Glucosinolate Degradation Products, Isothiocyanates, Nitriles, and Thiocyanates, Induce Stomatal Closure Accompanied by Peroxidase-Mediated Reactive Oxygen Species Production in Arabidopsis thaliana

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Isothiocyanates, nitriles, and thiocyanates are degradation products of glucosinolates in crucifer plants. In this study, we investigated the stomatal response to allyl isothiocyanate (AITC), 3-butenenitrile (3BN), and ethyl thiocyanate (ESCN) in Arabidopsis. AITC, 3BN, and ESCN induced stomatal closure in the wild type and the atrbohD atrbohF mutant. Stomatal closure was inhibited by catalase and salicylhydroxamic acid (SHAM). The degradation products induced extracellular reactive oxygen species (ROS) production in the rosette leaves, and intracellular ROS accumulation, NO production, and cytosolic free calcium concentration ([Ca²⁺]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 [Ca²⁺]cyt oscillation in Arabidopsis.

Key words: allyl isothiocyanate; 3-butenenitrile; ethyl thiocyanate; glucosinolates; reactive oxygen species

Isothiocyanates (ITCs), nitriles, and thiocyanates are degradation products of glucosinolate in crucifer plants. Degradation is catalyzed by myrosinases (EC 3.2.1.147).1,5) The myrosinase-glucosinolate system is involved in a range of biological activities, because ITCs have repellent effects on herbivores and insects, and biocidal activity,2) but nitriles and thiocyanates are not as bioactive as isothiocyanates, and consequently evidence of their physiological roles in plants is limited.

Guard cells surround stomatal pores in pairs and respond to various environmental stimuli. This regulates gas exchange, transpirational water loss, and the invasion of microorganisms.3) Glucosinolate-myrosinase system enhances abscisic acid (ABA)-induced stomatal closure,4) and allyl isothiocyanate (AITC) induces stomatal closure under methyl jasmonate (MeJA)-priming conditions in Arabidopsis thaliana.5) but it remains unknown whether nitriles and thiocyanates induce stomatal closure.

Stomatal closure requires the production of reactive oxygen species (ROS). This is mediated by plasma membrane NADPH oxidases and cell-wall peroxidases,6–8) NADPH oxidases AtRBOHD and AtRBOHF are involved in ABA- and MeJA-induced stomatal closure,6,7,9) and salicylhydroxamic acid (SHAM)-sensitive cell-wall peroxidases are involved in salicylic acid (SA)-, yeast elicitor (YEL)-, and chitosan (CHT)-induced stomatal closure.10–12) It has been reported that AITC induced ROS production in guard cells, and that this was slightly inhibited by an NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI),5) but it remains unsettled which enzyme mediates AITC signaling. Moreover, it remains unknown whether other degradation products induce ROS production, and what enzyme catalyzes 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,13) and SHAM (1 to 3 mM) and NaN₃ (300 μM to 1 mM) as inhibitors of cell-wall peroxidases.14) On the other hand, catalase (CAT) (100 to 500 units/mL) is widely used as an H₂O₂ scavenger,15) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide-3-oxide (cPTIO) (100 μM to 1 mM) as an NO scavenger.16)

ABA- and MeJA-induced ROS production results in the activation of Ca²⁺-permeable non-selective cation channels on the plasma membrane, leading to cytosolic free calcium concentration ([Ca²⁺]cyt) oscillation during stomatal closure.7,9) AITC-induced stomatal closure is also accompanied by [Ca²⁺]cyt oscillations in Arabidopsis guard cells.5) Moreover, whether nitriles and thiocyanates such as AITC induce stomatal closure and whether the production of ROS and the [Ca²⁺]cyt oscillations are induced in guard cells in response to nitriles and thiocyanates, remain to be clarified.

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

**Materials and Methods**

Plant materials and growth conditions. Arabidopsis (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\% relative humidity under a 16 h light/8 h dark regime. Water was applied 2–3 times per week with Hyponex solution (0.1\%) on the plant growth tray. Rosette leaves from 4- to 5-week-old plants were employed in assays. [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations in guard cells were measured using a Ca\textsuperscript{2+}-sensing fluorescent protein, Yellow Cameleon 3.6 (YC3.6).\textsuperscript{17}

**Measurement of stomatal aperture.** Stomatal aperture measurements were done as described previously.\textsuperscript{18} Excised rosette leaves were floated on a medium containing 5 mM KCl, 50\% CaCl\textsubscript{2}, and 10\% MES-Tрис (pH 6.15) for 2 h under light (80\% m\textsuperscript{-2} s\textsuperscript{-1}) to induce stomatal opening, and then were treated with 50\% AITC, 3BN, or ESCN. Stomatal apertures were measured after 2 h of incubation. CAT (100 units/mL), SHAM (1 mM), NaN\textsubscript{3} (1 mM), or DPI (20\% μM) was added 30 min before the application of AITC, 3BN, or ESCN. Leaves were blunted for 20\% s and epidermal tissues were collected. The epidermal tissues were mounted on a slide glass, and images of the stomatal apertures were captured using an Olympus IX71S87 microscope connected to a CS230 digital imaging color camera. Twenty stomatal apertures were measured in each experiment.

**Measurement of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and superoxide (O\textsuperscript{2−}) in whole leaves.** Rosette leaves of Arabidopsis plants were analyzed using 3,3’-diaminobenzidine tetrahydrochloride hydrate (DAB) (Tokyo Chemical Industries, Tokyo), as described previously.\textsuperscript{19} Excised rosette leaves were floated on a medium containing 5 mM KCl, 50\% CaCl\textsubscript{2}, and 10\% MES-Tрис (pH 6.15) with 0.05\% Tween20 and incubated for 2 h under light (80\% m\textsuperscript{-2} s\textsuperscript{-1}). Then the leaves were transferred to 1 mg/mL of 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 applied 30 min before AITC, 3BN, or ESCN application. After incubation, the leaves were cleared in boiling ethanol (99\%) for 10\% min. Localization of H\textsubscript{2}O\textsubscript{2} was visualized as a reddish-brown coloration. To detect O\textsuperscript{2−}, nitro blue tetrazolium (NBT) (Tokyo Chemical Industries) at 1 mg/mL was used instead of DAB. Localization of O\textsuperscript{2−} was visualized as a blue coloration. In both cases, the leaves were mounted on cover glasses and photographs were taken. The intensity of coloration was quantified using Adobe Photoshop CS2 software (Adobe Systems, San Jose, CA).

**Measurement of ROS and NO production in guard cells.** The production of ROS and NO in guard cells was examined using 50\% μM of 2,7’-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCF-DA) (Sigma, St. Louis, MO) and 10\% μM of 4,5-diaminofluorescein-2 diacetate (DAF-2DA) (Sigma) respectively, as described previously.\textsuperscript{20} For ROS detection, epidermal tissues were incubated in the light for 3 h in a medium containing 5 mM KCl, 50\% CaCl\textsubscript{2}, and 10\% MES-Tрис (pH 6.15), and then 50\% μM H\textsubscript{2}DCF-DA was added to the 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), SHAM (1 mM), or DPI (20\% μM) was added 30 min before the application of AITC, 3BN, or ESCN. To detect NO, 10\% μM DAF-2DA was added instead of 50\% μM H\textsubscript{2}DCF-DA. Fluorescent images of guard cells were captured using a fluorescence microscope (Biozero BZ-8000, Keyence, Osaka, Japan) with a filter: OP-6683SBZ filter GFP (excitation wavelength, 480/30 nm; emission wavelength, 510 nm; dichroic mirror wavelength, 505 nm). Fluorescent intensity was analyzed using ImageJ 1.42q software (NIH, Bethesda, MD).

**Measurement of [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations in the guard cells.** Yellow Cameleon 3.6 (YC3.6) was employed as a Ca\textsuperscript{2+} indicator to monitor [Ca\textsuperscript{2+}]	extsubscript{cyt} in Arabidopsis guard cells, as described previously.\textsuperscript{7} Abaxial epidermal peels of rosette leaves expressing YC3.6 were placed in a 6-well plate containing 5 mm KCl, 50\% CaCl\textsubscript{2}, and 10\% MES-Tрис (pH 6.15) for 2 h under light (80\% m\textsuperscript{-2} s\textsuperscript{-1}). Turgid guard cells were used in the measurement of [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillation. The excitation wavelength was 440 nm, and the emission wavelengths were 480 nm and 535 nm. Ratio-metric (F\textsubscript{480}/F\textsubscript{535}) images were obtained using a fluorescence microscope (IX71, Olympus, Tokyo) equipped with a dual-emission imaging system (W-View system; 440AF21 excitation filter, 454DRLP dichroic mirror and two emission filters, 480DF30 for cyan fluorescent protein and 535DF25 for yellow fluorescent protein; Hamamatsu Photonics, Hamamatsu, Japan) and a CCD camera (Hamamatsu ORCA-ER digital camera, Hamamatsu Photonics). The cyan fluorescent protein and yellow fluorescent protein fluorescence intensities of the guard cells were imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics).

**Statistical analysis.** The significance of differences between mean values was assessed by analysis of variance (ANOVA) with Tukey’s test. Differences in the frequency of [Ca\textsuperscript{2+}]	extsubscript{cyt} oscillations in the wild-type induced by glucosinolate degradation products were determined by χ\textsuperscript{2}-test. We regarded differences at the level of p < 0.05 as significant. Data are presented as means ± SE.

**Accession numbers.** The Arabidopsis genome initiative numbers for the genes discussed here are as follows: AtRBOHD (At5g47910), and AtRBOHF (At1g64060).

**Results**

**Glucosinolate degradation product-induced stomatal closure**

We examined to determine whether AITC, 3BN, and ESCN induce stomatal closure. AITC at 10\% μM and 50\% μM significantly induced stomatal closure in the wild-type plants (Fig. 1A), as reported previously.\textsuperscript{5} 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 the wild-type plants (Fig. 1A). A solvent control (0.1\% dimethyl sulfoxide, DMSO) did not affect stomatal aperture (data not shown).

We examined the effects of an H\textsubscript{2}O\textsubscript{2} scavenger, CAT, peroxidase inhibitors, SHAM and Na\textsubscript{3}N, and an NADPH oxidase inhibitor, DPI, on AITC-, 3BN-, and ESCN-induced stomatal closure (Fig. 1B). Degradation product-induced stomatal closure was significantly inhibited by 100 units/mL of CAT, by 1 mM SHAM, and by 1 mM Na\textsubscript{3}N, suggesting that glucosinolate degradation products induce stomatal closure via ROS production mediated by peroxidases. On the other hand, degradation product-induced stomatal closure was partially inhibited by 20\% μM DPI (Fig. 1B). CAT, SHAM, Na\textsubscript{3}N, and DPI did not affect stomatal aperture (data not shown).

The application of AITC, 3BN, and ESCN at 10\% μM and 50\% μM significantly induced stomatal closure in a dose-dependent manner in the atrbohD atrbohF mutant plants, as in the wild-type plants (Fig. 1C).

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

We examined histochemically the accumulation of H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2−} in rosette leaves using DAB\textsuperscript{18} and NBT\textsuperscript{20} respectively. AITC, 3BN, and ESCN at 50\% μM significantly induced H\textsubscript{2}O\textsubscript{2} production in the rosette leaves (Fig. 2A), which were significantly inhibited by
1 mM SHAM (Fig. 2A). AITC, 3BN, and ESCN at 50 mM significantly induced O$_2^-$/C0$_2$ production in the rosette leaves (Fig. 2B), which were also significantly inhibited by 1 mM SHAM (Fig. 2B).

Glucosinolate degradation product-induced intracellular ROS accumulation in guard cells

We examined to determine whether glucosinolate degradation products induce ROS accumulation in guard cells using fluorescent dye H$_2$DCF-DA. The application of 50 mM AITC significantly induced ROS accumulation in guard cells of the wild-type plants (Fig. 3A), as previous reported. Like AITC, 3BN and ESCN at 50 mM significantly induced ROS accumulation in guard cells of the wild-type plants. ROS accumulation was significantly inhibited by pretreatment with 100 units/mL of CAT and by 1 mM SHAM, but not by 20 mM DPI (Fig. 3A). AITC, 3BN, and ESCN at 50 mM also induced ROS accumulation in guard cells of the $\text{atrb}ohD$ $\text{atrb}ohF$ mutant plants (Fig. 3B). There were no significant differences in fluorescence intensity as between the wild type and the mutant (Fig. 3A and B).

Glucosinolate degradation product-induced NO production in guard cells

AITC-induced stomatal closure is accompanied by NO production. We examined the effects of an NO specific scavenger, cPTIO, on AITC-, 3BN-, and ESCN-induced stomatal closure in the wild-type plants (Fig. 4). Degradation product-induced stomatal closure was partially inhibited by cPTIO at 100 mM (Fig. 4A), whereas ROS accumulation in the guard cells was not
significantly inhibited by cPTIO (Fig. 4B). AITC, 3BN, and ESCN at 50 µM significantly induced NO production in the guard cells, but it was significantly reduced by SHAM at 1 mM (Fig. 4C).

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

We examined to determine whether degradation products would induce [Ca\(^{2+}\)]\(_{cyt}\) oscillations in the guard cells using YC3.6-expressing wild-type plants. AITC at 50 µM induced [Ca\(^{2+}\)]\(_{cyt}\) oscillation in 86% of the guard cells not treated by SHAM (n = 19 of 22 cells; Fig. 5A and C) as previously found,\(^{30}\) whereas AITC at 50 µM induced [Ca\(^{2+}\)]\(_{cyt}\) oscillation in 30% of the SHAM-treated wild-type guard cells (n = 6 of 20 cells; Fig. 5B and C). Like AITC, 3BN at 50 µM induced [Ca\(^{2+}\)]\(_{cyt}\) oscillation in 75% of the guard cells not treated by SHAM (n = 22 of 26 cells; Fig. 5D and F) while 3BN at 50 µM induced [Ca\(^{2+}\)]\(_{cyt}\) oscillation in 29% of the SHAM-treated guard cells (n = 7 of 24 cells; Fig. 5E and F). In turn, ESCN triggered [Ca\(^{2+}\)]\(_{cyt}\) oscillation in 85% of the guard cells not treated by SHAM (n = 22 of 26 cells; Fig. 5G and I) and in 27% of the SHAM-treated guard cells (n = 6 of 22 cells; Fig. 5H and I).

Discussion

Glucosinolate degradation product-induced stomatal closure

Glucosinolates are secondary metabolites in crucifer plants. Myrosinases are responsible for the degradation of glucosinolates, which results in the formation of a variety of products that are active against plant enemies (e.g., herbivores, insects, and pathogens).\(^{21}\) On the other hand, the amount of glucosinolate in young sprouts of broccoli was 70–100 µmol g\(^{-1}\) FW.\(^{20}\) and the amount of ITCs in ground leaves of Arabidopsis was approximately 10 µmol g\(^{-1}\) FW.\(^{22}\) Thus the amount of degradation

![Fig. 2. Glucosinolate Degradation Product-Induced ROS Production in Whole Leaves.](Image)

![Fig. 3. Glucosinolate Degradation Product-Induced ROS Accumulation in Guard Cells.](Image)
Glucosinolate Degradation Products Induce Stomatal Closure

(A) AITC-, 3BN-, and ESCN-induced stomatal closure was inhibited by 100 μM cPTIO. Rosette leaves of the wild-type plants were treated with cPTIO for 30 min before application of AITC, 3BN, or ESCN (n = 5, 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 (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 (n = 3, >60 stomata). The vertical scale represents the percentages of the fluorescence intensity for ROS and of the fluorescence intensity for NO when the fluorescence intensity of the treated cells is normalized to the control value, taken as 100%. Error bars represent SE.

products can reach the sub-mm level in the leaves, and the degradation products of glucosinolate can 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.7,9 In the present study, degradation products AITC, 3BN, and ESCN induced stomatal closure, which was inhibited by CAT and SHAM (Fig. 1B), and induced O$_2^-$ and H$_2$O$_2$ production in whole rosette leaves, which was inhibited by SHAM (Fig. 2), suggesting that the degradation products induce stomatal closure accompanied by extracellular peroxidase-mediated ROS production. In tobacco suspension-cultured cells and Vicia faba guard cells, SA-induced O$_2^-$ and H$_2$O$_2$ production was mediated by SHAM-sensitive extracellular peroxidases.21,22 Moreover, YEL- and CHT-induced ROS production is also mediated by SHAM-sensitive extracellular peroxidases.11,12 In sum, extracellular peroxidases might be a key enzyme in ROS production that induces stomatal closure.

SHAM inhibits peroxidases,10–12,14,22 but is not very specific in the inhibition of peroxidases, because it also inhibits alternative oxidases23 and lipoygenases.24 Hence it is impossible to exclude the involvement of alternative oxidases and lipoygenases in the degradation product-induced stomatal closure. However, another inhibitor of peroxidases, NaN$_3$,12,25 also inhibited the stomatal closure induced by the degradation products (Fig. 1), which confirms our conclusion that SHAM-sensitive peroxidases are involved in stomatal closure.

Arabidopsis has 73 class-III peroxidase genes (cell wall peroxidases),26 and a large number of class-III peroxidases are involved in the apoplastic oxidative burst for pathogen resistance in Arabidopsis.27 Class-III peroxidases might be involved in the ROS production induced by AITC, 3BN, and ESCN, but the involvement of other peroxidases cannot be excluded.

Two NADPH oxidases, AtRBOHD and AtRBOHF, are involved in ABA-induced ROS production in guard cells of Arabidopsis.5,9 On the contrary, neither the atrb0hD atrb0hF mutation nor the application of DPI impairs the degradation product-induced ROS production (Fig. 3), suggesting that degradation product-induced ROS production in guard cells is mediated by peroxidases, but not by NADPH oxidases. Nevertheless, DPI slightly inhibited degradation product-induced stomatal closure. This may have been because DPI is an inhibitor of flavoproteins, including NADPH oxidases. Furthermore, ITCs are potent inhibitors of leukocytic NADPH oxidases.28 Hence, it is unlikely that NADPH oxidases were responsible for the degradation product-induced stomatal closure that we observed.

Sharing of signal components between degradation product signaling and other signaling

AITC-, 3BN-, and ESCN-induced NO production was completely inhibited by SHAM (Fig. 4C), and AITC-, 3BN-, and ESCN-induced [Ca$^{2+}$]$_{cyt}$ oscillations was inhibited by SHAM (Fig. 5), suggesting that AITC, 3BN, and 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,12 indicating that SHAM-sensitive peroxidases are not involved in ABA signaling and degradation product signaling dose not completely overlap with ABA signaling.

Stomatal closure was induced by the degradation products of glucosinolate, YEL, CHT, and SA, which are inhibited by SHAM (Fig. 1B),10–12 suggesting that peroxidases are involved in these stomatal closure. The degradation products of glucosinolate-, YEL-, and CHT-
induced stomatal closure are accompanied by $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation (Fig. 5A, D and G),\textsuperscript{11,12} but SA-induced stomatal closure is not accompanied by $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation.\textsuperscript{10} Taken together, these results suggest that ROS production and $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation and elevation function not only in tandem but also in parallel in guard cell signaling.

In sum, degradation products can elicit extracellular ROS production followed by intracellular ROS accumulation and $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation and elevation, causing stomatal closure.

**Physiological significance of the degradation products of glucosinolates**

The hydrolysis of glucosinolates in plant damage results in the formation of ITCs, nitriles, and thiocyanates, and the types of degradation products that form vary with the pH, the chemical structure of the glucosinolates, and so on.\textsuperscript{1,28} For example, ITCs are generally produced at neutral pH, and nitriles at lower pH.\textsuperscript{28} In animal cells, ITCs strongly inhibit carcinogenesis and tumorigenesis, and induce apoptosis due to their electrophilicity.\textsuperscript{29} Moreover, animal cells are more
susceptible to ITCs than other degradation products, because ITCs are much more electrophilic than nitriles and thiocyanates.\(^{30}\)

Low concentrations of AITC increase glutathione S-transferase (GST) activity in Arabidopsis,\(^{31}\) although high concentrations (>1 mM) of exogenous AITC are harmful in Arabidopsis\(^ {31}\) and \textit{Lactuca sativa}.\(^ {32}\) In the present study, isothiocyanates, nitriles, and thiocyanates induced stomatal closure in Arabidopsis (Fig. 1A), suggesting that degradation products regulate physiological functions, including stomatal movements against the attack of insects, pathogens, and herbivores, regardless of the conditions as to glucosinolate degradation in crucifer plants.

**Conclusion**

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

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**References**