

Involvement of myrosinases in stomatal closure in Arabidopsis

September, 2025

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(Doctor's Course)
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Submitted by
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Abbreviations list

ABA, abscisic acid

AITC, allyl isothiocyanate

BCECF-AM, 2,7-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein-acetomethyl ester

[Ca²⁺]_{cyt}, cytosolic free calcium concentration

CHT, chitosan

DAF-2DA, 4,5-diaminobenzidine tetrahydrochloride hydrate

H₂DCF-DA, dichlorodihydrofluorescein diacetate

ITC, isothiocyanate

MeJA, methyl jasmonate

NO, nitric oxide

RCS, reactive carbonyl species

ROS, reactive oxygen species

SA, salicylic acid

SHAM, salicylhydroxamic acid

TGG, thioglucoside glucohydrolase

YC3.6, yellow cameleon 3.6

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CHAPTER 1

General Introduction

1.1 Stress signaling and response in plants

As sessile organisms, plants are continuously exposed to a wide range of external factors that can interfere with their normal physiological functions. Any such factor that adversely affects a plant's metabolism, growth, development, or productivity is broadly referred to as stress. These stressors are commonly categorized into two main types: abiotic and biotic. Biotic stress is caused by living organisms, including bacteria, viruses, fungi, insects, and other pests that interact with the plant in harmful ways. In contrast, abiotic stress arises from unfavorable environmental conditions, such as extreme temperatures, drought, high atmospheric carbon dioxide (CO₂) concentrations, salinity, and excessive or insufficient humidity (Zhang et al. 2023). These abiotic factors often result from climate variability or soil degradation and are recognized as major limitations to plant growth and agricultural productivity.

Both abiotic and biotic stresses can lead to significant yield losses and pose serious threats to global food security and ecosystem balance (Khan et al. 2025; Wani et al. 2016; Wang, Vincour, and Altman, 2003). In response to such stresses, plants deploy a complex array of adaptive mechanisms. These responses include alterations in gene expression, shifts in metabolic pathways, modifications in growth patterns, and physiological changes aimed at maintaining cellular homeostasis. Unlike animals, plants cannot escape unfavorable conditions, nor do they possess mobile immune cells. Instead,

they rely on highly sophisticated signaling networks to detect environmental cues and initiate appropriate defense responses (Khan et al. 2025).

Perception of stress signals triggers the production of specific signaling molecules, particularly plant hormones, which act as internal messengers. These hormones bind to dedicated receptors within plant cells, initiating intracellular signaling cascades that ultimately activate stress-responsive genes and defense mechanisms (Zhang et al. 2008; Müller et al. 2021; Roychoudhury and Aftab, 2021). Plants produce a range of hormones, small, bioactive compounds that function effectively even at low concentrations and are vital for their growth and development (Santner et al. 2009). Several of these hormones, such as abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA), play pivotal roles in regulating plant defense responses under environmental stress (Wang et al. 2021). One important response controlled by these hormones is stomatal closure, which helps plants minimize water loss and enhance stress tolerance (Murata et al. 2015; Zamora et al. 2021).

1.2 Stomata and guard cells signal transduction

Stomata are microscopic openings predominantly located on the surfaces of leaves and young stems, functioning as dynamic gateways between the internal tissues of the plant and the external environment. Each stoma is flanked by a pair of guard cells that precisely regulate its aperture in response to various internal signals and environmental cues. Through these pores, plants facilitate the uptake of carbon dioxide (CO₂) necessary for photosynthesis, while simultaneously releasing oxygen (O₂) and regulating water loss through transpiration. By modulating gas exchange and maintaining water balance, stomata play a central role in plant physiology, particularly in

enabling adaptation to changing environmental conditions (Shimazaki et al. 2007; Murata et al. 2015).

Guard cell signaling constitutes a sophisticated and tightly regulated network of molecular events that allows plants to perceive and respond to a wide range of internal and external stimuli by adjusting stomatal aperture. These dynamic responses, mediated by the guard cells, are critical for regulating gas exchange and transpiration key processes for photosynthetic efficiency, water conservation, and overall plant homeostasis. Under environmental stress conditions such as drought, high salinity, elevated CO₂ levels, or pathogen invasion, guard cells integrate multiple signals to control stomatal movement, optimizing physiological performance and enhancing stress tolerance (Driesen et al. 2020; Bergmann et al. 2017; Khokon et al. 2011).

At the heart of this regulation lies the process of signal transduction, in which environmental or hormonal signals such as variations in light intensity, humidity, or the presence of abscisic acid (ABA) are perceived by specific receptors located on the guard cell membrane (Murata et al., 2015). These signals are subsequently relayed through intracellular cascades involving secondary messengers such as calcium ions (Ca²⁺), reactive oxygen species (ROS), and phospholipids. This signaling cascade ultimately modulates ion channel activity, guard cell turgor, and gene expression. The intricate interplay between receptors, signaling molecules, and downstream effectors ensures precise control of stomatal behavior, allowing plants to balance carbon assimilation with water conservation. As such, guard cell signal transduction is a central component of the plant's adaptive strategy under fluctuating and often stressful environmental conditions.

1.3 Myrosinase

Myrosinase (thioglucoside glucohydrolase, TGG; EC 3.2.1.147) is a well-characterized enzyme predominantly found in cruciferous plants, where it plays a vital role in plant defense. Localized in specialized myrosin cells, this enzyme catalyzes the hydrolysis of glucosinolates into a range of biologically active compounds, such as isothiocyanates, which deter herbivores and inhibit pathogen growth (Zhao et al. 2008; Islam et al. 2009). In *Arabidopsis thaliana*, two myrosinase isoforms, TGG1 and TGG2, are especially abundant in guard cells and have been implicated in both glucosinolate metabolism and stress-related signaling pathways, including those regulating stomatal movement.

1.4 Signaling molecules implicated in plant stress responses

1.4.1 Allyl isothiocyanate

Isothiocyanates (ITCs) are sulfur-rich electrophilic compounds synthesized by Brassicaceae plants as part of their defense system. These molecules originate from the enzymatic breakdown of glucosinolates, where the unstable aglucone intermediate spontaneously rearranges to form ITCs without the need for additional proteins. One of the most studied ITCs is allyl isothiocyanate (AITC), which is generated from the hydrolysis of sinigrin and has been reported to trigger stomatal closure in *Arabidopsis thaliana* (Khokon et al. 2011; Hossain et al. 2013; Murata et al. 2015). In addition to its role in stomatal regulation, AITC exhibits broad-spectrum antimicrobial properties, contributing to plant defense against diverse pathogens (Hossain et al. 2013). Despite these findings, the precise molecular mechanisms by which guard cells perceive and respond to AITC remain to be fully elucidated.

1.4.2 Chitosan

Chitosan (CHT) is a well-characterized elicitor that acts as a molecular trigger of plant defense responses by resembling pathogen-associated signals and initiating immune-related physiological changes (Zhao et al. 2005). It is derived from the partial deacetylation of chitin, a structural polysaccharide found predominantly in the exoskeletons of insects and crustaceans, as well as in the cell walls of fungi (Bartnicki-Garcia, 1970). Structurally, chitosan is composed of β -(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine units arranged in a linear chain. CHT has been reported to induce stomatal closure in several plant species, including *Arabidopsis thaliana*, *Commelina communis*, and *Pisum sativum* (Lee et al. 1999; Srivastava et al. 2009; Salam et al. 2012). This response is associated with the accumulation of reactive oxygen species (ROS) in guard cells and SHAM-sensitive peroxidase-mediated ROS production in the apoplast (Khokon et al. 2010). In addition, chitosan triggers early guard cell signaling events such as cytosolic alkalization, calcium oscillations, and activation of S-type anion channels (Lee et al. 1999; Klüsener et al. 2002; Srivastava et al. 2009; Khokon et al. 2010; Salam et al. 2012). Despite these insights, the detailed molecular signaling pathways involved in guard cell responses to chitosan remain largely unresolved.

1.4.3 Salicylic acid

Salicylic acid (SA) is a key signaling molecule involved in the regulation of plant defense responses, particularly those triggered by pathogenic attacks. As a phenolic phytohormone, SA plays a central role in the activation of systemic acquired resistance (SAR), a long-lasting defense mechanism that enhances resistance throughout the plant (White, 1979; Ward et al. 1991; Uknes et al. 1992). Beyond pathogen defense, SA also contributes to abiotic stress tolerance, including enhanced drought resistance (Miura et al. 2013;

Okuma et al. 2014). Notably, several studies have shown that SA promotes stomatal closure (Lee, 1998; Mori et al. 2001; Zeng and He, 2010; Khokon et al. 2011; Hua et al. 2012), a critical response that limits water loss and pathogen entry. Within guard cells, SA signaling interacts with other signaling pathways, forming a complex network that underlies stomatal immunity (Melotto et al. 2017). However, the precise molecular mechanisms of SA action in guard cell signaling and its crosstalk with other pathways remain to be fully elucidated.

1.5 Second messengers in guard cell signaling

Second messengers are small intracellular molecules that transmit and amplify signals from receptors to target molecules within the cell. In plants, common second messengers such as calcium ions (Ca^{2+}), reactive oxygen species (ROS), and nitric oxide (NO) play vital roles in translating external cues like hormones or environmental stress into specific cellular responses. These molecules help regulate key processes including gene expression, ion transport, and enzyme activity. In guard cells, second messengers are central to signaling pathways that control stomatal movement, enabling plants to respond efficiently to changing environmental conditions.

1.5.1 Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules formed through the partial reduction of oxygen. Key types of ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), singlet oxygen ($^1\text{O}_2$), and hydroxyl radicals ($\bullet\text{OH}$) (Chen et al. 2010). Under normal conditions, ROS are generated at low levels in cellular organelles such as chloroplasts, mitochondria, peroxisomes, and the apoplast. However, under stress, their production increases significantly. The accumulation of ROS is determined by

the dynamic balance between their generation and scavenging (Mittler et al. 2004). Antioxidants like ascorbic acid (AsA) and glutathione (GSH), along with ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione peroxidase (GPX), play crucial roles in detoxifying ROS under both normal and stress conditions (Takahashi and Asada, 1998; Mittler et al. 2004). In guard cells, SOD facilitates the conversion of superoxide anion (O_2^-) into H_2O_2 , which can then act as a signaling molecule (Liochev et al. 2007). The presence of H_2O_2 in guard cells is often detected using the fluorescent probe H_2DCF -DA (Munemasa et al. 2019).

ROS are central to plant signaling under abiotic and biotic stress, including drought, salinity, and pathogen attack (Das et al. 2014). They trigger downstream responses by reversibly oxidizing cysteine residues in proteins, leading to conformational and functional changes (Waszczak et al. 2018). In guard cells, ROS are primarily produced via two pathways: NADPH oxidases located at the plasma membrane, and apoplastic class III peroxidases. While ABA and MeJA signaling rely on NADPH oxidase-mediated ROS production (Kwak et al. 2003; Jannat et al. 2011, 2012), SA, chitosan (CHT), and isothiocyanates (ITCs) induce ROS generation via SHAM-sensitive class III peroxidases (Hossain et al. 2013). Despite their well-established role, the exact mechanisms by which ROS integrate into guard cell signal transduction remain to be fully elucidated.

1.5.2 Nitric oxide

Nitric oxide (NO) is a small, lipophilic, and highly diffusible free radical that serves as a versatile signaling molecule in plants. Its ability to permeate cell membranes allows it to function effectively in both autocrine and paracrine signaling pathways (Khan et al. 2023). NO is involved in a wide range of

physiological and developmental processes, including seed germination, root architecture modulation, floral transition, fruit ripening, and stomatal movement (Beligni and Lamattina, 2000). It also plays a critical role in plant responses to various abiotic and biotic stresses such as drought, salinity, wounding, pathogen invasion, and temperature extremes.

In guard cells, NO acts as a secondary messenger in signal transduction pathways triggered by stress-related hormones such as abscisic acid (ABA) and methyl jasmonate (MeJA), mediating stomatal closure to reduce water loss (Munemasa et al. 2011). It also interacts with other signaling molecules, including reactive oxygen species (ROS), antioxidants, melatonin, and hydrogen sulfide, contributing to the fine-tuning of plant defense and adaptation mechanisms. In plants, NO is primarily produced through enzymatic pathways involving nitrate reductase (NR) and possibly nitric oxide synthase (NOS)-like enzymes, although the exact identity and regulation of NOS in plants remain a matter of debate (Yamasaki, 2000; Khan et al. 2023). Notably, while both NR and NOS contribute to NO production in ABA and MeJA-induced stomatal closure, jasmonate signaling specifically requires NOS-like activity, but not NR (Munemasa et al. 2011a). Despite significant progress, the precise mechanisms of NO generation and its integration into guard cell signaling networks continue to be areas of active research.

1.5.3 Calcium

Calcium ions (Ca^{2+}) play a vital role as secondary messengers in plant cells, mediating responses to a wide range of biotic and abiotic signals. In guard cells, changes in cytosolic Ca^{2+} concentration $[\text{Ca}^{2+}]_{\text{cyt}}$ are triggered by stimuli such as abscisic acid (ABA) and methyl jasmonate (MeJA), which activate Ca^{2+} -permeable channels in the plasma membrane and promote release from internal stores like the vacuole (Sanders et al. 2002; Roelfsema

et al. 2010). These signals generate $[Ca^{2+}]_{cyt}$ oscillations with distinct frequencies and amplitudes that are essential for downstream signaling (Munemasa et al. 2007, 2011b; Islam et al. 2010a, b). Calcium-dependent protein kinases (CDPKs) serve as key sensors that decode these Ca^{2+} signals and mediate physiological responses (Klimecka and Muszynska, 2007). Overall, $[Ca^{2+}]_{cyt}$ elevation functions as a critical regulator of stomatal movement in response to environmental and hormonal cues.

1.5.4 Cytosolic pH

Cytosolic pH plays a crucial role as a signaling component in various plant physiological responses, including stomatal movement (Irving et al. 1992; Fasano et al. 2001). An increase in guard cell cytosolic pH, or cytosolic alkalization, is closely associated with stomatal closure and is considered a key step in the signaling cascades induced by abscisic acid (ABA) and methyl jasmonate (MeJA) (Suhita et al. 2004). During ABA signaling, cytosolic alkalization contributes to H^+ efflux from guard cells and modulates ion channel activity specifically, by activating outward K^+ channels and inhibiting inward K^+ channels thereby promoting net K^+ efflux and stomatal closure, as demonstrated in *Vicia faba* (Blatt and Armstrong, 1993). These observations underline the significance of cytosolic pH dynamics as an integral regulator in guard cell signal transduction and stomatal regulation.

1.5.5 Reactive carbonyl species

In guard cell signaling, reactive oxygen species (ROS) initiate lipid oxidation, particularly targeting polyunsaturated fatty acids (PUFAs) in membranes, leading to the formation of lipid hydroperoxides (LOOH). As these oxidized lipids degrade through enzymatic and non-enzymatic, radical-driven reactions, they generate a range of reactive byproducts, including aldehydes,

ketones, and hydroxy acids (Müller, 2004; Mano, 2012). A specific subset of these carbonyl compounds, those with an α , β -unsaturated carbonyl structure are known as reactive carbonyl species (RCS), recognized for their high reactivity and ability to covalently modify proteins and nucleic acids (Mano et al. 2019). Under stress conditions, RCS levels rise significantly and have been linked to cellular damage and inhibited plant growth (Yin et al. 2010; Mano, 2012). However, RCS are not merely toxic byproducts; they also act as signaling molecules. Evidence shows that scavenging RCS with compounds like carnosine and pyridoxamine, or enhancing the expression of RCS-detoxifying enzymes, improves plant stress tolerance (Zhu et al. 2011; Biswas and Mano, 2016). In guard cells, RCS function downstream of ROS in the signaling pathways triggered by abscisic acid (ABA), methyl jasmonate (MeJA), and chitosan (CHT), suggesting they play a pivotal role in linking oxidative signals to stomatal movement and stress adaptation (Islam et al. 2016, 2019, 2020; Jahan et al. 2025).

1.6 Plant material used in this study

Arabidopsis thaliana is a widely used model plant, notable for its extensive collection of mutants impaired in various defense and stress-related signaling pathways. These genetic tools have enabled the functional analysis of numerous proteins involved in plant responses to environmental stimuli. In this study, we focused on myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147; TGG), a stomata-enriched enzyme encoded by two genes in the *Arabidopsis* genome TGG1 and TGG2 both of which have been implicated in abscisic acid (ABA) and methyl jasmonate (MeJA) signaling (Islam et al. 2009; Rhaman et al. 2020). To investigate their roles and specificity in guard cell signaling, we utilized the single mutants *tgg1-3*, *tgg2-1*, and the double mutant *tgg1-3 tgg2-1*.

1.7 Aims and Objectives of the study

The aim of this study is to deepen our understanding of the roles of the myrosinases TGG1 and TGG2 in guard cell signaling in *Arabidopsis thaliana*.

Our specific objectives are:

- To determine whether TGG1 and TGG2 are involved in AITC-induced stomatal closure by examining the stomatal responses of *tgg1*, *tgg2*, and *tgg1 tgg2* double mutants following AITC treatment.
- To investigate the responses of these mutants to chitosan (CHT) and salicylic acid (SA) in order to explore the roles of TGG1 and TGG2 in CHT- and SA-induced stomatal closure. This part of the study focused on their possible involvement in reactive carbonyl species (RCS)-mediated signaling pathways in guard cells.

CHAPTER 2

TGG1 and TGG2 mutations impair allyl isothiocyanate-mediated stomatal closure in Arabidopsis thaliana

2.1 Abstract

Myrosinase, referred to as thioglucoside glucohydrolase (TGG), plays a crucial role in plant physiology through catalyzing the hydrolysis of glucosinolates into bioactive isothiocyanates. In *Arabidopsis thaliana*, the myrosinases TGG1 and TGG2 are essential for abscisic acid- and methyl jasmonate-induced stomata closure. Allyl isothiocyanate (AITC), one of myrosinase products, triggers stomatal closure in *A. thaliana*. We investigated stomatal responses to AITC to clarify the role of TGG1 and TGG2 in *Arabidopsis* guard-cell signaling. Allyl isothiocyanate at 50 μ M and 100 μ M induced stomatal closure in the *tgg1* and *tgg2* single mutants but not in the *tgg1 tgg2* double mutant. Furthermore, AITC at 50 μ M induced the production of reactive oxygen species and nitric oxide, cytosolic alkalization, and oscillations in cytosolic free calcium concentration in guard cells of both wild-type and mutant plants. These findings suggest that TGG1 and TGG2 are involved in AITC signaling pathway through interaction with signal component(s) downstream of these signaling events, which is not accompanied by hydrolysis of glucosinolates because of the difference in subcellular localization between enzymes (myrosinases) and substrates (glucosinolates).

2.2 Introduction

Stomatal pores, bordered by pairs of specialized guard cells, are vital for regulating gas exchange and transpirational water loss. These guard cells are highly responsive to various biotic and abiotic signals, including plant hormones, light, drought, and pathogen attacks (Shimazaki et al. 2007; Murata et al. 2015; Ye et al. 2015).

Myrosinase (thioglucoside glucohydrolase (TGG), EC 3.2.1.147) can hydrolyze glucosinolates and is one of the most prominent proteins in cruciferous plants (Zhao et al. 2008; Islam et al. 2009). Isothiocyanates are most important products of glucosinolates in reaction to infliction by herbivores, insects, bacteria, and other stress factors (Bhat and Vyas 2019) and modulate stomatal movement triggered by light and phytohormones like abscisic acid (ABA) and methyl jasmonate (MeJA) (Zhao et al. 2008; Islam et al. 2009). In *Arabidopsis*, six myrosinase genes have been discovered, with TGG1 and TGG2 being enzymatically active (Barth and Jander 2006). Moreover, the *tgg* mutation decreased myrosinase activities in the rosette leaves in *Arabidopsis* based on the sinigrin assay and stomatal closure induced by ABA and MeJA was impaired in the *tgg* double mutant plants (Islam et al. 2009). Islam et al. (2009) concluded that TGG1 and TGG2 redundantly function downstream of ROS production and upstream of $[Ca^{2+}]_{cyt}$ in ABA and MeJA signaling pathways. Moreover, TGG1 deficiency decreased sensitivity to ABA and impaired response to wound-induced signals (Zhao et al. 2008).

Allyl isothiocyanate (AITC) is the hydrolysis product of the glucosinolate sinigrin and initiates stomatal closure in *Arabidopsis* (Khokon et al. 2011; Hossain et al. 2013; Murata et al. 2015). Furthermore, ITCs including AITC profoundly trigger stomatal closure in *Arabidopsis* (Afrin et al. 2020). Khokon et al. (2011) employed the 1,2-benzenedithiole-based cyclocondensation assay to show that TGG1 and TGG2 catalyze the hydrolysis reaction of

glucosinolates for ITC production in the grinded leaves of *Arabidopsis* although the cyclocondensation assay does not allow us to non-destructively measure ITCs in guard cells. Hence, if the myrosinases, TGG1 and TGG2, function as hydrolysis enzymes to produce ITCs in ABA and MeJA signaling, it can be expected that AITC induces stomatal closure in the *tgg1 tgg2* mutant because AITC is one of myrosinase reaction products. However, it may not be expected that ABA and MeJA promote the hydrolysis of glucosinolates in guard cells because myrosinases are spatially separated from their substrate glucosinolates in the plants (Bhat and Vyas 2019), that is, neither ABA nor MeJA is likely to induce the breakdown of glucosinolate mediated by TGG1 and TGG2 in *Arabidopsis*. To our knowledge, it remains to be clarified whether the myrosinases, TGG1 and TGG2, are involved in ITC-induced stomatal closure and whether AITC induces stomatal closure in the *tgg1 tgg2* mutants.

In this study, we examined stomatal responses of the *tgg* mutants to AITC in order to clarify whether TGG1 and TGG2 are involved in AITC-induced stomata closure and whether TGG1 and TGG2 have functions other than ITCs production in the guard-cell signaling pathways.

2.3 Materials and Methods

2.3.1 Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (WT) and *tgg1-3* (SAIL_786_B08), *tgg2-1* (SALK_038730), and *tgg1-3 tgg2-1* mutant lines were grown in a controlled environment. Plants were grown in 1:1 (v/v) mix of soil and vermiculite mix. The plant culture chamber maintained a $21 \pm 2^\circ\text{C}$ temperature and a 70% relative humidity, under a 16-hour light/8-hour dark cycle with a light intensity of $80 \mu\text{mol m}^{-2} \text{s}^{-1}$. Irrigation was done every 4 to 5 days using deionized water supplemented with HYPONeX (Hyponex Japan,

Osaka, Japan). Genomic PCR was used to confirm the mutant lines. For experimental use, 4- to 5-week-old rosette leaves were harvested.

2.3.2 Measurement of stomatal aperture

Stomatal aperture was measured according to the method described by Munemasa et al. (2019). For each experiment, at least 20 stomata were analyzed. Rosette leaves were detached and floated in a buffer solution containing 5 mM KCl, 50 μ M CaCl₂, and 10 mM MES-Tris (pH 5.6), and incubated under light for 2 hours to promote stomatal opening. After this initial incubation, 50 μ M AITC was added to the solution, and the leaves were incubated under light for an additional 2 hours. The leaves were then homogenized using a commercial blender, and epidermal fragments were collected by filtering through a nylon mesh. Stomatal images were captured using an inverted microscope (Olympus IX71; Tokyo, Japan), and aperture measurements were analyzed using WinRoof 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). To ensure reproducibility, all experiments were performed in triplicate.

2.3.3 Measurement of ROS and NO

Guard cells were exposed to 50 μ M AITC to induce the production of reactive oxygen species (ROS) and nitric oxide (NO). ROS generation was detected using 50 μ M H₂ DCF-DA (Sigma, St. Louis, MO, USA), and NO production was assessed using 10 μ M DAF-2DA (Sigma, St. Louis, MO, USA), following the protocol described by Munemasa et al. (2007).

For ROS detection, epidermal tissues were first incubated in a solution containing 5 mM KCl, 50 μ M CaCl₂, and 10 mM MES-Tris (pH 5.6) under light for 2 hours. After this, 50 μ M H₂ DCF-DA was added, and the tissues were incubated for 30 minutes at room temperature. Excess dye was then washed

out, and the dye-loaded tissues were treated with 50 μ M AITC for another 30 minutes. For NO detection, tissues were incubated with 10 μ M DAF-2DA under the same buffer conditions. Fluorescence signals were analyzed using ImageJ software, and all experiments were performed in triplicate.

2.3.4 Measurement of cytosolic pH

Cytosolic pH in guard cells was measured using the fluorescent dye BCECF-AM (Sigma, St. Louis, MO, USA), following the protocol described by Islam et al. (2010). Epidermal tissues were first incubated for 2 hours under light in an assay buffer containing 5 mM KCl, 50 μ M CaCl_2 , and 10 mM MES-Tris (pH 5.6). Subsequently, 20 μ M BCECF-AM was added, and the tissues were incubated in the dark at room temperature for 30 minutes. Excess dye was then washed out. Fluorescence signals were analyzed using ImageJ software, and all experiments were repeated three times to ensure reproducibility.

2.3.5 Measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations

Yellow Cameleon 3.6 (YC3.6) was used to monitor cytosolic free calcium concentration $[\text{Ca}^{2+}]_{\text{cyt}}$ in *Arabidopsis thaliana* guard cells, following the method of Islam et al. (2010b). Abaxial epidermal peels from rosette leaves were incubated for 2 hours under light in a solution containing 5 mM KCl, 50 μ M CaCl_2 , and 10 mM MES-Tris (pH 5.6). Turgid guard cells were selected for $[\text{Ca}^{2+}]_{\text{cyt}}$ measurements. Fluorescence was excited at 440 nm, and emissions were recorded at 480 nm and 535 nm. The 480/535 nm fluorescence ratio was calculated, and fluctuations greater than 0.1 were defined as calcium oscillations.

2.3.6 Statistical analysis

Analysis of variance (ANOVA) statistical analysis was employed using Tukey's test to assess the significance of mean values differences ($p < 0.05$). The χ^2 test was used to compare between WT and the *tgg* mutants in the elevation of $[Ca^{2+}]_{\text{cyt}}$ frequency induced by AITC. statistical significance was defined as $P < 0.05$.

2.3.7 Accession numbers

The genome initiative numbers for the genes of Arabidopsis mentioned in this article are: TGG1 (At5g26000) and TGG2 (At5g25980).

2.4 Results

2.4.1 stomatal responses to exogenous AITC in Arabidopsis Wild-type and *tgg* mutants

The stomatal responses to exogenous AITC at 50 μM and 100 μM were examined in wild-type (WT) Arabidopsis and in the *tgg1*, *tgg2*, and *tgg1 tgg2* mutants. The concentrations of 50 μM and 100 μM AITC were selected based on previous findings by Khokon et al. (2011a), which reported that ground Arabidopsis leaves contain approximately 10 $\mu\text{mol g}^{-1} \text{FW}^{-1}$ AITC. Treatments with AITC at both concentrations induced significant stomatal closure in WT ($p < 0.05$), consistent with earlier results (Khokon et al. 2011a), as well as in the *tgg1* ($p < 0.05$) and *tgg2* ($p < 0.05$) single mutants. However, no stomatal closure was observed in the *tgg1 tgg2* double mutant under the same conditions ($p < 0.05$) (Fig. 2.1). Notably, neither ABA nor MeJA induces stomatal closure in the *tgg1 tgg2* mutant, as reported by Islam et al. (2009).

These findings indicate that TGG1 and TGG2 function cooperatively in mediating stomatal closure in response to AITC, ABA, and MeJA.

2.4.2 AITC-induced ROS and NO accumulation in Arabidopsis wild-type and *tgg* mutants guard cells

To make it clear how TGG1 and TGG2 participate in the stomatal closure, we assessed the accumulation of reactive oxygen species (ROS) and nitric oxide (NO) in the wild type and *tgg* mutants.

Treatment with 50 μ M AITC induced significant ROS accumulation in the guard cells of *tgg1* ($p < 0.05$), *tgg2* ($p < 0.05$), and *tgg1 tgg2* ($p < 0.05$) mutants, as well as in wild-type plants ($p < 0.05$), consistent with earlier results (Khokon et al. 2011a) (Fig. 2.2). Nitric oxide accumulation in Arabidopsis guard cells was examined using DAF-2 DA. Treatment with 50 μ M AITC also significantly induced NO accumulation in the guard cells of wild type ($p < 0.05$), *tgg1* ($p < 0.05$), *tgg2* ($p < 0.05$), and *tgg1 tgg2* ($p < 0.05$) mutants (Fig. 2.3). Together, these results suggest that neither TGG1 nor TGG2 functions upstream of ROS and NO production in AITC signaling in guard cells.

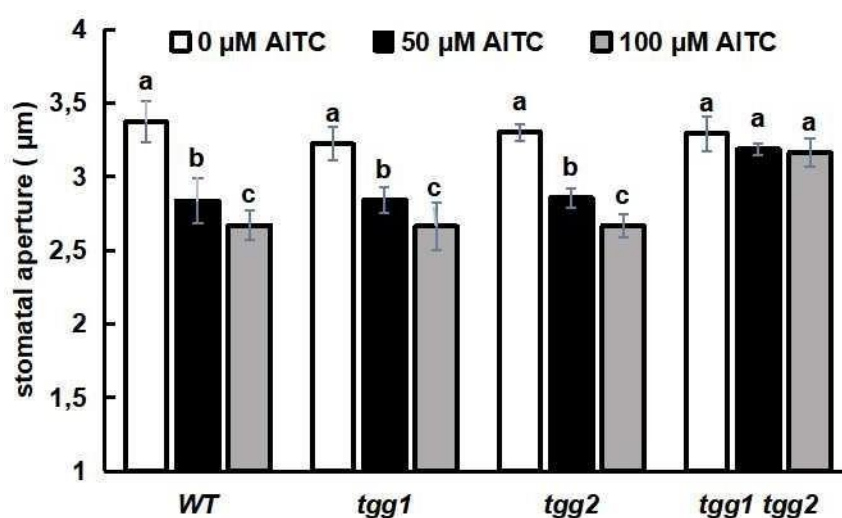


Fig. 2.1 Stomatal aperture in *Arabidopsis* in response to allyl isothiocyanates (AITC). Exogenous AITC induced stomatal closure at 50 μ M and 100 μ M in the wild-type, *tgg1*, and *tgg2* but not in the *tgg1 tgg2*. Data are presented as mean $\pm$ SE (n = 3, with >60 stomata). Bars with the same letter are not significantly different at P < 0.05

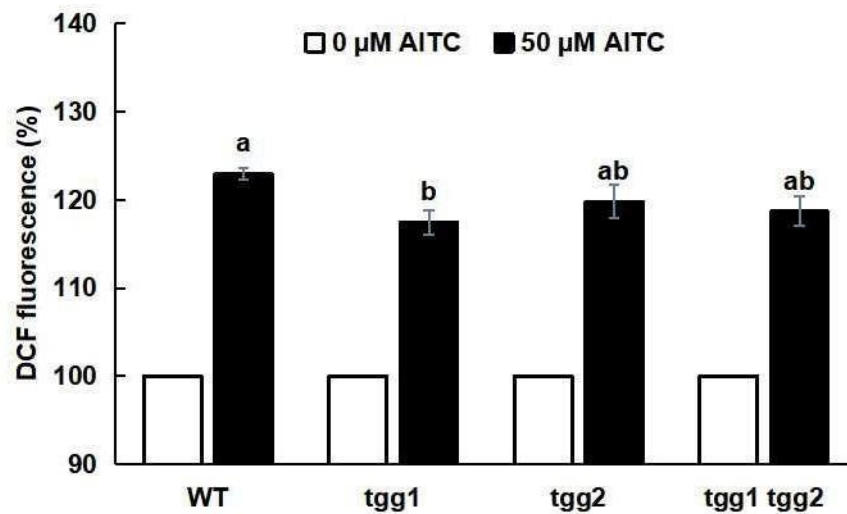


Fig. 2.2 Production of ROS in guard cells of the wild-type and *tgg* mutants induced by 50 μ M AITC. Reactive oxygen species in the wild-type and *tgg* mutants were measured using H₂DCF-DA. Data are presented as mean $\pm$ SE (n = 3, with >60 stomata). Bars sharing the same letter are not significantly different at P < 0.05

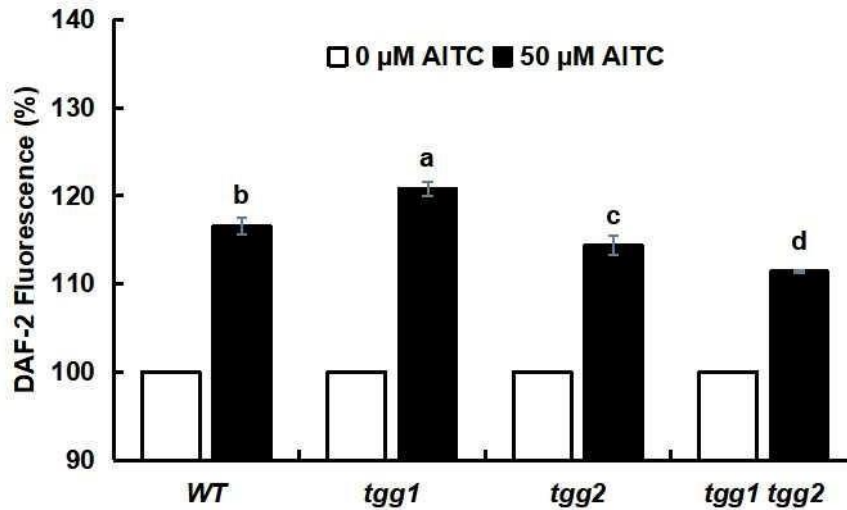


Fig. 2.3 Nitric oxide production induced by 50 μ M AITC in guard cells of the wild-type and *tgg* mutants. Nitric oxide in the wild-type and *tgg* mutants was measured using DAF-2DA. Data are presented as mean $\pm$ SE ($n = 3$, with >60 stomata). Bars sharing the same letter are not significantly different at $P < 0.05$

2.4.3 AITC-induced cytosolic alkalization in *Arabidopsis* wild-type and *tgg* mutants guard cells

ABA and MeJA induce cytosolic alkalization in guard cells, which contributes to stomatal closure (Shuita et al. 2004; Islam et al. 2010a, b). To investigate the involvement of TGG1 and TGG2 in AITC-induced cytosolic alkalization, we monitored cytosolic pH in guard cells of wild-type and *tgg* mutants using the fluorescent dye BCECF-AM. Exogenous application of 50 μ M AITC induced significant cytosolic alkalization in the guard cells of *tgg1* ($p < 0.05$), *tgg2* ($p < 0.05$), and *tgg1 tgg2* ($p < 0.05$) mutants, as well as in wild-type plants ($p < 0.05$) (Fig. 2.4). These results suggest that neither TGG1 nor TGG2 functions upstream of cytosolic alkalization in AITC signaling in guard cells (Fig. 2.4).

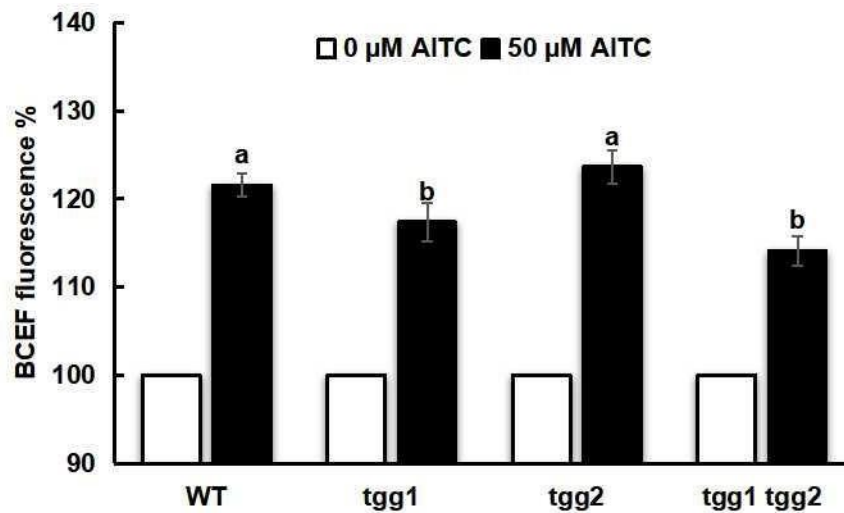


Fig. 2.4 Cytosolic alkalization induced by 50 μ M AITC in guard cells of the wild-type and *tgg* mutants. Cytosolic alkalization was measured using BCECF in the wild-type, *tgg1*, *tgg2*, and *tgg1 tgg2* plants. Data are presented as mean $\pm$ SE ($n = 3$, with >60 stomata). Bars with the same letter are not significantly different at $P < 0.05$

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

Elevation of cytosolic free calcium concentration $[Ca^{2+}]_{\text{cyt}}$ in guard cells was observed using the Ca^{2+} -sensing fluorescent protein YC3.6. When wild-type (WT) guard cells were treated with 50 μ M AITC, a transient elevation in $[Ca^{2+}]_{\text{cyt}}$ was detected in 86% of the cells ($n = 19$ out of 22; Fig. 2.5 E, F). Similarly, 82% of the *tgg1 tgg2* double mutant guard cells showed $[Ca^{2+}]_{\text{cyt}}$ elevation in response to 50 μ M AITC ($n = 15$ out of 18; Fig. 2.5 E, F). There was no significant difference in the frequency of $[Ca^{2+}]_{\text{cyt}}$ elevation between WT and the double mutant ($P = 0.789$), suggesting that neither TGG1 nor TGG2 functions upstream of $[Ca^{2+}]_{\text{cyt}}$ elevation in AITC signaling in guard cells.

At a concentration of 10 μ M, both ABA and MeJA significantly induced $[Ca^{2+}]_{cyt}$ elevation in WT guard cells, with 15 out of 20 cells responding to ABA (Fig. 2.5 B) and 15 out of 20 cells responding to MeJA (Fig. 2.5 C). The *tgg1 tgg2* double mutant exhibited a similar response, with 14 out of 19 cells responding to ABA (Fig. 2.5 B) and 13 out of 18 responding to MeJA (Fig. 2.5 C). The frequency of $[Ca^{2+}]_{cyt}$ elevation in the double mutant was not significantly different from that in the WT ($P = 0.925$ for ABA, $P = 0.846$ for MeJA). These results indicate that TGG1 and TGG2 do not act upstream of $[Ca^{2+}]_{cyt}$ elevation in guard cells in response to AITC, ABA, or MeJA.

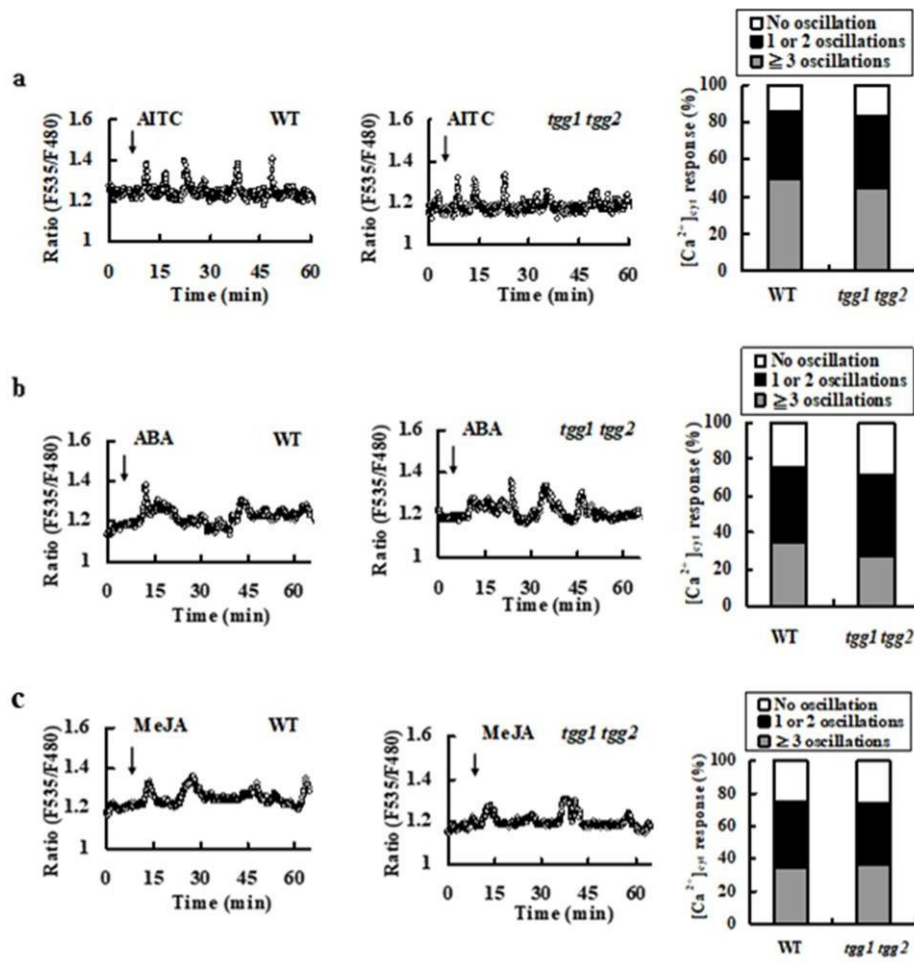


Fig. 2.5 Changes in $[Ca^{2+}]_{cyt}$ induced by AITC, ABA, and MeJA in Arabidopsis wild-type (WT) and *tgg1 tgg2* guard cells. (a) Representative $[Ca^{2+}]_{cyt}$ elevation ($F_{535\text{ nm}}/F_{480\text{ nm}}$) in 50 μM AITC-treated WT guard cells and *tgg1 tgg2* guard cells. A percentage bar chart illustrating the frequency of AITC-induced $[Ca^{2+}]_{cyt}$ oscillations in WT ($n = 22$) and *tgg1 tgg2* guard cells ($n = 18$). (b, c) 10 μM ABA- and MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in WT guard cells and *tgg1 tgg2* guard cells. A percentage bar chart illustrates the frequency of ABA- and MeJA-induced $[Ca^{2+}]_{cyt}$ oscillations in WT ($n = 20$) and *tgg1 tgg2* guard cells ($n = 19$). The arrow indicates when guard cells were treated with AITC, ABA or MeJA. Error bars represent SE.

2.5 Discussion

A hydrolysis product of sinigrin, AITC, triggers stomatal closure at physiological concentrations in one of glucosinolate-producing plants, *Arabidopsis thaliana* (Fig. 2.1), which is in agreement with our previous results (Khokon et al. 2011; Hossain et al. 2013). Furthermore, AITC induces stomatal closure in *Vicia faba*, which does not contain myrosinases or glucosinolates (Sobahan et al. 2015). These results suggest that ITCs including AITC are inducers to trigger stomatal closure regardless of biosynthesis of glucosinolates. ABA- and MeJA-induced ROS production is mediated by NADPH oxidases (Islam et al. 2010) while AITC-induced ROS depends on SHAM-sensitive apoplastic peroxidases (Hossain et al. 2013). The AITC-induced ROS production was not impaired in *tgg1 tgg2* mutant (Fig. 2.2), similar to ABA- and MeJA-induced ROS in this mutant (Islam et al. 2009), indicating that TGG1 and TGG2 do not function upstream of ROS production in guard-cell AITC signaling. RCS-induced stomatal closure, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ elevation are disrupted in the *tgg1 tgg2* mutant while AITC-induced alkalization and $[Ca^{2+}]^{cyt}$ elevation in the double mutant are not impaired (Fig. 2.4, 2.5; Rhaman et al. 2020), suggesting RCS signaling is not totally overlapped with AITC signaling in guard cells. ABA and MeJA also induce $[Ca^{2+}]_{cyt}$ elevation in both WT and *tgg1 tgg2* (Fig. 2.5), confirming that TGG 1 and TGG2 are not upstream of calcium signaling. Notably, the mutation impairs ABA- and MeJA-induced stomatal closure but not Ca^{2+} -induced closure (Islam et al. 2009; Rhaman et al. 2020), which can be attributed to differences in ROS production or upstream signals.

Myrosinase, TGG1, was outstandingly abundant guard cell proteins and the *tgg1* mutation suppressed the inhibition of plasma membrane inward-rectifying potassium channels by ABA (Zhao et al. 2008). Moreover,

TGG1 and TGG2 were redundantly involved in ABA and MeJA signal transduction pathways in guard cells of *A. thaliana* (Islam et al. 2009). However, most of plants do not contain myrosinases or glucosinolates but can close stomata in response to ABA and MeJA, indicating that myrosinases are not a common signaling component essential to ABA- and MeJA-induced stomatal closure. In plants such as Arabidopsis and brassica family, myrosinases hydrolyze glucosinolates to form toxic ITCs as defensive substances when bacteria, fungi, insects and herbivores attack the plants (Barth and Jander 2006; Andersson et al. 2009). However, neither ABA nor MeJA is likely to induce the hydrolysis of glucosinolate mediated by TGG1 and TGG2 in Arabidopsis because of the difference in subcellular localization (Bhat and Vyas 2019). Therefore, TGG1 and TGG2 may interact directly with downstream signal component(s) such as ion channels that are related to stomatal closure.

2.6 Conclusion

We can conclude that TGG1 and TGG2 play as a positive regulator downstream of production of ROS and NO, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ elevation, where TGG1 and TGG2 may directly interact with downstream signal component(s) such as ion channels to modulate their activities in Arabidopsis guard-cell signaling pathways.

CHAPTER 3

Myrosinases TGG1 and TGG2 redundantly function in Salicylic acid and Chitosan-mediated stomatal closure in *Arabidopsis thaliana*

3.1 Abstract

Myrosinase (β -thioglucoside glucohydrolase; EC 3.2.1.147), also known as TGG, is a highly abundant enzyme in *Arabidopsis* guard cells. The isoforms TGG1 and TGG2 function redundantly in stomatal closure responses to abscisic acid (ABA), methyl jasmonate (MeJA), and allyl isothiocyanate (AITC). To further investigate their roles in guard cell signaling, we examined their involvement in chitosan- and salicylic acid-mediated signaling pathways, an elicitor and a defense molecule, respectively. We analyzed the stomatal responses of *tgg* single mutants (*tgg1*, *tgg2*) and the double mutant (*tgg1 tgg2*). Both chitosan- and salicylic acid-induced stomatal closure in wild-type (WT) plants and in the single mutants, but not in the double mutant, indicating a redundant function of TGG1 and TGG2 in these pathways. Similarly, reactive carbonyl species (RCS) scavengers, carnosine and pyridoxamine triggered stomatal closure in WT and single mutants, but not in the double mutant. Although chitosan-induced reactive oxygen species (ROS) production occurred in all genotypes, RCS scavenger-mediated stomatal closure in response to chitosan was impaired in the double mutant. These findings suggest that TGG1 and TGG2 act redundantly downstream of ROS production and at or downstream of RCS signaling in the chitosan- and salicylic

acid-mediated signal transduction pathways in *Arabidopsis* guard cells. Overall, our results confirm that the myrosinases TGG1 and TGG2 participate in multiple signaling pathways in guard cells, warranting further molecular studies to elucidate their precise mechanisms of action.

3.2 Introduction

Stomatal pores, each flanked by a pair of specialized guard cells, are essential for controlling gas exchange and water loss through transpiration in plants. Guard cells integrate a wide array of environmental and endogenous cues to regulate stomatal aperture, responding dynamically to light, drought, pathogens, and plant hormones such as abscisic acid and jasmonates. This highly coordinated responsiveness allows plants to balance carbon dioxide uptake for photosynthesis with water conservation under fluctuating environmental conditions (Munemasa et al. 2007; Hossain et al. 2011; Ye et al. 2015).

Salicylic acid (SA) is a phenolic phytohormone that serves as a central regulator of plant immunity and stress responses. Best known for its role in activating systemic acquired resistance (SAR), a durable, broad-spectrum defense mechanism, SA enhances the plant's ability to resist future pathogenic attacks (White, 1979; Ward et al. 1991; Uknes et al. 1992). Beyond its function in biotic stress responses, SA also contributes to abiotic stress tolerance, including improved drought resistance (Miura et al. 2013; Okuma et al. 2014). One key mechanism through which SA confers protection is by promoting stomatal closure (Lee, 1998; Mori et al. 2001; Zeng and He, 2010; Khokon et al. 2011; Hua et al. 2012; Prodhan et al. 2018; Akter et al. 2020), thereby limiting water loss and restricting pathogen entry. Within guard cells, SA interacts with other hormonal and environmental signaling pathways,

forming an intricate regulatory network that underpins both stomatal immunity and adaptive stress responses (Melotto et al. 2017).

Chitosan (CHT) is a well-known elicitor derived from the partial deacetylation of chitin, a structural polysaccharide abundantly found in fungal cell walls, arthropod exoskeletons, and crustacean shells. According to Zhao et al. (2005), elicitors are molecules of biological or chemical origin that mimic microbial attacks and trigger a range of physiological and defense responses in plants. Chitosan has been widely reported to possess antifungal and antimicrobial activities (Kumar et al. 2005; Xing et al. 2005) and to promote plant growth and yield (Khan et al. 2002; Chibu et al. 2003; Gornik et al. 2008). Furthermore, it induces the expression of numerous defense-related genes involved in plant immune responses (Loschke et al. 1989; Doares et al. 1995). Beyond its antimicrobial and growth-promoting effects, chitosan has been shown to induce stomatal closure in various plant species, including *Solanum lycopersicum* and *Commelina communis* (Lee et al. 1999), *Pisum sativum* (Srivastava et al. 2009), and *Arabidopsis thaliana* (Khokon et al. 2010; Salam et al. 2012; Jahan et al. 2024), suggesting a role in early defense signaling and regulation of water loss.

Myrosinase (thioglucoside glucohydrolase, TGG; EC 3.2.1.147) is a prominent enzyme in cruciferous plants, especially abundant in guard cells where it contributes to stress-responsive signaling (Zhao et al. 2009; Islam et al. 2010; Rhaman et al. 2020). In *Arabidopsis thaliana*, TGG1 and TGG2 are highly expressed in guard cells and play an important role in mediating stomatal closure in response to signals such as abscisic acid (ABA) and methyl jasmonate (MeJA). Through this function, TGG enzymes help regulate stomatal aperture, enabling the plant to minimize water loss and enhance defense against environmental stress.

In this study, we examined the stomatal responses of *tgg* mutants to chitosan and salicylic acid to clarify whether TGG1 and TGG2 are involved in CHT- and SA-induced stomatal closure, and to determine if TGG1 and TGG2 directly interact with other signaling components within guard-cell pathways.

3.3 Materials and Methods

3.3.1 Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (WT) and *tgg1-3* (SAIL_786_B08), *tgg2-1* (SALK_038730), and *tgg1-3 tgg2-1* mutant lines were grown in a controlled environment, genomic PCR was used to confirm the mutant lines, and for experimental use, 4- to 5-week-old rosette leaves were harvested.

3.3.2 Stomatal aperture measurements

Stomatal aperture was determined using the method described by Munemasa et al. (2019). For each experiment, at least 20 stomatal apertures were measured. Excised rosette leaves were floated on a solution containing 5 mM KCl, 50 μ M CaCl₂, and 10 mM MES-Tris (pH 5.6), and incubated under light for 2 hours to promote stomatal opening. Subsequently, chitosan (10 and 50 μ g/mL) and salicylic acid (0.5 and 1 mM) were added to the assay solution and incubated under light for an additional 2 hours. Reactive carbonyl species (RCS) scavengers, carnosine (1 mM) and pyridoxamine (0.5 mM), were applied 30 minutes prior to chitosan and salicylic acid treatment. After the 2-hour incubation, the leaves were shredded using a commercial blender, and the epidermal tissues were collected through a nylon mesh. Images of stomata were captured using an inverted microscope (Olympus IX71; Tokyo, Japan),

and stomatal apertures were analyzed with WinRoof 3.0 software (Mitani Corporation, Fukui and Tokyo, Japan). To ensure data reliability, all measurements were performed in three independent experiments.

3.3.3 Measurement of ROS in guard cells

Rosette leaves were excised and homogenized using a blender, and epidermal tissues were collected through a nylon mesh. The tissues were incubated for 2 hours in stomatal assay solution containing 5 mM KCl, 50 μ M CaCl_2 , and 10 mM MES-Tris (pH 5.6). To detect reactive oxygen species (ROS) induced by 10 μ g/mL chitosan, 50 μ M $\text{H}_2\text{DCF-DA}$ dye (Sigma, St. Louis, MO, USA) was added, followed by a 30-minute incubation at room temperature in the dark. Afterward, the tissues were washed, recollected using nylon mesh, and resuspended in fresh assay solution. Chitosan (50 μ g/mL) was then applied, and samples were incubated for an additional 20 minutes in the dark. Reactive carbonyl species (RCS) scavengers, carnosine (1 mM) and pyridoxamine (0.5 mM), were applied 30 minutes prior to chitosan treatment to assess their effect on ROS production and to further investigate the role of TGG in the chitosan signaling pathway. ROS-associated fluorescence in guard cells was observed using a fluorescence microscope (Bio Zero BZ-8000; KEYENCE) equipped with an OP-66835 BZ filter (excitation 480/30 nm, emission 510 nm, dichroic mirror 505 nm). Fluorescence intensity was quantified using ImageJ software (version 1.42q; National Institutes of Health, USA).

3.3.4 Chitosan preparation

Low molecular weight chitosan (CAT no.448869) was purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA) and prepared as described previously with slight modifications (Hadwiger and Beckman, 1980).

3.3.5 Statistical analysis

Analysis of variance (ANOVA) statistical analysis was employed using Tukey's test to assess the significance of mean values differences ($p < 0.05$). statistical significance was defined as $P < 0.05$.

3.3.6 Accession numbers

The genome initiative numbers for the genes of *Arabidopsis* mentioned in this article are: TGG1 (At5g26000) and TGG2 (At5g25980).

3.4 Results

3.4.1 Stomatal responses to chitosan and salicylic acid in *Arabidopsis* wild-type and *tgg* mutants

To determine the involvement of TGG1 and TGG2 in chitosan- and salicylic acid-induced stomatal closure, stomatal responses to exogenous chitosan (10 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$) and salicylic acid (0.5 mM and 1 mM) were examined in wild-type (WT) *Arabidopsis thaliana* and in the *tgg1*, *tgg2*, and *tgg1 tgg2* mutant lines. The concentrations of chitosan and salicylic acid were selected based on previous studies by Jahan et al. (2025) and Akter et al. (2020), which demonstrated their efficacy in inducing stomatal closure in *Arabidopsis* guard cells. Both concentrations of chitosan and salicylic acid triggered significant stomatal closure in WT plants ($p < 0.05$), as well as in the *tgg1* and *tgg2* single mutants ($p < 0.05$). In contrast, no stomatal closure was observed in the *tgg1 tgg2* double mutant under the same treatment conditions ($p < 0.05$) (Fig. 3.1). Consistent with previous reports, the double mutant also failed to respond to ABA, MeJA, and AITC, which are known to induce

stomatal closure in WT plants. These findings suggest that TGG1 and TGG2 function redundantly to mediate stomatal closure in response to multiple signaling molecules, including chitosan, salicylic acid, AITC, ABA, and MeJA.

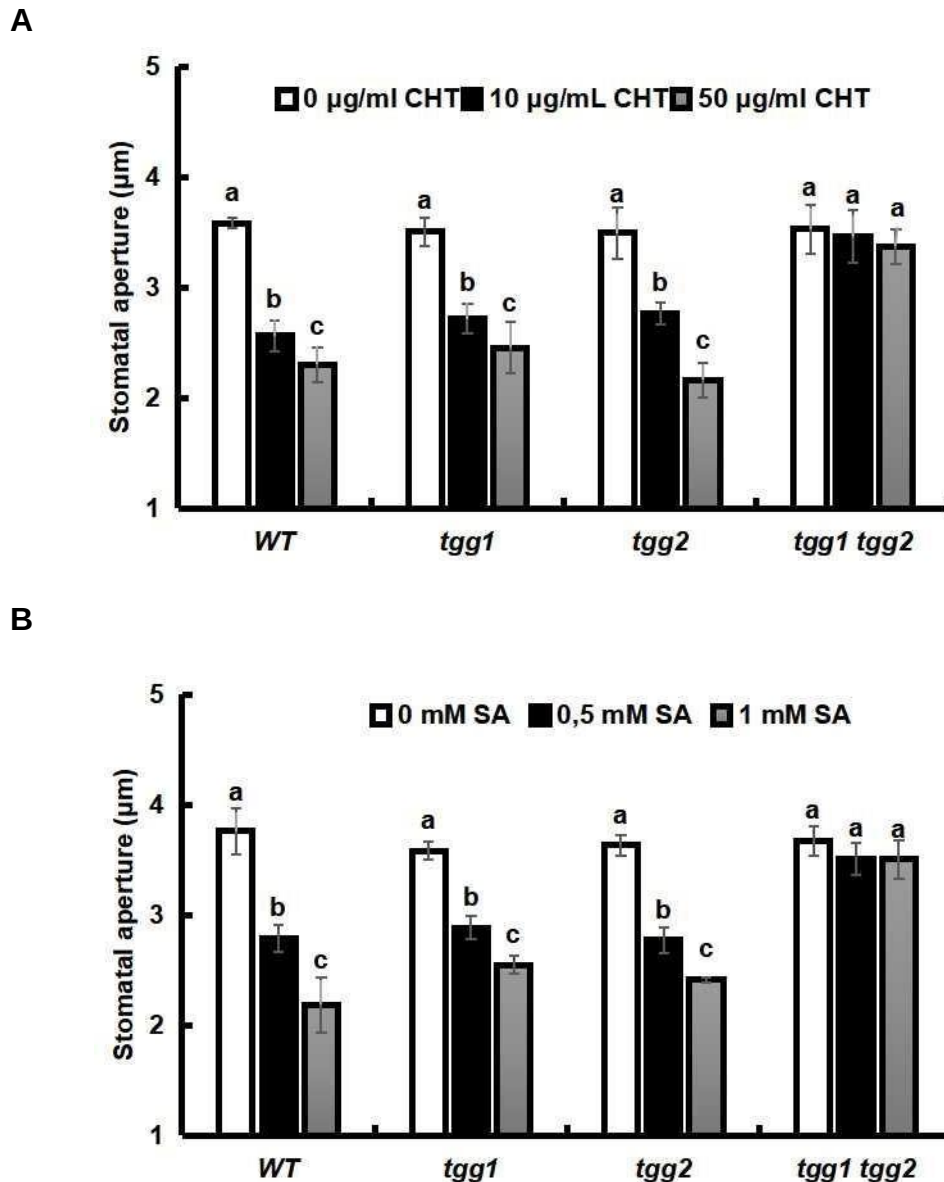


Fig. 3.1 Stomatal aperture in Arabidopsis in response to chitosan (CHT) and salicylic acid (SA). (A) Exogenous CHT-induced stomatal closure at 10 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ in the wild-type, *tgg1*, and *tgg2* but not in the *tgg1 tgg2*. (B) Exogenous SA-induced stomatal closure at 0.5 mM and 1 mM in the wild-type,

tgg1, and *tgg2* but not in the *tgg1 tgg2*. Data are presented as mean $\pm$ SE (n = 3, with >60 stomata). Bars with the same letter are not significantly different at $P < 0.05$

3.4.2 Impairment of chitosan- and salicylic acid-induced stomatal closure by application of reactive carbonyl species (RCS) scavengers in wild-type and *tgg* mutants

To further investigate the role of TGG1 and TGG2 in chitosan (CHT) and salicylic acid (SA) signaling pathways, and their potential interaction with downstream signaling components, we assessed the effects of reactive carbonyl species (RCS) scavengers, carnosine and pyridoxamine, on CHT- and SA-induced stomatal closure in *Arabidopsis* WT, *tgg1*, *tgg2* single mutants, and the *tgg1 tgg2* double mutant. Treatment with carnosine (1 mM) or pyridoxamine (0.5 mM) significantly inhibited stomatal closure induced by CHT (50 μ g/mL; Fig 3.2) and SA (1 mM; Fig 3.3) in both WT and mutant lines. These findings indicate that RCS are involved in CHT- and SA-mediated stomatal signaling and that scavenging RCS impairs this response. The inhibition of stomatal closure by RCS scavengers in WT and *tgg* mutants suggests that TGG1 and TGG2 contribute to RCS-related signaling in both the CHT and SA pathways.

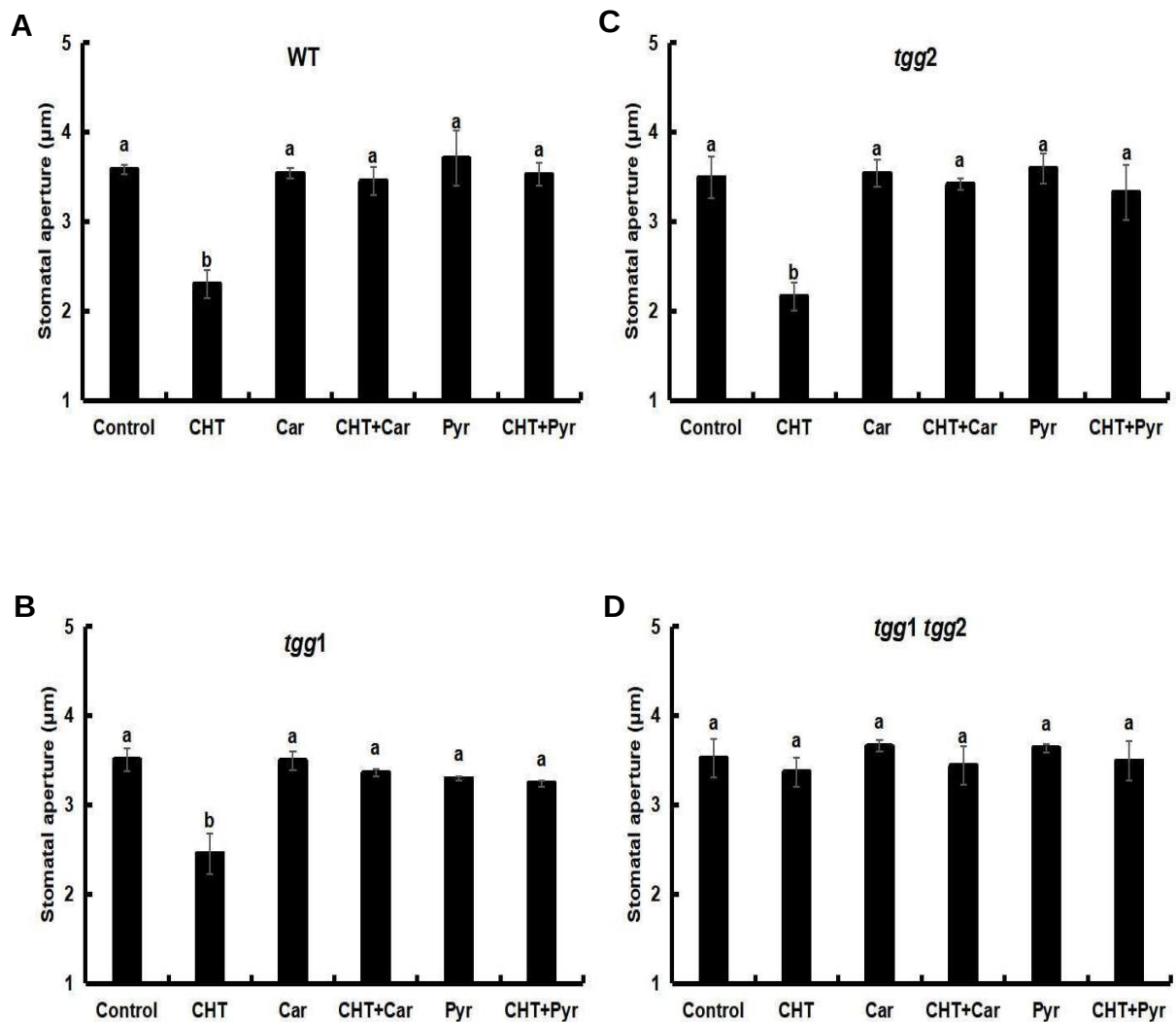


Fig. 3.2 Effects of RCS scavengers, carnosine and pyridoxamine; on CHT-induced Stomatal closure in *Arabidopsis* WT, *tgg1*, *tgg2*, and *tgg1 tgg2* mutants. 50 μg/mL CHT was added after 2 h incubation under light condition and the RCS scavengers (1 mM carnosine and 0.5 pyridoxamine) were added 30 min prior to CHT application. Data are presented as mean ± SE (n = 3, with >60 stomata). Bars with the same letter are not significantly different at $P < 0.05$

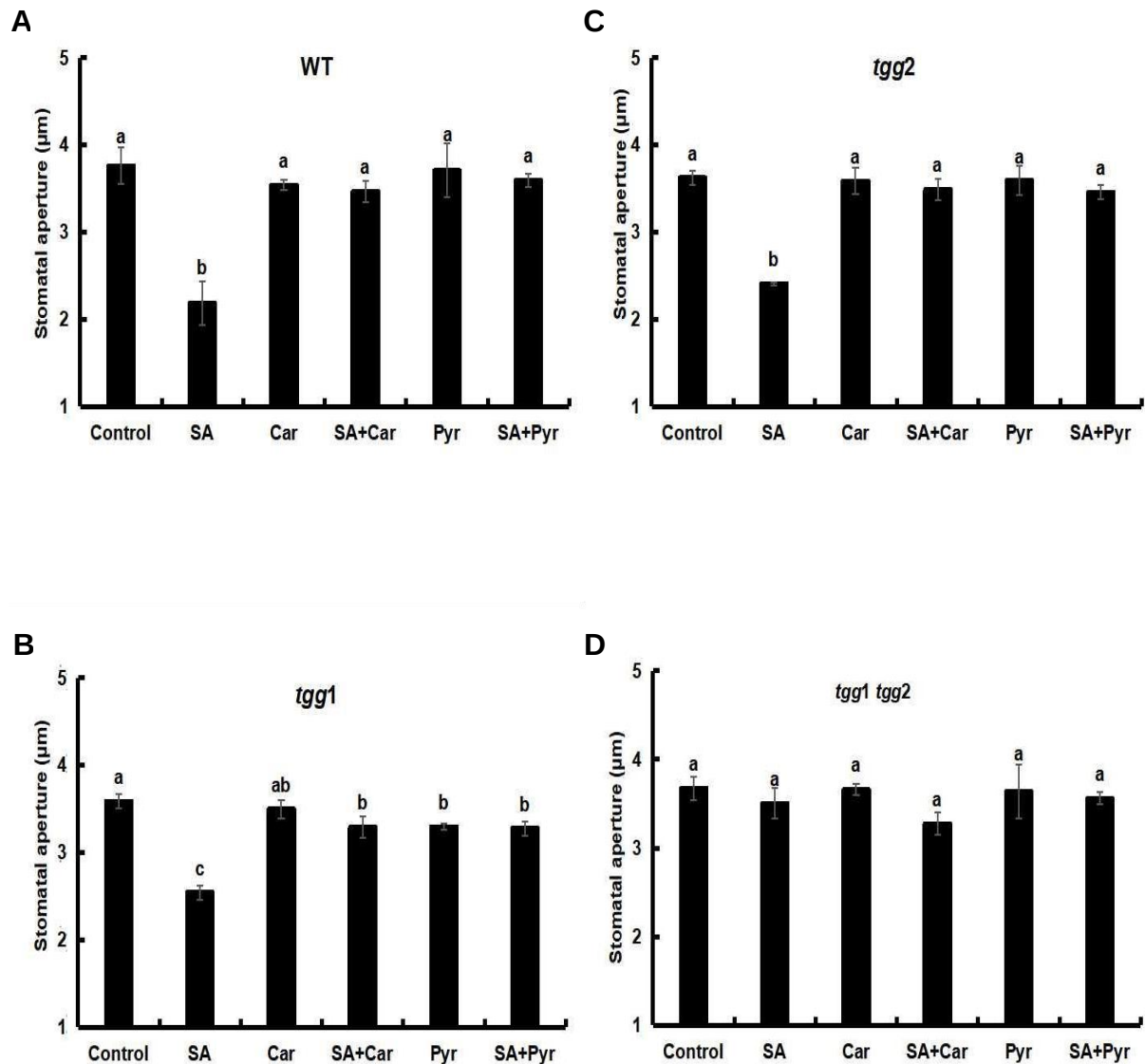


Fig. 3.3 Effects of RCS scavengers, carnosine and pyridoxamine; on SA-induced Stomatal closure in Arabidopsis. 1 mM SA was added after 2 h incubation under light condition and the RCS scavengers (1 mM carnosine and 0.5 pyridoxamine) were added 30 min prior to SA application. Data are presented as mean $\pm$ SE (n = 3, with >60 stomata). Bars with the same letter are not significantly different at $P < 0.05$

3.4.3 Chitosan-induced ROS production in wild-type and *tgg* mutants

Treatment with 50 $\mu\text{g/mL}$ chitosan (CHT) induced significant ROS accumulation in guard cells of *tgg1* ($p < 0.05$), *tgg2* ($p < 0.05$), and *tgg1 tgg2* ($p < 0.05$) mutants, as well as in wild-type plants ($p < 0.05$), consistent with previous findings (Jahan et al. 2025) (Fig. 3.4). These results indicate that neither TGG1 nor TGG2 acts upstream of ROS production in the CHT signaling pathway within guard cells.

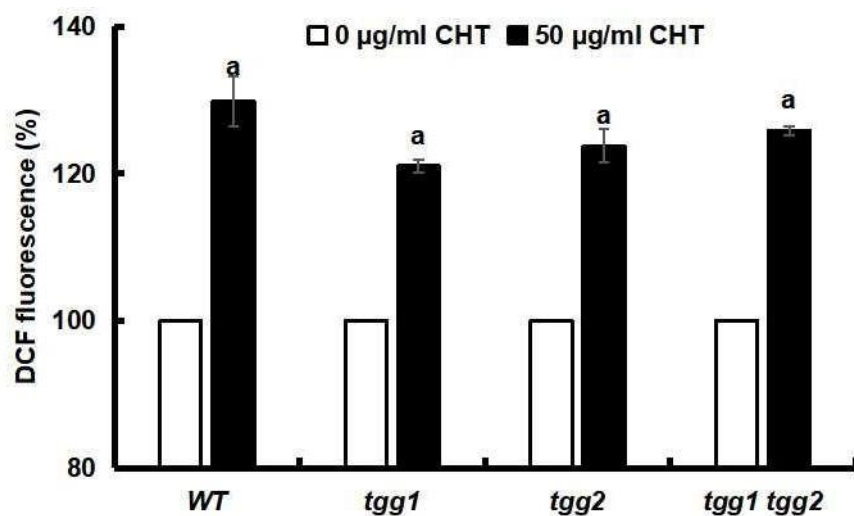


Fig. 3.4 Production of ROS in guard cells of the wild-type and *tgg* mutants induced by 50 $\mu\text{g/mL}$ CHT. Reactive oxygen species in the wild-type and *tgg* mutants were measured using $\text{H}_2\text{DCF-DA}$. Data are presented as mean $\pm$ SE ($n = 3$, with >60 stomata). Bars sharing the same letter are not significantly different at $P < 0.05$

3.4.4 Reactive carbonyl species (RCS) scavengers did not affect chitosan-induced ROS production in wild-type and *tgg* mutants

To clarify that TGG1 and TGG2 function downstream of ROS production, we investigated chitosan (CHT)-induced reactive oxygen species (ROS)

accumulation in wild-type (WT) and *tgg1*, *tgg2*, and *tgg1 tgg2* mutant plants in the presence of reactive carbonyl species (RCS) scavengers, carnosine and pyridoxamine. Our results showed that treatment with these RCS scavengers did not affect ROS production in either WT or the *tgg* mutants (Fig. 3.5). This suggests that TGG1 and TGG2 function downstream of ROS generation in the CHT-induced stomatal closure pathway. Consequently, while ROS production is an early event in chitosan signaling, TGG1 and TGG2 likely act at a later signaling stage, potentially mediating responses triggered by reactive carbonyl species.

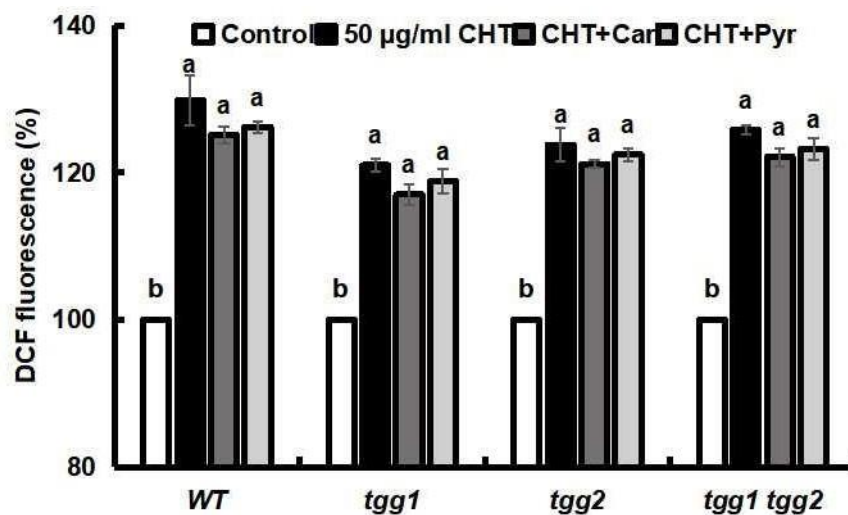


Fig. 3.5 Effects of RCS scavengers, carnosine and pyridoxamine; on CHT-induced ROS production in *Arabidopsis* guard cells. 1 mM SA was added after 2 h incubation under light condition and the RCS scavengers (1 mM carnosine and 0.5 pyridoxamine) were added 30 min prior to CHT application. Data are presented as mean $\pm$ SE ($n = 3$, with >60 stomata). Bars with the same letter are not significantly different at $P < 0.05$

3.4.5 TGG1 and TGG2 are involved in acrolein-induced stomatal closure in *Arabidopsis* guard cells

Acrolein has been shown to inhibit light-induced stomatal opening (Islam et al. 2015) and to induce stomatal closure in *Arabidopsis* (Islam et al. 2019). To investigate the involvement of TGG1 and TGG2 in reactive carbonyl species (RCS)-induced stomatal closure, we examined the stomatal responses to acrolein in *tgg1*, *tgg2*, and *tgg1 tgg2* mutants. Acrolein at 10 μ M triggered significant stomatal closure in WT plants ($p < 0.05$), as well as in the *tgg1* and *tgg2* single mutants ($p < 0.05$). In contrast, no stomatal closure was observed in the *tgg1 tgg2* double mutant under the same treatment conditions ($p < 0.05$) (Fig. 3.6).

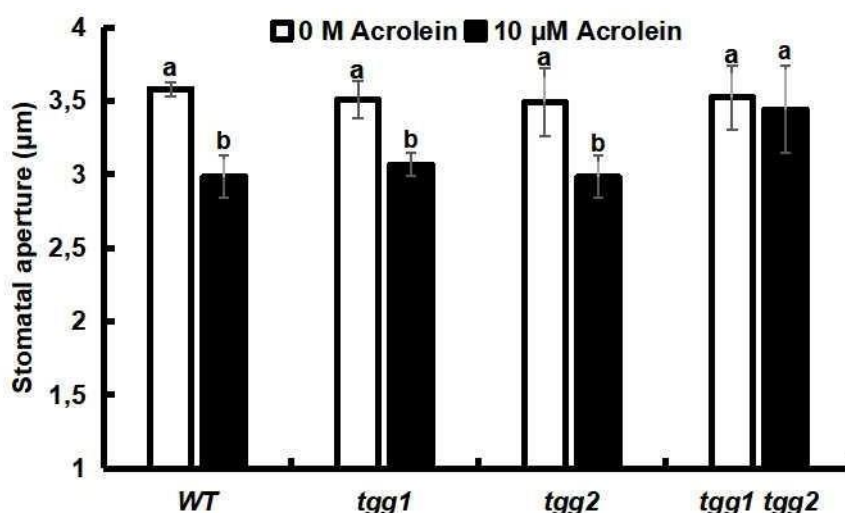


Fig. 3.6 Effects of acrolein, an RCS representative, on stomatal aperture in *Arabidopsis* guard cells. 10 μ M acrolein-induced stomatal aperture in WT and *tgg* single mutants but did not affect *tgg* double mutant. Data are presented as mean $\pm$ SE ($n = 3$, with >60 stomata). Bars with the same letter are not significantly different at $P < 0.05$

3.5 Discussion

Myrosinases are prominent components of the stomatal complex, with TGG1 is one of the most abundantly expressed proteins in guard cell. They play critical roles in stomatal movement triggered by environmental and hormonal cues such as light, ABA, and MeJA and both TGG1 and TGG2 have been shown to function redundantly in ABA and MeJA signaling pathways in *Arabidopsis thaliana* guard cells (Islam et al. 2009). Functionally, they hydrolyze glucosinolates into biologically active compounds such as isothiocyanates (ITCs), particularly in response to biotic stress (Zhao et al. 2008; Bhat and Vyas 2019). In the previous chapter, we explored the involvement of myrosinase-derived allyl isothiocyanate (AITC) in stomatal regulation and found that TGG1 and TGG2 act redundantly in mediating AITC-induced stomatal closure. However, the precise positioning of these enzymes within the broader AITC signaling cascade remained unclear.

In this chapter, we expanded our investigation to two additional stress-related pathways, chitosan (CHT) and salicylic acid (SA) signaling, to determine whether the role of TGG1 and TGG2 extends beyond ABA, MeJA, and AITC signaling. Our hypothesis was that TGG1 and TGG2 serve as central components in guard cell signaling downstream of ROS and possibly RCS production, regardless of the stress signaling molecule involved.

Our results showed that the application of CHT (10 μ g/mL and 50 μ g/mL) and SA (0.5 mM and 1 mM) induced stomatal closure in WT, *tgg1*, and *tgg2* single mutants, but not in the *tgg1 tgg2* double mutant (Fig. 1). This suggests that TGG1 and TGG2 are redundantly required for both CHT- and SA-induced stomatal closure. Moreover, the application of reactive carbonyl species (RCS) scavengers, carnosine and pyridoxamine significantly inhibited CHT- and SA-induced stomatal closure in WT plants (Fig 2). Consistently, exogenous

application of acrolein (10 μ M), a representative RCS, induced stomatal closure in WT, *tgg1*, and *tgg2*, but not in the *tgg1 tgg2* double mutant (Fig. 5). These results are in agreement with previous findings by Rhaman et al. (2020), which demonstrated that TGG1 and TGG2 are essential for RCS-induced stomatal closure in the ABA signaling pathway. Our present findings suggest that this functional requirement extends to SA and CHT signaling pathways, indicating a possible common involvement of TGG1 and TGG2 in RCS-mediated stomatal closure across different signaling pathways.

ROS accumulation was observed in all genotypes following CHT treatment, indicating that ROS production is independent of TGG1 and TGG2 (Fig. 3). This is consistent with reports that CHT-induced ROS production is mediated by SHAM-sensitive peroxidases in guard cells. Importantly, RCS scavengers did not alter ROS levels (Fig. 4), suggesting that ROS acts upstream of RCS production. This supports our current model in which TGG1 and TGG2 operate downstream of ROS and at or downstream of RCS generation in the CHT signaling pathway. These results are consistent with previous reports that, in ABA signaling, TGG1 and TGG2 function downstream of ROS and RCS and upstream of cytosolic Ca^{2+} elevation and pH changes in guard cells (Rhaman et al. 2020). Similarly, Jahan et al. (2025) showed that RCS is produced downstream of ROS in CHT signaling in both *Arabidopsis* and *Nicotiana tabacum*, suggesting a conserved ROS-RCS-TGG module in stress-induced stomatal regulation.

In SA signaling, it is well established that SA induces stomatal closure in *Arabidopsis* WT plants in association with ROS production. Here, SHAM-insensitive apoplastic peroxidases mediate extracellular ROS generation, producing mainly superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). These ROS subsequently migrate into guard cells through the

apoplastic pathway (Allan and Fluhr 1997; Khokon et al. 2011), where they oxidize membrane lipids, resulting in the formation of RCS. Our findings suggest that SA-induced RCS production is likely involved in stomatal closure, as this response was suppressed by carnosine and pyridoxamine (Fig. 2.2). Previous reports demonstrated that ABA- and MeJA-induced stomatal closure and RCS accumulation were also blocked by RCS scavengers (Islam et al. 2016, 2019), supporting the idea that RCS are common downstream mediators of multiple hormonal signaling pathways leading to stomatal closure. HPLC-based quantification of RCS levels in WT and mutant plants under CHT and SA treatments is needed to further clarify these interactions.

3.6 Conclusion

Taken together, our current findings demonstrate that TGG1 and TGG2 play a central, redundant role in stomatal closure induced by chitosan and salicylic acid, in addition to their previously characterized roles in ABA, MeJA, and AITC responses. These enzymes act downstream of ROS production and at or downstream of RCS generation, suggesting a conserved function in integrating oxidative and carbonyl signaling in guard cells. Their involvement in multiple signaling pathways underscores their significance as multifunctional regulators of plant defense and stress adaptation.

Chapter 4

General summary

Stomata are microscopic pores on the leaf surface of higher plants that regulate gas exchange and water loss, with their aperture controlled by pairs of guard cells. These guard cells perceive and integrate various environmental and endogenous signals, such as light, drought, CO₂ levels, phytohormones including abscisic acid (ABA) and methyl jasmonate (MeJA), as well as microbial cues, to modulate stomatal movement.

In cruciferous plants like *Arabidopsis thaliana*, the myrosinase-glucosinolate system contributes to both plant defense and stomatal regulation. Myrosinases catalyze the hydrolysis of glucosinolates into bioactive compounds such as isothiocyanates (ITCs), which act as deterrents against herbivores and pathogens. The myrosinase isoforms TGG1 and TGG2 are highly expressed in guard cells and are implicated in stomatal regulation in response to phytohormones and defense-related molecules. In *Arabidopsis thaliana*, TGG1 and TGG2 are essential for ABA- and MeJA-induced stomatal closure. Allyl isothiocyanate (AITC), a hydrolysis product of glucosinolates, also induces stomatal closure in *Arabidopsis*, a process associated with reactive oxygen species (ROS) production mediated by SHAM-sensitive peroxidases. Therefore, as a myrosinase-derived compound, AITC is presumed to induce stomatal closure even in the *tgg1 tgg2* double mutant. Similarly, salicylic acid (SA) and chitosan (CHT) trigger stomatal closure in *Arabidopsis* through reactive oxygen species (ROS) production involving SHAM-sensitive peroxidases. In both ABA- and CHT-induced signaling pathways, reactive carbonyl species (RCS) act downstream of ROS in *Arabidopsis* guard cells. Various stress conditions lead to the overproduction

of ROS in plants. The accumulated ROS oxidizes lipids, particularly polyunsaturated fatty acids (PUFAs), resulting in the formation of highly reactive and cytotoxic compounds known as RCS. At low concentrations, RCS can function as signaling molecules. However, whether TGG1 and TGG2 play a role in RCS signaling in response to CHT and SA is still not well understood.

In order to clarify whether TGG1 and TGG2 are involved in AITC-induced stomata closure we examined stomatal responses of the *tgg* mutants to AITC and whether TGG1 and TGG2 have functions other than ITCs production in the guard-cell signaling pathways. Specifically, I examined stomatal movement, ROS and nitric oxide (NO) production, cytosolic alkalization, and cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevation in response to AITC, a known product of myrosinase activity (Chapter 2). Application of 50 μ M AITC significantly induced stomatal closure in the wild type (WT) and in the single mutants *tgg1* and *tgg2*, but not in the *tgg1 tgg2* double mutant, indicating that TGG1 and TGG2 function redundantly in AITC-induced stomatal closure. However, AITC-induced ROS production was not impaired in the *tgg1 tgg2* mutant, consistent with observations for ABA- and MeJA-induced ROS responses in the same mutant. Similarly, AITC-induced NO accumulation occurred normally in all lines, suggesting that TGG1 and TGG2 do not act upstream of ROS or NO production in guard cell signaling. AITC also triggered cytosolic alkalization in both WT and mutant lines. Additionally, AITC, ABA, and MeJA all induced $[Ca^{2+}]_{cyt}$ oscillations in both WT and *tgg1 tgg2* mutants, indicating that TGG1 and TGG2 are not involved upstream of calcium signaling or cytosolic pH regulation during stomatal closure. Collectively, these findings indicate that while TGG1 and TGG2 are positive regulators of AITC-induced stomatal closure, they do not mediate early signaling events such as ROS and NO production, cytosolic alkalization, or calcium oscillation in guard cells.

Interestingly, although TGG1 and TGG2 also function redundantly in ABA- and MeJA-induced stomatal closure in *Arabidopsis*, most plant species lacking myrosinases and glucosinolates still exhibit stomatal responses to these hormones. This suggests that the myrosinase-glucosinolate pathway is not essential for hormone-induced stomatal closure in general. Furthermore, due to the distinct subcellular localization of TGG enzymes and glucosinolates, it is unlikely that ABA or MeJA directly trigger glucosinolate hydrolysis. Instead, TGG1 and TGG2 may contribute to stomatal closure through non-enzymatic mechanisms, possibly by interacting with downstream effectors such as ion channels.

To further clarify the roles of TGG1 and TGG2 in guard cell signaling pathways, I examined the stomatal response to chitosan (CHT), a biotic elicitor, and to salicylic acid (SA) (Chapter 3). Treatment with 10 and 50 $\mu\text{g/mL}$ CHT or 0.5- and 1-mM SA induced stomatal closure in wild-type (WT), *tgg1*, and *tgg2* single mutants, but not in the *tgg1 tgg2* double mutant, indicating that TGG1 and TGG2 function redundantly in both signaling pathways. Application of reactive carbonyl species (RCS) scavengers, 1 mM carnosine and 0.5 mM pyridoxamine, inhibited stomatal closure in all genotypes in response to both CHT and SA. In contrast, treatment with 10 μM acrolein, a representative RCS, induced stomatal closure in WT, *tgg1*, and *tgg2*, but not in the *tgg1 tgg2* mutant, suggesting that TGG1 and TGG2 are required for RCS-mediated stomatal signaling. Additionally, treatment with 50 $\mu\text{g/mL}$ CHT induced ROS accumulation in guard cells, and this ROS production was unaffected by the application of RCS scavengers, indicating that ROS generation occurs independently of or upstream of RCS production. Taken together, these results suggest that TGG1 and TGG2 act downstream of ROS production and upstream or at the level of RCS production in the signaling pathway. TGG1

and TGG2 are thus required for stomatal closure in response to both CHT and SA.

TGG1 and TGG2 are essential and functionally redundant components of guard cell signaling in *Arabidopsis thaliana*, required for stomatal closure in response to the myrosinase-derived metabolite AITC, the elicitor chitosan, the defense hormone salicylic acid, as well as the previously reported phytohormones abscisic acid and methyl jasmonate. These findings indicate that TGG1 and TGG2 integrate specific biotic stress signals into stomatal regulation independently of their enzymatic role in glucosinolate hydrolysis, highlighting their unique function in linking plant defense to stomatal movement.

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