060).

1.12 Purposes of the study

The aim of this study is to obtain novel information about the effects of glucosinolate degradation products, AITC, 3-butenenitrile (3BN), and ethyl thiocyanate (ESCN) on stomatal movement in Arabidopsis to clarify the roles of degradation products of glucosinolates in *planta.*

Specific aims were as follows:

1. To examine the effects of glucosinolate degradation products, AITC, 3BN, and ESCN, on stomatal movement, ROS and NO production, and $[Ca^{2+}]_{cyt}$ oscillation in guard cells to elucidate AITC, 3BN, and ESCN signaling in Arabidopsis guard cells.
2. To investigate function of myrosinases in guard cell signaling to examine stomatal movement, ROS and NO production, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillations in guard cells of *tgg* mutants in response to AITC.

Chapter 2

Glucosinolate degradation products, isothiocyanates, nitriles, and thiocyanates, induce stomatal closure accompanied by peroxidase-mediated reactive oxygen species production in *Arabidopsis thaliana*

2.1. Abstract

Isothiocyanates, nitriles, and thiocyanates are degradation products of glucosinolates in crucifer plants. In this study, I investigated stomatal response to allyl isothiocyanate (AITC), 3-butenenitrile (3BN), and ethyl thiocyanate (ESCN) in Arabidopsis. AITC, 3BN, and ESCN induced stomatal closure in wild type and *atrbohD atrbohF* mutant. The stomatal closure was inhibited by catalase and salicylhydroxamic acid (SHAM). The degradation products induced extracellular reactive oxygen species (ROS) production in rosette leaves, and intracellular ROS accumulation, NO production, and cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) oscillations in guard cells, which were inhibited by SHAM. These results suggest that glucosinolate degradation products induce stomatal closure accompanied by extracellular ROS production mediated by SHAM-sensitive peroxidases, intracellular ROS accumulation, and $[Ca^{2+}]_{cyt}$ oscillation in Arabidopsis.

2.2 Introduction

Isothiocyanates (ITCs), nitriles, and thiocyanates are degradation products of glucosinolate in crucifer plants and the degradation are catalyzed by myrosinases (EC 3.2.1.147) (Bones and Rossiter 1996; Wittstock and Halkier 2002). The myrosinase-glucosinolate system is involved in a range of biological activities because ITCs have repellent effect on herbivores and insects and biocidal activity (Lambrix et al. 2001; Mewis et al. 2005; Yan and Chen 2007). However, nitriles and thiocyanates are not as bioactive as isothiocyanates and consequently evidences of their physiological roles in plants are limited.

Guard cells in pairs surround stomatal pores and response to various environmental stimuli, which regulate gas exchange, transpirational water loss, and invasion of microorganisms (Israelsson et al. 2006; Melotto et al. 2006; Shimazaki et al. 2007). Glucosinolate-myrosinase system enhances abscisic acid (ABA)-inhibition of stomatal opening, (Zhao et al. 2008) and allyl isothiocyanate (AITC) induces stomatal closure under methyl jasmonate (MeJA)-priming conditions in *Arabidopsis thaliana* (Khokon et al. 2011a). However, it remains unknown whether nitriles and thiocyanates induce stomatal closure.

Stomatal closure requires production of reactive oxygen species (ROS) which is mediated by plasma membrane NADPH oxidases and cell-wall peroxidases (Bolwell et al. 1998; Pei et al. 2000; Torres et al. 2002; Kwak et al. 2003; Bindschedler et al. 2006). NADPH oxidases, AtRBOHD and AtRBOHF, are involved in ABA- and MeJA-induced stomatal closure, (Pei et al. 2000; Kwak et al. 2003; Munemasa et al. 2007; Islam et al. 2010a, b) and salicylhydroxamic acid (SHAM)-sensitive cell wall peroxidases are involved in salicylic acid (SA)-, yeast elicitor (YEL)-, and chitosan (CHT)-induced stomatal closure (Mori et al. 2001; Khokon et al. 2010a, b, 2011b). It has been reported

that AITC induced ROS production in guard cells, which was slightly inhibited by an NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) (Khokon et al. 2011a). However, it is still unsettled which enzyme mediates AITC signaling. Moreover, it remains unknown whether other degradation products induce ROS production or which enzyme catalyzes the ROS production in guard cells.

Various enzymes are involved in ROS production in plant cells. DPI (50 to 200 μM) is widely used as an inhibitor of plasma membrane NADPH oxidases, (Cross and Jones 1986) and SHAM (1 to 3 mM) and NaN_3 (300 μM to 1 mM) as inhibitors of cell wall peroxidases (Martinez et al. 1998; Garrido et al. 2012). On the other hand, catalase (CAT) (100 to 500 units/mL) is widely used as an H_2O_2 scavenger, (Kono and Fridovich 1982) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3- oxide (cPTIO) (100 μM to 1mM) as an NO scavenger (Correa-Aragunde et al. 2004).

ABA- and MeJA-induced ROS production results in activation of Ca^{2+}-permeable non-selective cation channels on the plasma membrane, leading to $[Ca^{2+}]_{cyt}$ oscillation during stomatal closure (Islam et al. 2010b; Munemasa et al. 2007; 2011b). AITC-induced stomatal closure is also accompanied by $[Ca^{2+}]_{cyt}$ oscillations in Arabidopsis guard cells (Khokon et al. 2011a). Moreover, whether nitriles and thiocyanates like AITC induce stomatal closure and whether production of ROS and $[Ca^{2+}]_{cyt}$ oscillations are induced in guard cells in response to nitriles and thiocyanates remain to be clarified.

In this study, I examined effects of glucosinolate degradation products, AITC, 3-butenenitrile (3BN), and ethyl thiocyanate (ESCN) on stomatal movement, ROS and NO production, and $[Ca^{2+}]_{cyt}$ oscillation in Arabidopsis in order to clarify the roles of degradation products of glucosinolates in crucifer plants.

2.3 Materials and Methods

2.3.1 Plant materials and growth conditions

Arabidopsis thaliana wild type, ecotype Columbia-0 (Col-0) and *atrbohD atrbohF* plants were grown on soil containing a mixture of 70% (v/v) vermiculite (Asahi-Kogyo, Okayama, Japan) and 30% (v/v) Kureha soil (Kureha Chemical, Tochigi, Japan) in a growth chamber at 22±2˚C, 80 μmol m^{-2} s^{-1} light intensity, and 60±10% relative humidity under a 16 h light/8 h dark regime. Water was applied two to three times per week with Hyponex solution (0.1%) on the plant growth tray. Rosette leaves from 4- to 5-week-old plants were employed for assays. $[Ca^{2+}]_{cyt}$ oscillations in guard cells was measured using a Ca^{2+}-sensing fluorescent protein, Yellow Cameleon 3.6 (YC3.6) (Nagai et al. 2004).

2.3.2 Measurement of stomatal aperture

Stomatal aperture measurements were performed as described previously (Murata et al. 2001). Excised rosette leaves were floated on medium containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 μmol m^{-2} s^{-1}) to induce stomatal opening and then were treated with 50 μM AITC, 3BN, or ESCN. Then, stomatal apertures were measured after 2 h incubation. CAT (100 units mL^{-1}), SHAM (1 mM), NaN_3 (1 mM), or DPI (20 μM) were added 30 min before application of AITC, 3BN, or ESCN. Leaves were blended for 20 s, and epidermal tissues were collected. The epidermal tissues were mounted on a slide glass and images of stomatal apertures were captured using an Olympus IX71S87 microscope connected to a CS230 digital imaging color camera and were analyzed with image analysis software (WinROOF V3.61, MITANI Corporation, Osaka, Japan). Twenty stomatal apertures were measured in each individual experiment.

2.3.3 Measurement of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in whole leaves

The rosette leaves of Arabidopsis plants were analyzed using 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB) (Tokyo Chemical Industries, Tokyo, Japan) as described previously (Khokon et al. 2011b). Excised rosette leaves were floated on medium containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) with 0.05% Tween20 and incubated for 2 h under light (80 μmol m^{-2} s^{-1}). Then, the leaves were transferred in 1 mg mL^{-1} DAB solution and gently infiltrated in a vacuum for 2 h. AITC, 3BN, or ESCN at 50 μM was added and infiltrated for 4 h. SHAM at 1 mM was added 30 min before application of AITC, 3BN, or ESCN. After incubation, the leaves were cleared in boiling ethanol (99%) for 10 min. Localization of H_2O_2 is visualized as a reddish-brown coloration. For detection of O_2^-, nitro blue tetrazolium (NBT) (Tokyo Chemical Industries) at 1 mg mL^{-1} was used instead of DAB. Localization of O_2^- is visualized as a blue coloration. In both cases, the leaves were mounted on cover glass and pictures were taken. The intensity of coloration was quantified using Adobe Photoshop CS2 software (Adobe Systems Inc., San Jose, CA, USA).

2.3.4 Measurement of ROS and NO production in guard cells

Production of ROS and NO in guard cells were examined using 50 μM of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma, St Louis, MO, USA) and 5 μM of 4,5-diaminofluorescein-2diacetate (DAF-2DA) (Sigma), respectively, as described previously (Munemasa et al. 2007). For ROS detection, epidermal tissues were incubated in the light for 3 h in medium containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15), and then 50 μM H_2DCF-DA was added to this medium. The epidermal tissues were incubated for 30 min at room temperature, and then the excess dye was washed out. The dye-loaded tissues were treated with 50 μM AITC, 3BN, or ESCN for 30 min. CAT (100 units mL^{-1}), SHAM (1 mM), or DPI (20 μM) were added 30 min

before application of AITC, 3BN, or ESCN. For NO detection, 5 μM DAF-2DA was added instead of 50 μM H_2DCF-DA. Fluorescent images of guard cells were captured using a fluorescence microscope (Biozero BZ-8000, Keyence, Osaka, Japan) with filter: OP-66835BZ filter GFP (excitation wavelength, 480/30 nm; emission wavelength, 510 nm; and dichroic mirror wavelength, 505 nm). The fluorescence intensity was analyzed using ImageJ 1.42q software (NIH, Bethesda, MD, USA).

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

Yellow Cameleon 3.6 (YC3.6) was employed as a Ca^{2+} indicator to monitor cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in Arabidopsis guard cells as described previously (Islam et al. 2010b). The abaxial epidermal peels of rosette leaves expressing YC3.6 were placed in a 6-well plate containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES-Tris (pH 6.15) for 2 h under light condition (80 μmol m^{-2} s^{-1}). Turgid guard cells were used for the measurement of $[Ca^{2+}]_{cyt}$ oscillation. Excitation wavelengths were 440 nm and emission wavelengths were 480 nm and 535 nm. The ratio-metric (F_{535}/F_{480}) images were obtained using 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 cyan fluorescent protein (CFP) and 535DF25 for yellow fluorescent protein (YFP); Hamamatsu Photonics, Hamamatsu, Japan] and a CCD camera (Hamamatsu ORCA-ER digital camera, Hamamatsu Photonics). The CFP and YFP fluorescence intensities of guard cells were imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics).

2.3.6 Statistical analysis

The significance of differences between mean values was assessed using analysis

of variance (ANOVA) with Tukey's test. Differences in the frequency of $[Ca^{2+}]_{cyt}$ oscillations in the wild type induced by glucosinolate degradation products were determined by χ^2-test. We regarded differences at the level of $P < 0.05$ as significant. Data are means ± SE.

2.3.7 Accession numbers

Arabidopsis genome initiative numbers for the genes discussed in this article are as follows: *AtRBOHD* (At5g47910), *AtRBOHF* (At1g64060).

2.4. Results

2.4. 1 Glucosinolate degradation product-induced stomatal closure

I examined whether AITC, 3BN, and ESCN induce stomatal closure. AITC at 10 μM and 50 μM significantly induced stomatal closure in wild-type plants (Fig. 2.1A) as reported previously (Khokon et al. 2011a). Application of 3BN at 10 μM and 50 μM significantly induced stomatal closure and application of ESCN at 10 μM and 50 μM significantly induced stomatal closure in wild-type plants (Fig. 2.1A). Solvent control (0.1% dimethyl sulfoxide, DMSO) did not affect stomatal aperture (data not shown).

I examined the effects of an H_2O_2 scavenger, CAT, peroxidase inhibitors, SHAM and NaN_3, and an NADPH oxidase inhibitor, DPI, on AITC-, 3BN-, and ESCN-induced stomatal closure (Fig. 2.1B). The degradation product-induced stomatal closure were significantly inhibited by 100 units/mL CAT, 1 mM SHAM, and 1 mM NaN_3, suggesting that the glucosinolate degradation products induce stomatal closure *via* ROS production mediated by peroxidases. On the other hand, the degradation product-induced stomatal closure were partially inhibited by 20 μM DPI (Fig. 2.1B). CAT, SHAM, NaN_3**,** and DPI did not affect stomatal aperture by themselves (data not shown).

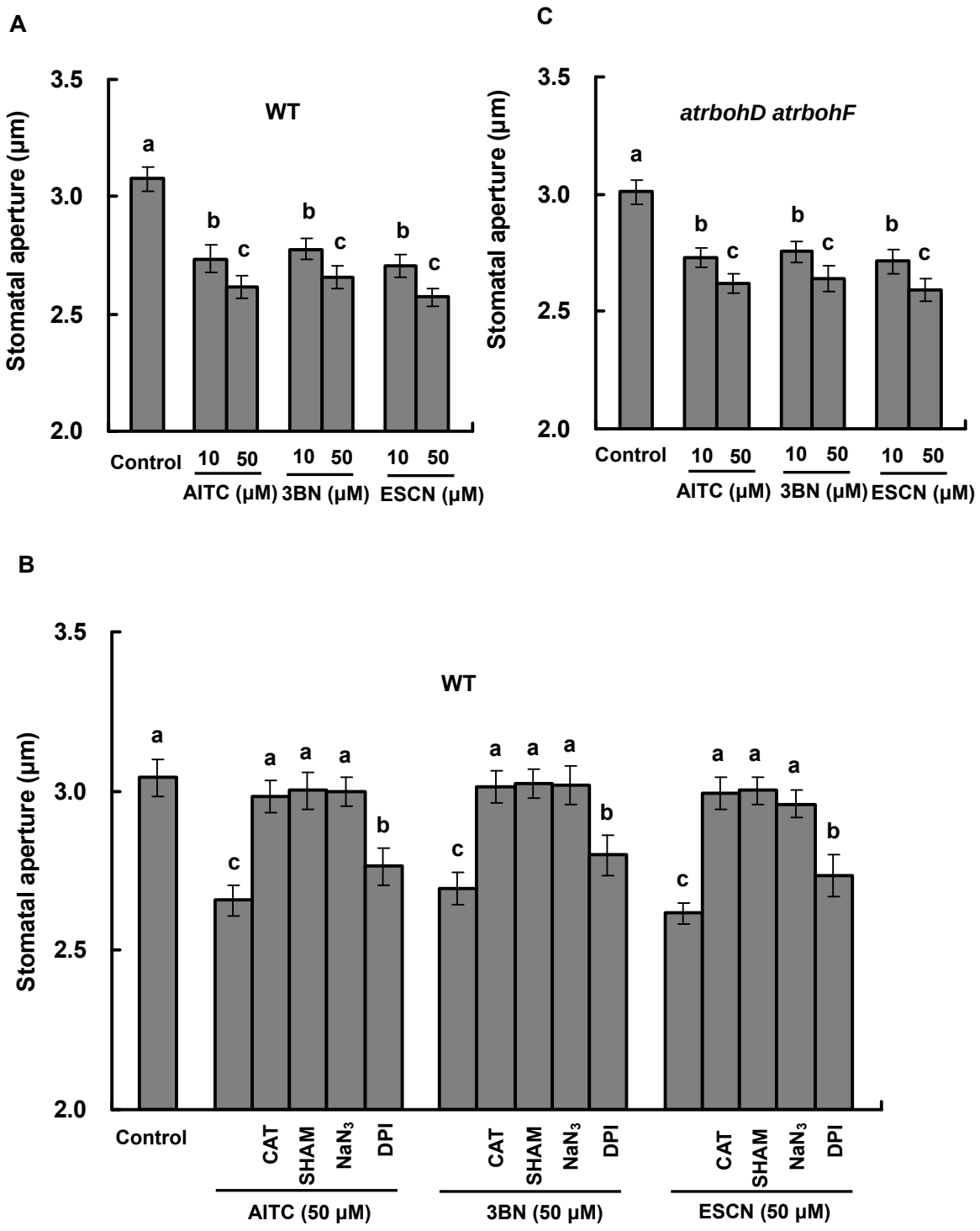

Fig. 2.1 Glucosinolate degradation product-induced stomatal closure in Arabidopsis. (A) AITC, 3BN, and ESCN at 50 μM induced stomatal closure in wild-type plants (n = 3, 60 stomata for each). (B) AITC-, 3BN-, and ESCN-induced stomatal closure were inhibited by 100 units mL^{-1} CAT, 1 mM SHAM, 1 mM NaN_3, and 20 μM DPI for 30 min (n = 3, 60

stomata for each). (C) AITC, 3BN, and ESCN at 50 μM induced stomatal closure in *atrbohD atrbohF* mutant plants (n = 3, 60 stomata for each). Error bars represent SE.

Application of AITC, 3BN, and ESCN at 10 μM and 50 μM significantly induced stomatal closure in a dose-dependent manner in *atrbohD atrbohF* mutant plants as wild-type plants (Fig. 2.1C).

2.4.2 Glucosinolate degradation product-induced extracellular ROS production in whole leaves

I histochemically examined accumulation of H_2O_2 and O_2^- in rosette leaves using DAB (Thordal-Christensen et al. 1997) and NBT (Doke 1983), respectively. AITC, 3BN, and ESCN at 50 μM significantly induced H_2O_2 production in rosette leaves (Fig. 2.2A), which were significantly inhibited by 1 mM SHAM (Fig. 2.2A). AITC, 3BN, and ESCN at 50 μM significantly induced O_2^- production in rosette leaves (Fig. 2.2B), which were also significantly inhibited by 1 mM SHAM (Fig. 2.2B).

2.4.3 Glucosinolate degradation product-induced intracellular ROS accumulation in guard cells

I examined whether glucosinolate degradation products induce ROS accumulation in guard cells using the fluorescent dye H_2DCF-DA. Application of 50 μM AITC significantly induced ROS accumulation in guard cells of wild-type plants (Fig. 2.3A) as previous reported (Khokon et al. 2011a). Like AITC, 3BN and ESCN at 50 μM significantly induced ROS accumulation in guard cells of wild-type plants. The ROS accumulation was significantly inhibited by pretreatment with 100 units/mL CAT and 1 mM SHAM but not by 20 μM DPI (Fig. 2.3A). AITC, 3BN, and ESCN at 50 μM also

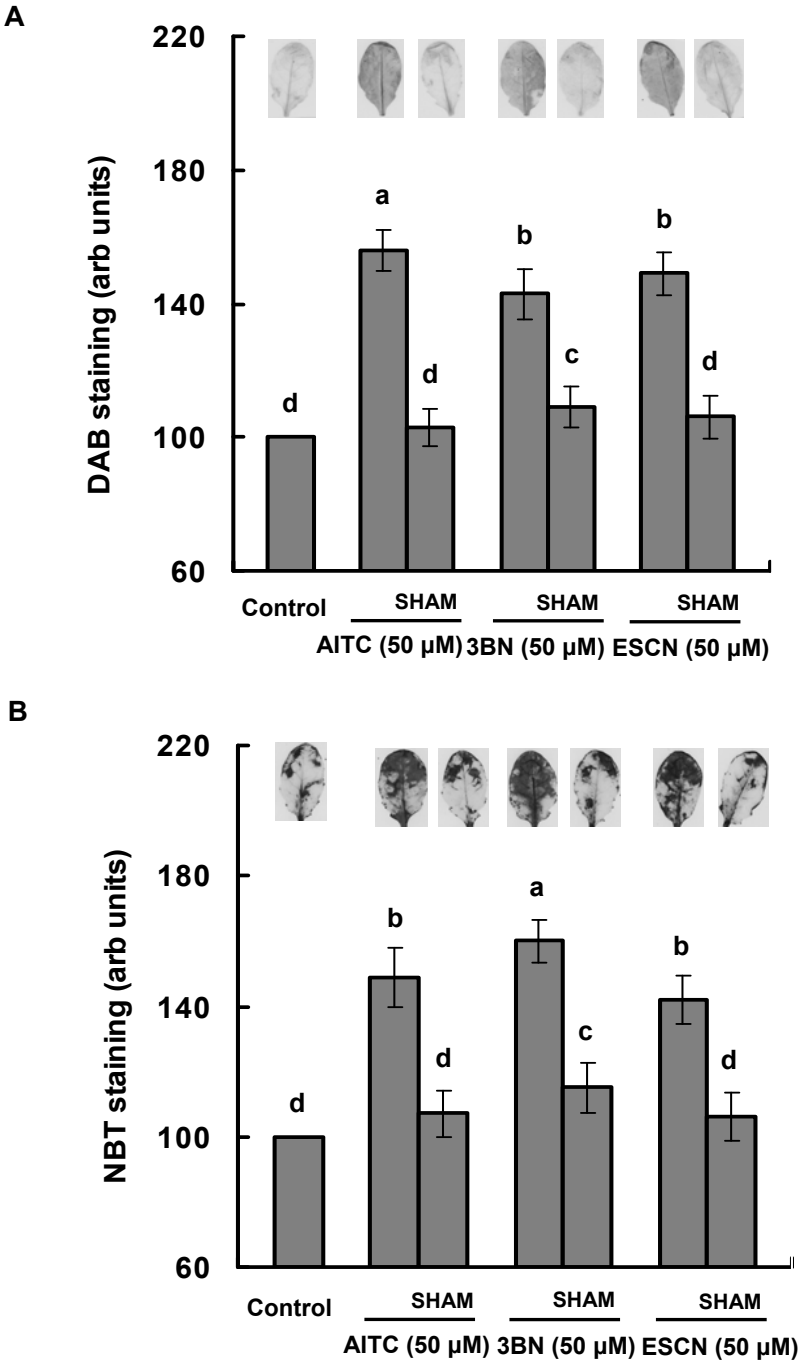

Fig. 2.2 ROS production in whole leaves by degradation products of glucosinolate. (A) AITC, 3BN, and ESCN at 50 μM induced H_2O_2 production in leaves. The degradation product-induced H_2O_2 accumulation was inhibited by 1 mM SHAM for 30 min. (B) AITC, 3BN, and ESCN at 50 μM induced superoxide (O_2^-) production in leaves. The degradation product-induced O_2^- production was inhibited by 1 mM SHAM for 30 min. Both DAB and

NBT staining were quantified based on arbitrary value of pixel intensity of stained area of leaves. Average from three independent experiments (12 leaves per bar) are shown. Error bars represent SE.

induced ROS accumulation in guard cells of *atrbohD atrbohF* mutant plants (Fig. 2.3B). There are no significant differences in fluorescence intensity between the wild type and the mutant (Fig. 2.3A, B).

2.4.4 Glucosinolate degradation product-induced NO production in guard cells

AITC-induced stomatal closure is accompanied by NO production (Khokon et al. 2011a). I examined the effect of an NO specific scavenger, cPTIO, on AITC-, 3BN-, and ESCN-induced stomatal closure in wild-type plants (Fig. 2.4). The degradation product-induced stomatal closure were partially inhibited by cPTIO at 100 μM (Fig. 2.4A) whereas the ROS accumulation in guard cells were not significantly inhibited by cPTIO (Fig. 2.4B). AITC, 3BN, and ESCN at 50 μM significantly induced NO production in guard cells, which were significantly abolished by SHAM at 1 mM (Fig. 2.4C).

2.4.5 Glucosinolate degradation product-induced cytosolic Ca^{2+} oscillations in guard cells

I examined whether the degradation products induce $[Ca^{2+}]_{cyt}$ oscillations in guard cells using YC3.6-expressing wild-type plants. AITC at 50 μM induced $[Ca^{2+}]_{cyt}$ oscillation in 86% of SHAM-untreated guard cells (n = 19 of 22 cells; Fig. 2.5A, C) as previously shown (Khokon et al. 2011a) whereas AITC at 50 μM induced $[Ca^{2+}]_{cyt}$ oscillation in 30% of SHAM-treated wild-type guard cells (n = 6 of 20 cells; Fig. 2.5B, C).

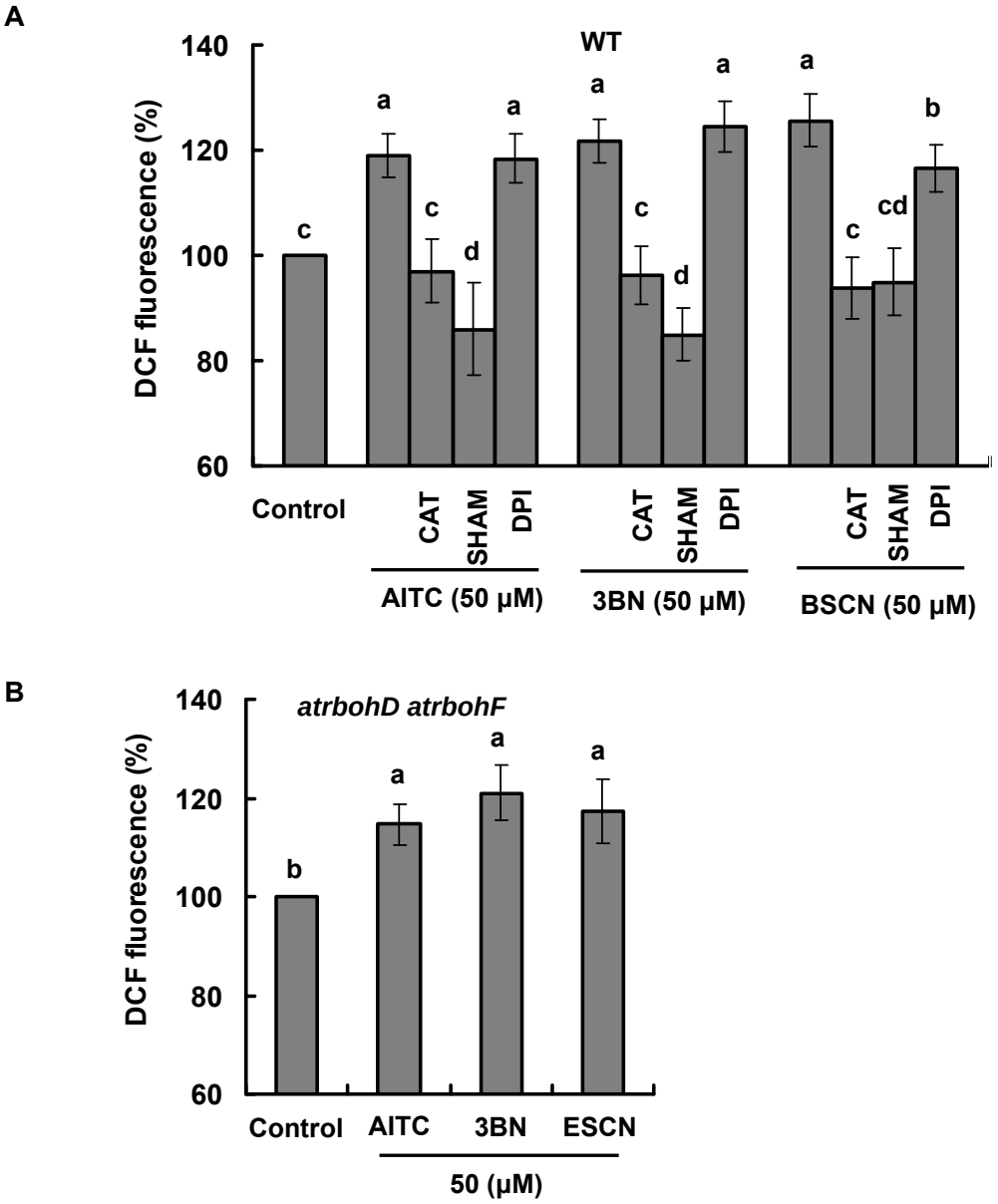

Fig. 2.3 Glucosinolate degradation product-induced ROS accumulation in guard cells. (A) AITC, 3BN, and ESCN at 50 μM induced ROS accumulation in guard cells of wild-type plants. ROS accumulation was inhibited by 100 units mL^{-1} CAT, and 1 mM SHAM for 30 min but not by 20 μM DPI (n = 3, > 60 stomata). (B) AITC, 3BN, and ESCN at 50 μM induced ROS accumulation in guard cells of *atrbohD atrbohF* plants (n = 3, > 60 stomata). The vertical scale represents the percentage of DCF fluorescent levels when fluorescent intensities of treated cells are normalized to the control value taken as 100%. Error bars represent SE.

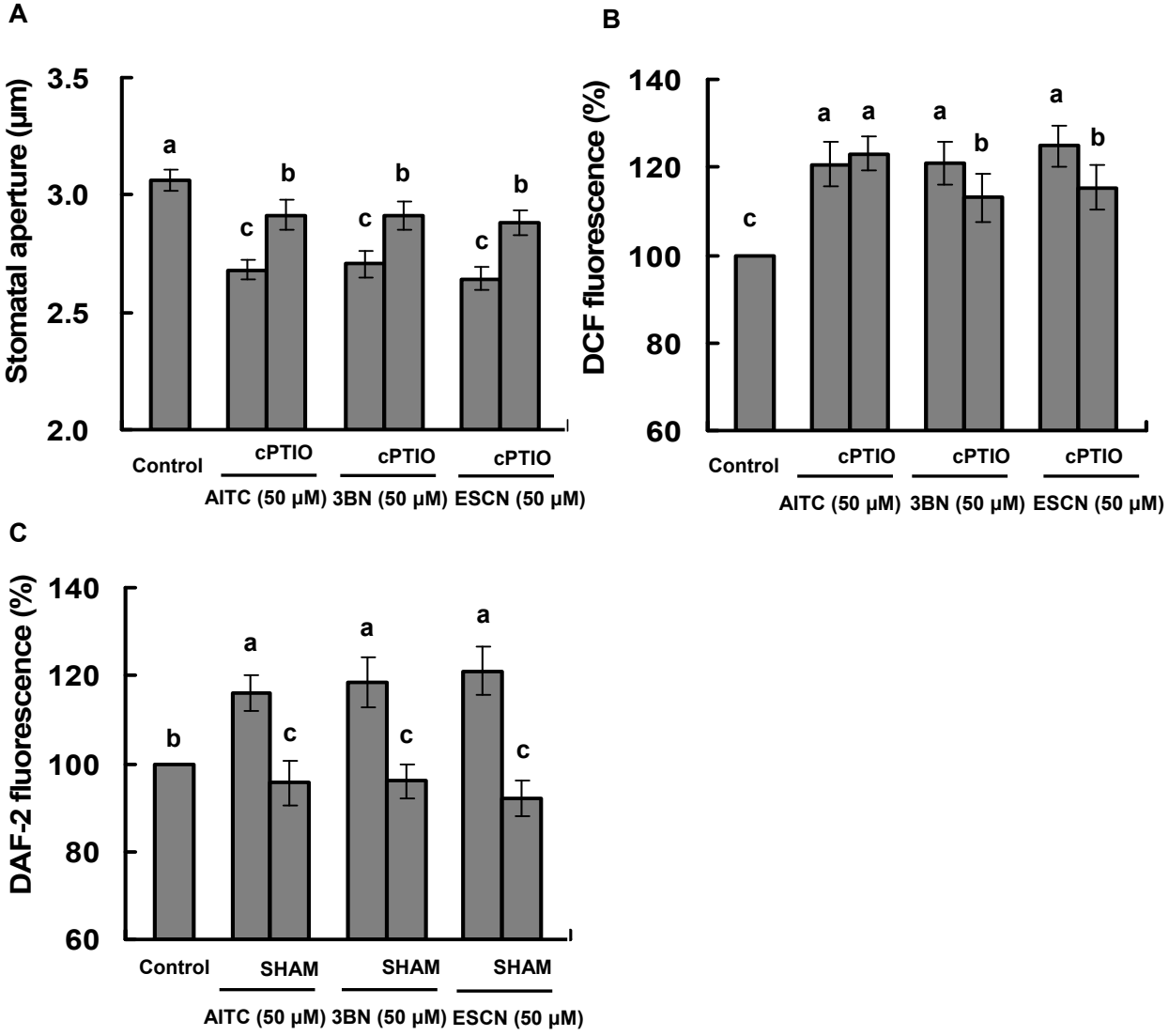

Fig. 2.4 Glucosinolate degradation product-induced NO production in guard cells. (A) AITC-, 3BN-, and ESCN-induced stomatal closure was inhibited by 100 μM cPTIO for 30 min (n = 3, 60 stomata for each). (B) AITC, 3BN, and ESCN at 50 μM induced ROS accumulation in guard cells. ROS accumulation was not inhibited by 100 μM cPTIO for 30 min (n = 3, > 60 stomata). (C) AITC, 3BN, and ESCN at 50 μM induced NO accumulation in guard cells. NO accumulation was inhibited by 1 mM SHAM for 30 min (n = 3, > 60 stomata). The vertical scale represents the percentage of DCF fluorescence levels for ROS and of DAF-2 fluorescent levels for NO when the fluorescence intensities of treated cells are normalized to the control value taken as 100%. Error bars represent SE.

Like AITC, 3BN at 50 μM induced $[Ca^{2+}]_{cyt}$ oscillation in 75% of SHAM-untreated guard cells (*n* = 22 of 26 cells; Fig. 2.5D, F) while 3BN at 50 μM induced $[Ca^{2+}]_{cyt}$ oscillation in 29% of SHAM-treated guard cells (*n* = 7 of 24 cells; Fig. 2.5E, F). In turn, ESCN triggered $[Ca^{2+}]_{cyt}$ oscillation in 85% of SHAM-untreated guard cells (*n* = 22 of 26 cells; Fig. 2.5G, I) and 27% of SHAM-treated guard cells (*n* = 6 of 22 cells; Fig. 2.5H, I).

2.5 Discussion

2.5.1 Glucosinolates degradation products, isothiocynates, nitriles, and thiocyanates, induce stomatal closure

Glucosinolates are secondary metabolities in crucifer plants. Myrosinases are responsible for the degradation of glucosinolates, resulting in the formation of a variety of products that are active against plant enemies (e.g. herbivores, insects, and pathogens) (Brader et al. 2001; Halkier and Gershenzon 2006). On the other hand, contents of glucosinolate in young sprouts of broccoli 70-100 $\mu molg^{-1}FW$ (Fahey et al. 2001), and contents of ITCs in grinded leaves of Arabidopsis were approximately 10 $\mu molg^{-1}FW$ (Khokon et al. 2011a). Hence, contents of the degradation products can reach sub-mM level in leaves and the degradation products of glucosinolate may induce stomatal closure in plants attacked by enemies such as herbivores.

ROS functions as a second messenger in stomatal closure. ABA and MeJA induce ROS production in guard cells mediated by NADPH oxidases, resulting in stomatal closure (Pei et al. 2000; Murata et al. 2001; Munemasa et al. 2007). In the present study, the degradation products, AITC, 3BN, and ESCN, induced stomatal closure, which were inhibited by CAT and SHAM (Fig. 2.1B), and induced O_2^- and H_2O_2 production in whole rosette leaves, which were inhibited by SHAM (Fig. 2.2), suggesting that the degradation products induce stomatal closure accompanied by extracellular peroxidase-mediated ROS

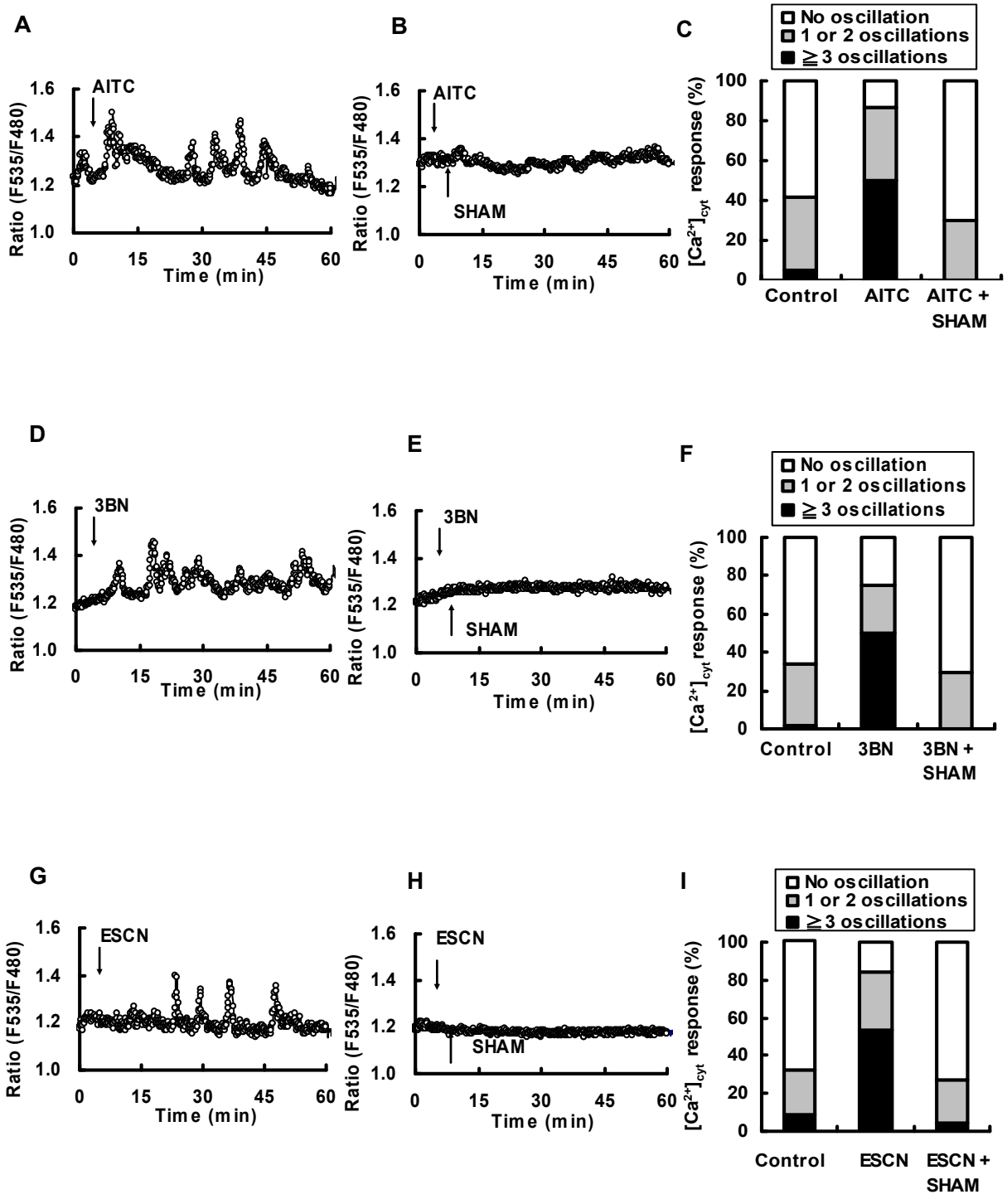

Fig. 2.5 AITC-, 3BN-, and ESCN-induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells of wild-type (WT) plants. Representative data of fluorescence emission ratio (535/480 nm) showing 50 μM AITC-induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells untreated with SHAM (A) (n = 19 of 22 cells; 86%) and treated with SHAM (B) (n = 6 of 20 guard cells; 30%). (C) Stack

column representation of AITC-induced $[Ca^{2+}]_{cyt}$ oscillation (%) in WT guard cells treated with DMSO (control; *n* = 20), AITC (*n* = 22), and SHAM + AITC (*n* = 20). (D) 3BN at 50 μM induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells untreated with SHAM (*n* = 22 of 26 guard cells; 75%) and treated with SHAM (E) (*n* = 7 of 24 cells; 29%). (F) Stack column representation of 3BN-induced $[Ca^{2+}]_{cyt}$ oscillation (%) in WT guard cells treated with DMSO (control; *n* = 18), 3BN (*n* = 20), and SHAM + 3BN (*n* = 24). (G) ESCN at 50 μM induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells untreated with SHAM (*n* = 22 of 26; 85%) and treated with SHAM (H) (*n* = 6 of 22 cells; 27%). (I) Stack column representation of ESCN-induced $[Ca^{2+}]_{cyt}$ oscillation (%) in WT guard cells treated with DMSO (control; *n* = 20), ESCN (*n* = 26), and SHAM + ESCN (*n* = 22). Vertical arrow indicates when guard cells were treated with 50 μM AITC, 3BN, and ESCN. Error bars represent SE.

production. In tobacco suspension cultured cells and *Vicia faba* guard cells, SA-induced O_2^- and H_2O_2 production are mediated by SHAM-sensitive extracellular peroxidases (Kawano and Muto 2000; Mori et al. 2001). Moreover, yeast elicitor (YEL)- and chitosan (CHT)-induced ROS production are also mediated by SHAM-sensitive extracellular peroxidases (Khokon et al. 2010a, b). Taken together, extracellular peroxidases may be a key enzyme in ROS production that induces stomatal closure.

SHAM inhibits peroxidases (Martinez et al. 1998; Mori et al. 2001; Khokon et al. 2010a, b, 2011b) but is not so specific to inhibition of peroxidases because SHAM also inhibits alternative oxidases (Ikeda-Saito et al. 1991) and lipoxygenases (Magnani et al. 1989). Hence, it is impossible to exclude the involvement of alternative oxidases and lipoxygenases. However, another inhibitor of peroxidases, NaN_3 (Martinez et al. 1998; Garrido et al. 2012) also inhibited the stomatal closure induced by the degradation products (Fig. 2.1), which supports our conclusion that SHAM-sensitive peroxidases are

involved in the stomatal closure.

Arabidopsis has 73 classes III peroxidase genes (cell wall peroxidase) (Tognolli et al. 2002) and a large number of class III peroxidases are involved in the apoplastic oxidative burst for pathogen resistance in Arabidopsis (Bindschedler et al. 2006). The class III peroxidases may be involved in the ROS production induced by AITC, 3BN, and ESCN but involvement of other peroxidases cannot be excluded.

NADPH oxidases, AtRBOHD and AtRBOHF, are involved in ABA-induced ROS production in guard cells of Arabidopsis (Pei et al. 2000; Kwak et al. 2003; Munemasa et al. 2007). On the contrary, neither *atrbohD atrbohF* mutation nor application of DPI impairs the degradation product-induced ROS production (Fig. 2.3), suggesting that the degradation product-induced ROS production in guard cells is mediated by peroxidases but not by NADPH oxidases. However, DPI slightly inhibited the degradation product-induced stomatal closure. This may be because DPI is an inhibitor of flavoprotein including NADPH oxidases.

Furthermore, ITCs are potent inhibitors of leukocytic NADPH oxidases (Miyoshi et al. 2004). Hence, it is unlikely that NADPH oxidases are responsible for the degradation product-induced stomatal closure.

2.5.2 Sharing signal components with other signaling

The AITC-, 3BN-, and ESCN-induced NO production were completely inhibited by SHAM (Fig. 2.4C) and AITC, 3BN, and ESCN induced $[Ca^{2+}]_{cyt}$ oscillations, which were inhibited by SHAM (Fig. 2.5), suggesting that AITC, or 3BN, or ESCN signaling shares signal components downstream of ROS production with ABA and MeJA signaling. However, ABA-induced stomatal closure and ROS production were not inhibited by SHAM (Khokon et al. 2010a), indicating that SHAM-sensitive peroxidases are not

involved in ABA signaling and that the degradation product signaling is not completely overlapped with ABA signaling.

Stomatal closure induced by degradation products of glucosinolates, YEL, CHT, and SA, which are inhibited by SHAM (Fig. 2.1B, Khokon et al. 2010a,b, 2011b), suggesting involvement of activation of peroxidases in these stomatal closure. However, degradation products of glucosinolate-, YEL-, and chitosan-induced stomatal closure are accompanied by $[Ca^{2+}]_{cyt}$ oscillation (Fig. 2.5A, D, G, Khokon et al. 2010a, b) but SA-induced stomatal closure is not accompanied by $[Ca^{2+}]_{cyt}$ oscillation (Khokon et al. 2011b). Taken together, these results suggest that ROS production and $[Ca^{2+}]_{cyt}$ oscillation/elevation function not only in tandem but also in parallel in guard cells signaling.

In summary, the degradation products may elicit extracellular ROS production, followed by intracellular ROS accumulation and $[Ca^{2+}]_{cyt}$ oscillation/elevation, causing stomatal closure.

2.5.3 Physiological significance of degradation products of glucosinolates

Hydrolysis of glucosinolates on plant damage results in formation of ITCs, nitriles, and thiocyanates and what type of degradation products forms depend on pH, chemical structure of glucosinolates, and so on (Bones and Rossiter 1996; Husebye et al. 2002). For example, ITCs are generally produced at neutral pH, while nitriles at lower pH (Macleod and Rossiter 1986; Galletti et al. 2001). In animal cells, ITCs strongly inhibit carcinogenesis and tumorigenesis and induce apoptosis because of their electrophilicity (Miyoshi et al. 2007; Hayes et al. 2008). Moreover, animal cells are more susceptible to ITCs than other degradation products because ITCs are much more electrophilic than other degradation products, nitriles and thiocyanates (Duncan and Milne 1993; Hayes et

al. 2008).

Low concentration of AITC increases glutathione *S*-transferase (GST) activity in Arabidopsis (Hara et al. 2010), although high concentration (> 1 mM) of exogenous AITC is harmful in Arabidopsis (Hara et al. 2010) and *Lactuca sativa* (Brown and Morra 1996). In this study, isothiocyanates, nitriles, and thiocyanates induce stomatal closure in Arabidopsis (Fig. 2.1A), suggesting that the degradation products regulate physiological functions including stomatal movements against attack of insects, pathogens, and herbivores regardless of the condition for glucosinolate degradation in crucifer plants.

2.5.4 Conclusion

The degradation products of glucosinolates, ITCs, nitriles, and thiocyanates induce stomatal closure accompanied by SHAM-sensitive peroxidases-dependent ROS accumulation, NO production, and $[Ca^{2+}]_{cyt}$ oscillations in guard cells.

Chapter 3

Allyl isothiocyanate-induced stomatal closure impaired by *tgg1 tgg2* mutation in Arabidopsis

3.1 Abstract

Myrosinase (thioglucoside glucohydrolase, TGG) is an enzyme that catalyzes the hydrolysis of glucosinolates to form isothiocyanates (ITCs) and is involved in abscisic acid (ABA)- and methyl jasmonate (MeJA)-induced stomatal closure in Arabidopsis. One of myrosinase products, allyl isothiocyanate (AITC), induces stomatal closure in Arabidopsis. I investigated function of myrosinases in guard cell signaling and examined AITC signal pathway in guard cells. AITC induced stomatal closure in the *tgg1* and the *tgg2* single mutants but not in the *tgg1 tgg2* double mutant, suggesting that TGG1 and TGG2 have other functions than hydrolysis of glucosinolates in guard cell signaling. It also caused production of reactive oxygen species (ROS) and nitric oxide (NO), cytosolic alkalization and oscillation of the cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) in guard cells of the wild type and all mutants, suggesting that TGG1 and TGG2 function downstream of these signal events or independently from them. AITC-induced stomatal closure was not impaired in an NADPH oxidase mutant (*atrobhD atrobohF*) but was inhibited by a peroxidase inhibitor, salicylhydroxamic acid (SHAM), suggesting that AITC-induced ROS production is mediated by SHAM-sensitive peroxidases.

3.2 Introduction

Stomata, which are formed by pairs of guard cells, mediate transpiration and carbon dioxide uptake. Guard cells can shrink and swell in response to various physiological stimuli, resulting in stomatal closure and opening (Shimazaki et al. 2007; Kim et al. 2010).

Myrosinases, β-thioglucoside glucohydrolases (TGGs), (EC 3.2.1.147) abundantly present in crucifer plants and catalyze degradation of glucosinolates, resulting in the formation of variety of products such as isothiocyanates (ITCs) that have repellent effect on insects and herbivores (Lambrix et al. 2001; Yan and Chen 2007). Six *TGG* genes have been identified and two functional myrosinase genes, *TGG1* and *TGG2*, have been found in the Arabidopsis genome (Xue et al. 1995; Husebye et al. 2002; Barth and Jander 2006). Abscisic acid (ABA)- and methyl jasmonate (MeJA)-induced stomatal closure were impaired in the *tgg1 tgg2* mutants (Islam et al. 2009). However, it remains unclear whether TGG1 and TGG2 function as enzyme myrosinase in ABA and MeJA signaling in guard cells. Recently, we found that one of glucosinolate hydrolysates, allyl isothiocyanate (AITC), induces stomatal closure, which is accompanied by reactive oxygen species (ROS) and nitric oxide (NO) production, and cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) oscillation in guard cells (Khokon et al. 2011a).

ABA and MeJA induce stomatal closure accompanied by production of ROS in guard cells, which is mediated by NADPH oxidases (Kwak et al. 2003; Suhita et al. 2004). In contrast, salicylic acid-, yeast elicitor-, and chitosan-induced ROS production are mediated by salicylhydroxamic acid (SHAM)-sensitive cell wall peroxidases (Mori et al. 2001; Khokon et al. 2010a, b, 2011b). AITC-induced ROS production was slightly

inhibited by an NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) (Khokon et al. 2011a), but it is unsettled which enzyme mediates ROS production in guard cell AITC signaling.

ABA- and MeJA-induced ROS production results in activation of Ca^{2+}-permeable non-selective cation channels on the plasma membrane, leading to $[Ca^{2+}]_{cyt}$ oscillation during stomatal closure (Pei et al. 2000; Kwak et al. 2003; Munemasa et al. 2011b). Moreover, TGG1 and TGG2 function downstream of ROS production in ABA and MeJA signaling (Islam et al. 2009). Furthermore, AITC also induces ROS production and $[Ca^{2+}]_{cyt}$ oscillation in guard cells (Khokon et al. 2011a).

In order to clarify whether TGG1 and TGG2 function as enzyme myrosinases to hydrolyze glucosinolates in guard cell ABA and MeJA signaling, I investigated whether one of myrosinase products, AITC, induces stomatal closure in the *tgg* mutants or I examined stomatal movement, ROS and NO production, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillations in guard cells of *tgg* mutants in response to AITC. Additionally, in order to clarify which enzymes, NADPH oxidases or cell wall peroxidases, mediate AITC-induced ROS production, we examined response to AITC in the *atrbohD atrbohF* mutant and effects of SHAM on AITC-induced stomatal responses.

3.3 Materials and Methods

3.3.1 Plant materials and growth conditions

Arabidopsis thaliana wild type (WT) (ecotype Columbia-0) and *tgg1* (At5g26000), *tgg2* (At5g25980), *tgg1 tgg2*, and *atrbohD atrbohF* mutant plants were grown in a growth chamber following previous method discussed in chapter 2. $[Ca^{2+}]_{cyt}$ oscillations in guard cells was measured using a Ca^{2+}-sensing fluorescent protein,

Yellow Cameleon 3.6 (YC3.6) (Nagai et al. 2004; Mori et al. 2006). To obtain YC3.6-expressing plants, wild type and *tgg1 tgg2* double mutant plants were crossed with a Col-0 plant that had previously been transformed with YC3.6.

3.3.2 Measurement of stomatal aperture

Stomatal apertures were measured as described previously in chapter 2 (Murata et al. 2001). Note that inhibitor, salicylhydroxamic acid (SHAM) at 1 mM, was added 30 min before application of AITC.

3.3.3 Measurement of ROS and NO production

ROS and NO production in guard cells was examined by using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma, St Louis, MO, USA) and 5 μM of 4,5-diaminofluorescein-2diacetate (DAF-2DA) (Sigma), respectively, as described previously in chapter 2 (Munemasa et al. 2007).

3.3.4 Measurement of cytosolic pH

Cytosolic pH in guard cells was examined using 2′,7′-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Islam et al. 2010a). The epidermal tissues were incubated for 3 h in an assay solution containing 50 mM KCl and 10 mM MES-KOH (pH 6.5), and then 20 μM BCECF-AM was added to the sample. The epidermal tissues were incubated for 30 min in the dark at room temperature, and then the excess dye was washed out with the assay solution. The image was obtained and analyzed as described above.

3.3.5 Measurement of $[Ca^{2+}]_{cyt}$ oscillations

Four- to 6- week-old wild-type and *tgg1 tgg2* plants expressing YC3.6 were used for the measurement of guard cell $[Ca^{2+}]_{cyt}$ oscillations as described previously in chapter 2 (Islam et al. 2010b).

3.3.6 Statistical analysis

The significance of differences between mean values was assessed using Student's *t*-test. Differences in the frequency of $[Ca^{2+}]_{cyt}$ oscillations in the wild type and *tgg* mutants induced by AITC was determined by χ^2-test. Differences were considered significant for *P* values < 0.05. Data are means ± SE.

3.3.7 Accession numbers

Arabidopsis genome initiative numbers for the genes discussed in this article are as follows: *TGG1* (At5g26000) and *TGG2* (At5g25980).

3.4 Results

3.4.1 Stomatal response of *tgg1 tgg2* mutant

I examined stomatal response to AITC in *tgg1*, *tgg2*, and *tgg1 tgg2* mutants. Here, I tested 50 μM and 100 μM AITC because our previous results showed that AITC contents in grinded leaves of Arabidopsis were approximately 10 $\mu molg^{-1}FW^{-1}$ (Khokon et al. 2011a). AITC at 50 μM and 100 μM induced stomatal closure in the wild type ($P < 0.004$ and $P < 10^{-3}$) (Fig. 3.1A), in agreement with previous results (Khokon et al. 2011a). The application of AITC at 50 μM and 100 μM induced stomatal closure in the *tgg1* mutant ($P < 0.004$ and $P < 10^{-3}$) and the *tgg2* mutant ($P < 0.002$ and $P < 10^{-3}$) but not in the *tgg1 tgg2* mutant ($P = 0.17$ and $P = 0.08$) (Fig. 3.1A). Neither ABA nor MeJA

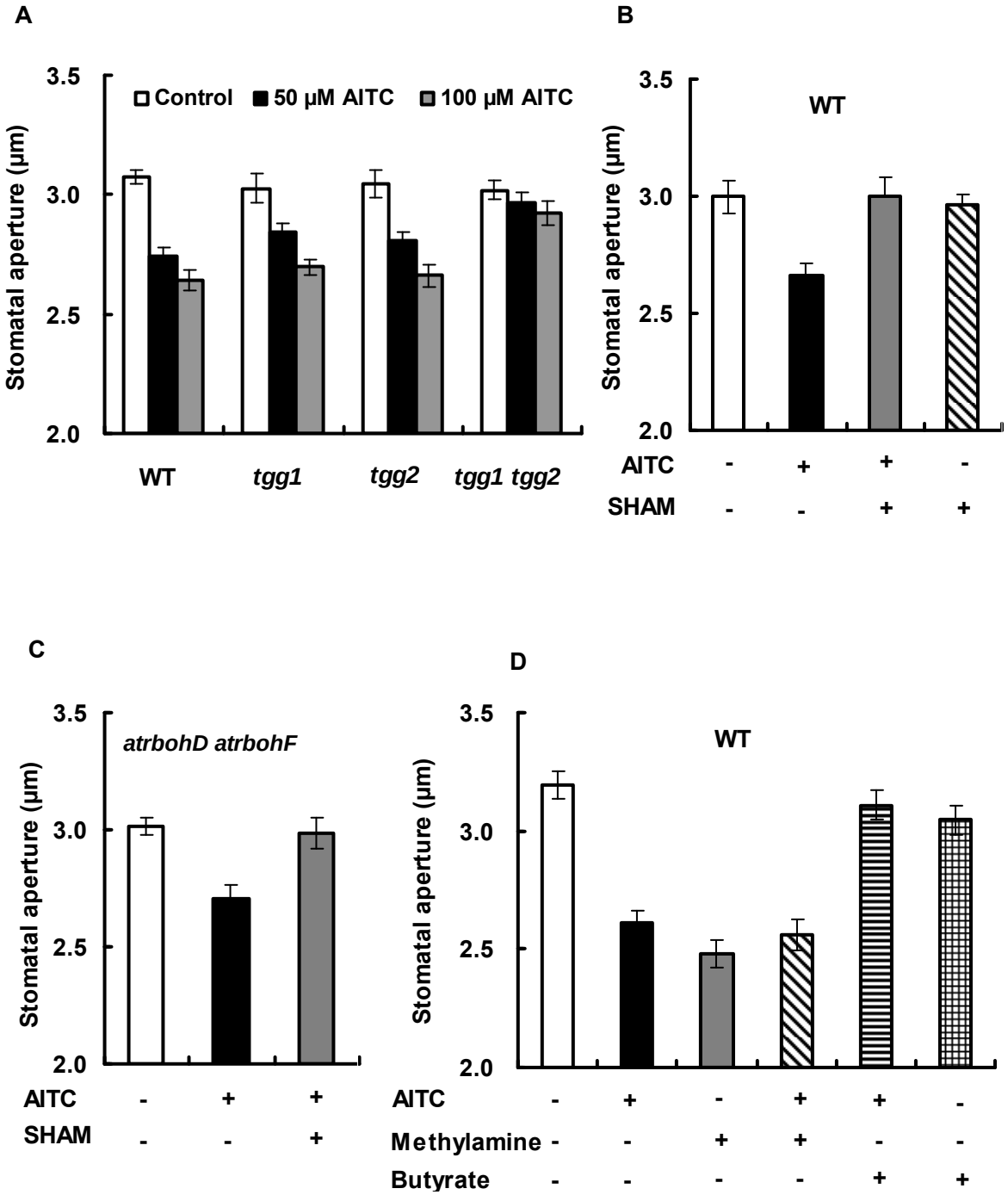

Fig. 3.1 Stomatal movement in Arabidopsis in response to allyl isothiocyanate (AITC). (A) AITC at 50 μM (black bars) and 100 μM (ash bars) induced stomatal closure in wild type, *tgg1-3*, and *tgg2-1,* but not in *tgg1-3 tgg2-1*. (B) Effect of 1 mM salicylhydroxamic acid (SHAM) (gray bars) on 50 μM AITC-induced stomatal closure

in wild-type plants (black bars). (C) AITC at 50 μM (black bars) induced stomatal closure in *atrbohD atrbohF* mutant. AITC-induced stomatal closure was inhibited by 1 mM SHAM (gray bars). (D) Rosette leaves of wild-type plants were incubated for 3 h in the light and then were treated with sodium butyrate (0.5 mM) or methylamine (2 mM). Then, stomatal apertures were measured after 3 h incubation. AITC at 50 μM was added 10 min after butyrate or methylamine application. Averages from three independent experiments (60 stomata per bar) are shown. Error bars represent SE.

induces stomatal closure in the *tgg1 tgg2* mutant (Islam et al. 2009). These results indicate that TGG1 and TGG2 cooperatively function in stomatal closure in response to AITC, ABA, and MeJA.

I investigated which enzymes, cell wall peroxidases or plasma membrane NADPH oxidases, mediate ROS production in guard cell AITC signaling. SHAM at 1 mM inhibited AITC-induced stomatal closure in the wild type ($P < 0.003$) (Fig. 3.1B). Moreover, AITC induced stomatal closure in the *atrbohD atrbohF* mutant ($P < 10^{-3}$), which was inhibited by SHAM ($P < 0.006$) (Fig. 3.1C). These results suggest that AITC-induced stomatal closure is mediated by cell wall peroxidases but not by plasma membrane NADPH oxidases.

I examined the effects of an alkalinizing agent, methylamine, and an acidifying agent, butyrate, on AITC-induced stomatal closure in wild-type plants. AITC-induced stomatal closure was inhibited by 0.5 mM butyrate but not by 2 mM methylamine ($P = 0.19$) (Fig. 3.1D). These results suggest that AITC-induced stomatal closure is accompanied by cytosolic alkalization in guard cells.

3.4.2 AITC-induced ROS and NO accumulation in guard cells

I examined whether AITC induced ROS and NO accumulation in guard cells of the wild type and *tgg* mutants, to investigate involvement of TGG1 and TGG2 in AITC-induced ROS and NO accumulation in guard cells. AITC at 50 μM induced ROS accumulation in guard cells of the wild type (Fig. 3.2A), in agreement with previous results (Khokon et al. 2011a). The application of 50 μM AITC induced ROS accumulation in guard cells of the *tgg1* ($P < 0.003$), *tgg2* ($P < 10^{-3}$), and *tgg1 tgg2* mutants ($P < 0.003$) (Fig. 3.2A).

Production of NO in guard cells was examined using DAF-2DA. AITC at 50 μM induced NO production in guard cells of the wild type (Fig. 3.2B) as reported previously (Khokon et al. 2011a). Like wild type, AITC at 50 μM induced NO production in guard cells of the *tgg1* ($P < 0.002$), *tgg2* ($P < 10^{-3}$), and *tgg1 tgg2* mutants ($P < 0.007$) (Fig. 3.2B). Together, these results suggest that neither TGG1 nor TGG2 function upstream of ROS and NO production in guard cells of AITC signaling.

3.4.3 AITC-induced cytosolic alkalization in guard cells

ABA and MeJA induce cytosolic alkalization in guard cells, which leads to stomatal closure (Suhita et al. 2004; Gonugunta et al. 2008; Islam et al. 2010a, b). To investigate involvement of TGG1 and TGG2 in AITC-induced cytosolic alkalization in guard cells, I examined cytosolic pH in guard cells of the wild type and *tgg* mutants using a pH-sensitive fluorescent dye, BCECF-AM. The application of AITC at 50 μM induced cytosolic alkalization in guard cells of the *tgg1* ($P < 10^{-3}$), *tgg2* ($P < 10^{-3}$), *tgg2* ($P < 10^{-3}$), *tgg1 tgg2* ($P < 0.007$) mutants as well as wild-type plants ($P < 10^{-3}$) (Fig. 3.3). These results suggest that either TGG1 or TGG2 does not function upstream of

cytosolic alkalization in guard cells in response to AITC.

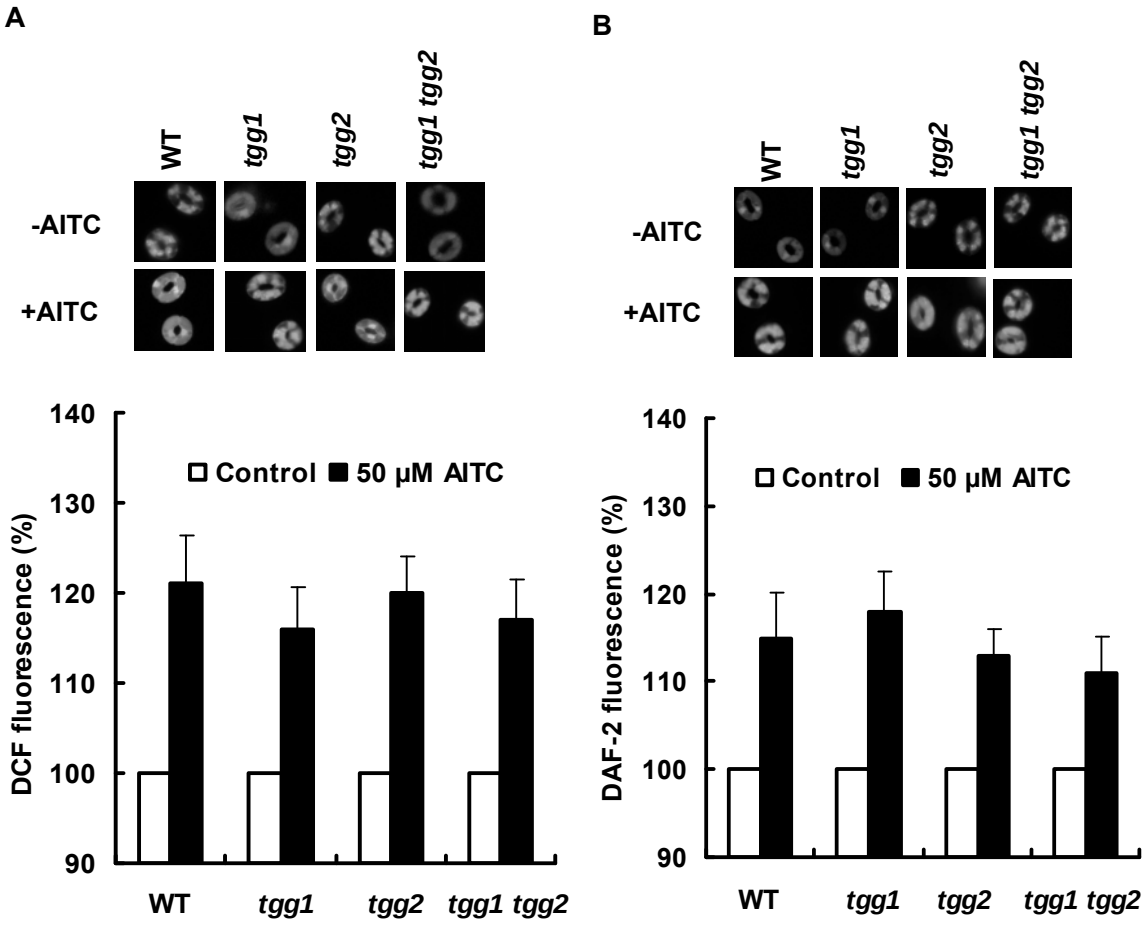

Fig. 3.2 AITC-induced ROS and NO accumulation in guard cells of wild type and *tgg* mutants. (A) Representative gray-scale DCF fluorescence images (upper panel). ROS accumulation as shown by DCF fluorescence in wild type and *tgg* mutants (lower panel). (B) Representative gray-scale DAF-2 fluorescence images wild type and *tgg* mutants (upper panel). NO accumulation as shown by DAF-2 fluorescence in wild type and *tgg* mutants (lower panel). In each graph, fluorescence intensity was normalized to the control values. Data were obtained from more than 60 guard cells. Error bars represent SE.

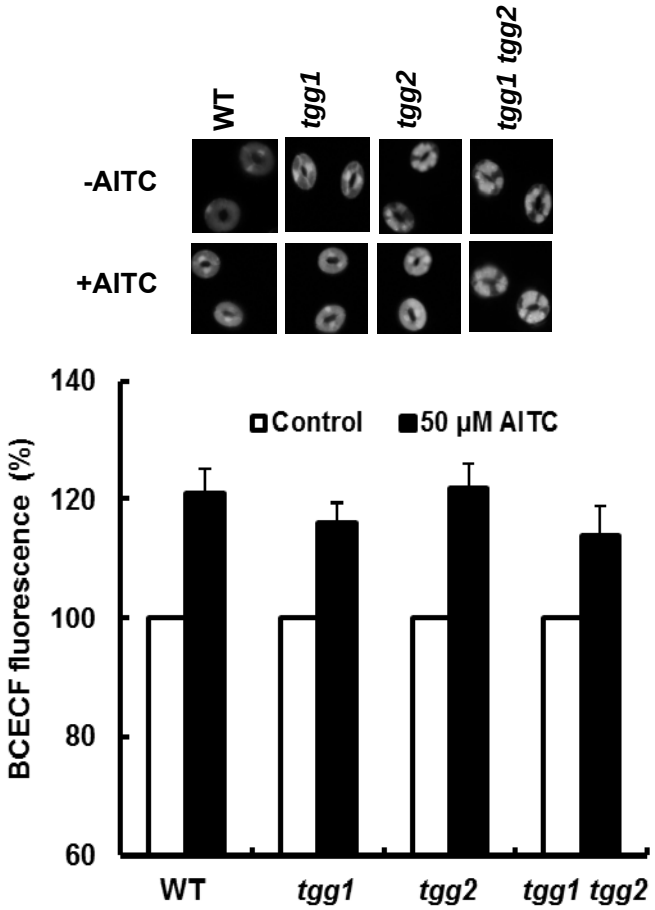

Fig. 3.3 Allyl isothiocyanate (AITC)-induced cytosolic alkalization in guard cells of wild type (WT) and *tgg* mutants. Representative gray-scale BCECF fluorescence images (upper panel). Cytosolic alkalization as shown by BCECF fluorescence in WT, *tgg1*, *tgg2*, and *tgg1 tgg2* (lower panel). In each graph, fluorescence intensity was normalized to the control values. Data were obtained from more than 60 guard cells. Error bars represent SE.

3.4.4 AITC-induced $[Ca^{2+}]_{cyt}$ oscillations in *tgg1 tgg2* guard cells

I monitored $[Ca^{2+}]_{cyt}$ oscillations in guard cells using a Ca^{2+}-sensing fluorescent protein, YC3.6. When the wild type (WT) guard cells were treated with 50 μM AITC,

86% of the guard cells showed $[Ca^{2+}]_{cyt}$ transient elevation(s), hereafter $[Ca^{2+}]_{cyt}$ oscillations (n = 19 of 22 cells; Fig. 3.4A and C). There was no significant difference in the number of AITC-induced $[Ca^{2+}]_{cyt}$ oscillation between this study and our previous study (Khokon et al. 2011a) (P = 0.861, χ^2 = 0.0308). When the double mutant guard cells were treated with 50 μM AITC, 82% of the guard cells showed $[Ca^{2+}]_{cyt}$ oscillations (n = 15 of 18 cells; Fig. 3.4B and C). There was no significant difference in the frequency of $[Ca^{2+}]_{cyt}$ oscillations between the WT and the double mutant (P = 0.789), suggesting that neither TGG1 nor TGG2 functions upstream of $[Ca^{2+}]_{cyt}$ oscillation in guard cells in response to AITC.

ABA at 10 μM significantly induced $[Ca^{2+}]_{cyt}$ oscillation in the WT (n = 15 of 20 cells; Fig. 3.4D and F) and in the *tgg1 tgg2* mutant (n = 14 of 19 cells; Fig. 3.4E and F), while MeJA at 10 μM also significantly induced $[Ca^{2+}]_{cyt}$ oscillation in the WT (n = 15 of 20 cells; Fig. 3.4G and I) and in the *tgg1 tgg2* mutant (n = 13 of 18 cells; Fig. 3.4H and I). There were no significant differences in the frequency of $[Ca^{2+}]_{cyt}$ oscillations between the WT and the double mutant (P = 0.925 for ABA, and P = 0.846 for MeJA), suggesting that neither TGG1 nor TGG2 functions upstream of $[Ca^{2+}]_{cyt}$ oscillation in guard cells in response to ABA and MeJA, in agreement with our previous results (Islam et al. 2009).

3.5 Discussion

Myrosinases including TGG1 and TGG2 catalyze degradation of glucosinolates and isothiocyanates are one of the degradation products. The *tgg1 tgg2* mutation impairs ABA- and MeJA-induced stomatal closure (Islam et al. 2009) and one of myrosinase products, AITC, induces stomatal closure in a dose dependent manner in

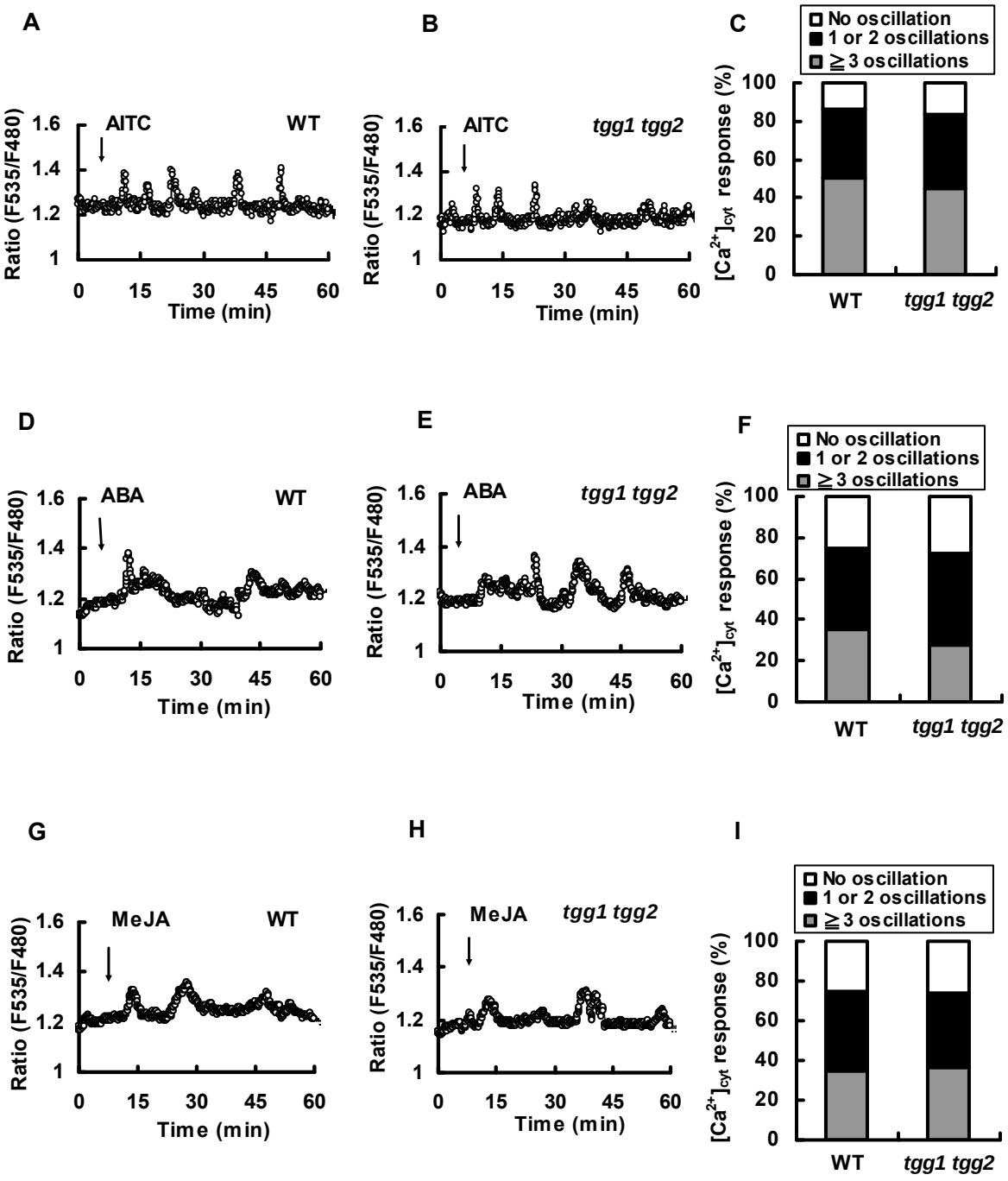

Fig. 3.4 $[Ca^{2+}]_{cyt}$ oscillations elicited by allyl isothiocyanate (AITC) in Arabidopsis wild-type (WT) and *tgg1 tgg2* guard cell. A, B, representative fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in 50 μM AITC-treated WT guard cells (n = 19 of 22 cells; 86%) (A) and *tgg1 tgg2* guard cells (n = 15 of 18 cells; 82%)

(B). (C) Percentage bar chart showing the number of AITC-induced $[Ca^{2+}]_{cyt}$ oscillations in the WT (n = 22) and the *tgg1 tgg2* guard cells (n = 18). D, E, ABA at 10 μM induced $[Ca^{2+}]_{cyt}$ oscillation in WT guard cells (n = 15 of 20 cells; 75%) (D) and *tgg1 tgg2* guard cells (n = 14 of 19 cells; 73%) (E). (F) Percentage bar chart showing the number of ABA-induced $[Ca^{2+}]_{cyt}$ oscillations in the WT (n = 20) and the *tgg1 tgg2* guard cells (n = 19). G, H, MeJA at 10 μM induced $[Ca^{2+}]_{cyt}$ oscillation in WT guard cells (n = 15 of 20 cells; 75%) (G) and *tgg1 tgg2* guard cells (n = 13 of 18 cells; 72%) (H). (I) Percentage bar chart showing the number of MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in the WT (n = 20) and the *tgg1 tgg2* guard cells (n = 18). The arrow indicates when guard cells were treated with AITC, ABA or MeJA. Error bars represent SE.

Arabidopsis (Khokon et al. 2011a). These results can make us expect that ITCs such as AITC mediate ABA- and MeJA-induced stomatal closure. However, AITC-induced stomatal closure was impaired in the *tgg1 tgg2* double mutant but not in the single mutants (Fig. 1A), indicating that TGG1 and TGG2 positively regulate AITC-induced stomatal closure even though AITC is one of products of myrosinases. Hence, myrosinases, TGG1 and TGG2, are likely to have certain functions other than hydrolysis of glucosinolates in ABA and MeJA signaling in Arabidopsis guard cells.

ROS act as important second messengers in ABA and MeJA signaling in guard cells (Pei et al. 2000; Murata et al. 2001; Munemasa et al. 2007; Islam et al. 2010b). AITC-induced stomatal closure is also accompanied by ROS accumulation in guard cells (Khokon et al. 2011a). Our previous study shows that DPI slightly inhibited AITC-induced stomatal closure (Khokon et al. 2011a) and this study shows that AITC induced stomatal closure in wild type and *atrbohD atrbohF* mutant (Fig. 1B, C) and that

AITC-induced stomatal closure was completely inhibited by SHAM (Fig. 1B, C). There is a discrepancy in enzymes mediating ROS production between these results. However, the partial inhibition by DPI of AITC-induced stomatal closure may be caused by side effects of DPI because DPI can inhibit flavoproteins including NADPH oxidases.

ABA and MeJA increase cytosolic alkalization in guard cells to induce stomatal closure (Irving et al. 1992; Suhita et al. 2004; Gonugunta et al. 2008; Islam et al. 2010a, b) and ABA activates vacuolar H^+ATPase activity in *Mesembryanthemum*, leading to cytosolic alkalization (Barkla et al. 1999). AITC-induced stomatal closure was inhibited by the acidifying reagent (butyrate) but not by the alkalizing reagent (methylamine) (Fig. 1D) and AITC-induced stomatal closure was accompanied by cytosolic alkalization (Fig. 3.3), suggesting that AITC activates vacuolar H^+ATPase activity, resulting in cytosolic alkalization. The *tgg1 tgg2* mutation did not affect cytosolic alkalization, suggesting that TGG1 and TGG2 function downstream of cytosolic alkalization in AITC signaling.

Cytosolic free calcium concentration oscillation/elevation occurs during ABA- and MeJA-induced stomatal closure (Allen et al. 2000, 2001; Hossain et al. 2011a, b) and is closely related with production of ROS and activation of Ca^{2+}-permeable cation channels in guard cells (Pei et al. 2000; Kwak et al. 2003). In this study, AITC induced ROS production (Fig. 3.2A) and $[Ca^{2+}]_{cyt}$ oscillation (Fig. 3.4) in guard cells of both wild type plants and *tgg1 tgg2* mutants. However, Ca^{2+} induces stomatal closure but H_2O_2 does not induce stomatal closure in the *tgg1 tgg2* mutants (Islam et al. 2009). On the other hand, long-term Ca^{2+} programmed stomatal closure requires stimulus-specific calcium oscillations, that is, certain specific Ca^{2+} signatures inhibit stomatal re-opening after Ca^{2+} (reactive) stomatal closure (Allen et al. 2000, 2001). Therefore, the H_2O_2-independent $[Ca^{2+}]_{cyt}$ oscillations in the *tgg1 tgg2* double mutant may induce

stomatal closure and may be attributed to malfunction of $[Ca^{2+}]_{cyt}$ homeostasis due to the double mutation. In addition, application of Ca^{2+} induces $[Ca^{2+}]_{cyt}$ elevation in guard cells, which is mediated by Ca^{2+} influx from extracellular space (Allen et al. 2000) and/or Ca^{2+} release from intracellular stores (Ward and Schroeder 1994). Taken together, these results suggest that TGG1 and TGG2 function in stomatal closure induced by external Ca^{2+}. In animal cells, $[Ca^{2+}]_{cyt}$ elevation can regulate activation of downstream components of signaling cascades (Dolmetsch et al. 1998; Himmelbach et al. 1998). These results suggest that TGG1 and TGG2 function downstream of $[Ca^{2+}]_{cyt}$ elevation in response to AITC.

OST1 activates SLAC1 activity *via* phosphorylation leading stomatal closure (Vahisalu et al. 2010), while *ost1* mutation does not affect AITC-induced stomatal closure (Khokon et al. 2011a), suggesting that OST1 is not a signal component in AITC signal pathway. On the other hand, ITCs including AITC have many target compounds in animal cells because ITCs are so electrophilic that they can easily react with nucleophilic targets. Taken together, AITC may affect several signal components like calcium-dependent protein kinases (CDPKs), which activate S-type anion channels (Geiger et al. 2010; Brandt et al. 2012).

ABA and MeJA at 50 μM reduce stomatal aperture by 30%, while AITC at 100 μM reduced by 15 to 20%. Therefore, AITC-induced stomatal closure can reduce transpiration to some extent. It is suggested that ITCs do not only repel insects and herbivores but also induce stomatal closure to suppress water loss caused by their bite.

In conclusion, 1) TGG1 and TGG2, have certain functions other than hydrolysis of glucosinolates in ABA and MeJA signal pathways in guard cells, 2) TGG1 and TGG2 also function in AITC-induced stomatal closure even though AITC is a

product of myrosinases, 3) TGG1 and TGG2, positively function in AITC-induced stomatal closure but are not involved in AITC-induced ROS and NO production, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillation in guard cells, and 4) AITC-induced stomatal closure is accompanied by ROS production mediated by SHAM-sensitive peroxidases but not by NADPH oxidases.

General summary

Plants produce a multitude of secondary metabolites to survive in a complex biotic environment. Abiotic stresses, such as drought, salinity, cold, high temperature, chemical toxicity and oxidative stresses lead to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity. Glucosinolates are plant secondary metabolites present in crucifer plants. Isothiocyanates (ITCs), nitriles, and thiocyanates are degradation products of glucosinolate and the degradation are catalyzed by myrosinases. The myrosinase-glucosinolate system is involved in a range of biological activities because ITCs have repellent effect on herbivores and insects. Moreover, animal cells are more susceptible to ITCs than other degradation products because ITCs are much more electrophilic than other degradation products, nitriles and thiocyanates. In plant, nitriles and thiocyanates are not as bioactive as isothiocyanates and consequently evidences of their physiological roles are limited. Stomatal closure is not only induced by phytohormones but also by isothiocyanates. It has been reported that allyl isothiocyanate (AITC) induces stomatal closure in Arabidopsis. But, mechanism of this isothiocyanate-, and other degradation products, nitrile- and thiocyanate-induced stomatal closure are still unknown. Plasma membrane NADPH oxidases and cell-wall peroxidases are two major sources of reactive oxygen species (ROS) production. Abscisic acid (ABA)- and methyl jasmonate (MeJA)-induced stomatal closure are accompanied by ROS production mediated by plasma membrane NADPH oxidases. Salicylic acid-, yeast elicitor-, and chitosan-induced stomatal closure are accompanied by extracellular ROS production mediated by cell-wall peroxidases. However, it is unknown whether the degradation products induce ROS production in plants or which

enzymes catalyze the ROS production in guard cells.

Guard cells surrounding stomatal pores can respond to various environmental stimuli. To regulate gas exchange and transpirational water loss, plants control volume of guard cells resulting in opening and closing of stomata in response to phytohormones and various environmental signals. To clarify the roles of degradation products of glucosinolates in Arabidopsis guard cells, I investigated whether glucosinolate degradation products, AITC, 3BN, and ESCN, induce stomatal closure, ROS production, and $[Ca^{2+}]_{cyt}$ oscillation in wild-type plants (Chapter 2). The degradation products induced stomatal closure in a dose dependent manner in wild type and *atrbohD atrbohF* mutant. The glucosinolates degradation product-induced stomatal closure requires ROS production depending on peroxidases but not on NADPH oxidases as stomatal closure were completely inhibited by a peroxidase inhibitor, salicylhydroxamic acid (SHAM) in wild-type plants. For further confirmation, peroxidase inhibitor sodium azide (NaN_3) was used and showed similar response like SHAM. Moreover, a histochemical study showed that degradation products induced both hydrogen peroxide (H_2O_2) and super oxide (O_2^-) production in whole leaves and production of these ROS was dependent on SHAM-sensitive peroxidase. I also measured the degradation product-induced ROS accumulation in guard cells of both plants and production of these ROS was completely inhibited by catalase (CAT) and SHAM but not by DPI or the *atrbohD atrbohF* mutation. Furthermore, the degradation products induced $[Ca^{2+}]_{cyt}$ oscillation in guard cells and degradation products-induced $[Ca^{2+}]_{cyt}$ oscillation were significantly suppressed by SHAM. Together, these results indicate that glucosinolate degradation products, isothiocyanates, nitriles, and thiocyanates, induce stomatal closure accompanied by extracellular ROS production mediated by SHAM-sensitive

peroxidases, intracellular ROS accumulation, and $[Ca^{2+}]_{cyt}$ oscillation in Arabidopsis.

Myrosinases (thioglucoside glucohydrolases, TGGs) are cytosolic and vacuolar enzymes, and are involved in ABA- and MeJA-induced stomatal closure in Arabidopsis. To clarify the function of two myrosinases, TGG1 and TGG2, I examined the effects of glucosinolate hydrolysates, AITC, on stomatal movement, ROS and NO production, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillations in guard cells of wild type and *tgg* mutants (Chapter 3). I found that AITC induced stomatal closure in the *tgg1* and the *tgg2* single mutants but not in the *tgg1 tgg2* double mutant. These results indicate that TGG1 and TGG2 cooperatively function in stomatal closure in response to AITC. I also measured AITC-induced ROS and NO production in guard cells using H_2DCF-DA and DAF-2DA. AITC induced ROS and NO production in *tgg1*, *tgg2*, *tgg1 tgg2* mutants as well as wild type. ABA and MeJA also induced ROS and NO production in wild type and *tgg* mutants. Moreover, AITC-induced stomatal closure was inhibited by SHAM in wild type and *atrbohD atrbohF* mutant. AITC-induced stomatal closure requires ROS production mediated by peroxidases. I also measured AITC-induced cytosolic alkalization in guard cells of wild type and *tgg* mutants. I found that AITC induced cytosolic alkalization in guard cell of *tgg1*, *tgg2*, *tgg1 tgg2* mutants as well as wild-type plants. Furthermore, AITC-induced stomatal closure was inhibited by butyrate but not by methylamine, indicating that AITC-induced stomatal closure was accompanied by cytosolic alkalization. AITC induced $[Ca^{2+}]_{cyt}$ oscillation in both wild type and *tgg1 tgg2* mutants and ABA and MeJA also induced $[Ca^{2+}]_{cyt}$ oscillation in both wild type and *tgg1 tgg2* mutants, indicating that neither TGG1 nor TGG2 functions upstream of $[Ca^{2+}]_{cyt}$ oscillation in guard cells in response to AITC, ABA, and MeJA. Thus my findings indicate that TGG1 and TGG2 positively function in AITC-induced stomatal

closure but are not involved in AITC-induced ROS and NO production, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ oscillation in guard cells, and AITC-induced stomatal closure is accompanied by ROS production mediated by SHAM-sensitive peroxidases but not by NADPH oxidases.

Taken together, my findings suggest that glucosinolate degradation products function as a signaling molecule in Arabidopsis under stress conditions and glucosinate-myrosinase system regulates physiological function including stomatal movements against attack of insects, pathogens, and herbivores regardless of the condition for glucosinolate degradation in crucifer plants.

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