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Chitosan signaling in guard cells requires endogenous salicylic acid

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An elicitor chitosan (CHT) induces stomatal closure but the mechanism remains to be clarified. A phytohormone salicylic acid (SA) is crucial for elicitor-induced defense signaling in plants. Here we investigated whether endogenous SA is required for CHT signaling in guard cells. In the SA-deficient *nahG* mutant, treatment of CHT did not induce either apoplastic reactive oxygen species (ROS) production or stomatal closure but co-treatment of CHT and SA induced both apoplastic ROS production and stomatal closure, indicating the involvement of endogenous SA in CHT-induced apoplastic ROS production and CHT-induced stomatal closure. Furthermore, CHT induced transient cytosolic free calcium concentration increments in the *nahG* mutant in the presence of exogenous SA but not in the absence of exogenous SA. These results provide evidence that endogenous SA is a crucial element in CHT-induced stomatal closure.

Key words: stomatal closure; chitosan; salicylic acid; reactive oxygen species; cytosolic Ca^{2+} oscillations

A pair of guard cells forms a stomatal pore, which is the most important port of entry for pathogens, gaseous exchange and transpiration. Guard cells respond to a variety of biotic and abiotic stimuli such as elicitors, salicylic acid (SA), and abscisic acid (ABA) and consequently control innate immunity, exchange of CO_2 and O_2, and transpirational loss of water.[1)]

Elicitor is a chemical or biological molecule that mimics microbial attack and causes physiological changes in plants.[2)] Chitosan (CHT), a fungal elicitor, is a deacylated derivative of chitin which is present mainly in the exoskeleton of insects, crustaceans, and cell wall of fungi.[3)] CHT induces stomatal closure in plants,[4–6] but the mechanism remains unclear.

Previous studies have suggested that SA is important for CHT signaling in plants. For instances, CHT oligosaccharide induces defense responses via the SA-mediated signaling pathway,[7)] and induces methyl-salicylate accumulation in plants.[8)] Hossain et al.[9)] reported that methyl jasmonate (MeJA)-induced stomatal closure requires endogenous ABA whereas Issak et al.[10)] reported that SA-, yeast elicitor (YEL)- or CHT-induced stomatal closure requires neither endogenous ABA nor endogenous JA in Arabidopsis. However, whether CHT signaling in guard cells requires endogenous SA are to be investigated.

It is well-known that reactive oxygen species (ROS) play a role as a second messenger in guard cell signaling[11,12)] and that ROS production is crucial for signal integration in guard cells.[1,13,14)] CHT signaling in guard cells is accompanied by ROS production which is mediated by salicylhydroxamic acid (SHAM)-sensitive peroxidases.[4–6)]

Calcium (Ca^{2+}) functions as an important second messenger in signaling in guard cells.[11,15,16)] Elicitors trigger ROS production which activates non-selective Ca^{2+} channels leading to elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$).[5,6,17,18)] CHT elicits $[Ca^{2+}]_{cyt}$ oscillations for the induction of stomatal closure in Arabidopsis.[6)]

To elucidate the roles of endogenous SA in CHT signaling in guard cells, here we analyzed CHT-induced apoplastic ROS production, $[Ca^{2+}]_{cyt}$ oscillations, and stomatal closure in the SA-deficient *nahG* mutant. We found that treatment of CHT did not induce either apoplastic ROS production or stomatal closure but co-treatment of CHT and SA induced both apoplastic ROS production and stomatal closure. In addition, CHT elicited transient $[Ca^{2+}]_{cyt}$ increments in the presence of SA but not in the absence of SA in the *nahG* mutants. Based on the results, we concluded that endogenous SA is an important signal transducer in CHT signaling in Arabidopsis guard cells.

Materials and methods

Plants and their growth conditions. Arabidopsis ecotype Columbia-0 (Col-0) was used as wild-type plant. The *nahG*,[19)] was used as an SA-deficient

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Abbreviations: CHT, chitosan; SA, salicylic acid; ABA, abscisic acid; ROS, reactive oxygen species; $[Ca^{2+}]_{cyt}$, concentration of cytosolic Ca^{2+}; MeJA, methyl jasmonate; JA, Jasmonic acid; YEL, yeast elicitor; SHAM, salicylhydroxamic acid

mutant. Plants were grown in vermiculite:soil (v/v) 1:1 mixture in controlled growth conditions under a photoperiod of 16-h-light/8-h-dark with photon flux density of 80 μmol m^{-2} s^{-1}. The relative humidity and temperature were 70% and 21 °C. Hyponex (0.1%) nutrient solution was provided to the plants twice per week. Four-week-aged plants were used for the experiments.

Analysis of stomatal apertures. Stomatal apertures were measured as described previously.[20] Young fully expanded leaves were floated on assay buffer (5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES, pH 6.15) for 2 h in the light to induce stomatal opening. The solutions of chemicals such as CHT (50 μg/mL), SA (50 μM), and catechol (50 to 500 μM) were directly added to the buffer solution either individual or in combination. After again 2 h incubation in the light, the leaves were blended in tap water in a commercial blender for 25 to 30 s. The blended-epidermal tissues were collected using a nylon mesh (pore size 100 μm) and were mounted on glass slides for microscopic observation. Twenty stomatal apertures were measured for each individual experiment.

Measurement of apoplastic ROS production. The generation of ROS was measured by the Thordal-Christensen et al.[21] method. The leaves were floated on assay buffer containing 5 mM KCl, 50 μM $CaCl_2$, 10 mM MES, and 0.1% Tween 20, pH 6.15 in the light for 3 h. Then 3,3′-diaminobenzidine (DAB) at 1 mg/mL was added to the assay buffer and gently infiltrated for 2 h in a vacuum. In the presence or absence of SA, CHT was then added to the buffer solution and again incubated for 2 h. The leaves were depigmented by boiling in ethanol. Hydrogen peroxide (H_2O_2) was visualized as reddish-brown color which was quantified using Image-J software (National Institutes of Health, USA).

Measurement of $[Ca^{2+}]_{cyt}$ oscillations in guard cells. Four- to six-week-old wild-type and *nahG* plants expressing a Ca^{2+}-reporter fluorescence protein, Yellow Cameleon 3.6 (YC3.6) were used for the observation of guard cell $[Ca^{2+}]_{cyt}$ oscillations as described previously.[9,22] The lower surface of the excised leaf was attached on a glass slide using medical adhesive (Stock No. 7730; Hollister). Then the upper surface and mesophyll tissues of the leaves were removed. Isolated lower epidermal peels were incubated in the solution containing 5 mM KCl, 50 μM $CaCl_2$, 10 mM MES (pH 6.15-Tris) under light for 2 h to enhance stomatal opening. The $[Ca^{2+}]_{cyt}$ oscillations were monitored in the turgid guard cells. Guard cells were treated with 50 mg/mL CHT solution by using a peristaltic pump in the 5 min after monitoring and 50 μM SA was added just after CHT application. For dual-emission ratio imaging of YC3.6, a 445DRLP dichroic mirror, a 440AF21 excitation filter, and two emission filters, 480DF30 for cyan fluorescent protein (CFP) and 535DF25 for yellow fluorescent protein (YFP) were used. The CFP and YFP fluorescence intensities of guard cells were imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics). Similar exposure time was applied for both CFP and YFP.

Statistical analysis. Two-way ANOVA was performed to compare the significance of differences of mean values of stomatal apertures and ROS production between the groups (e.g. between wild-type and *nahG* plants) and *t*-test was performed to compare that within

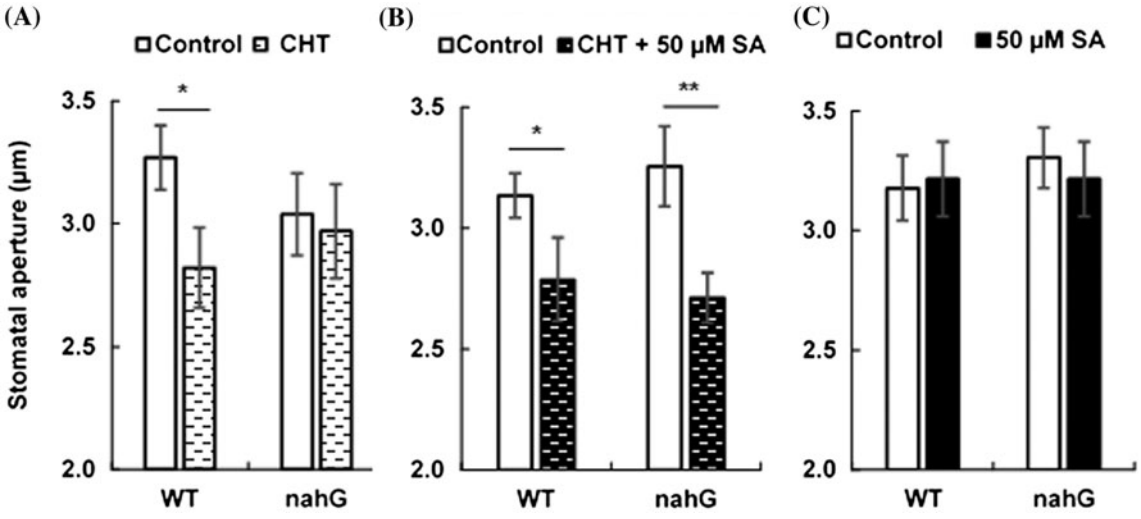

Fig. 1. CHT-induced responses of stomatal apertures in Arabidopsis wild-type (WT) and *nahG* plants. (A) CHT at 50 μg/mL induced stomatal closure in the WT plant but not in the *nahG* mutant. (B) Stomatal closure induced by co-treatment of 50 μg/mL CHT and 50 μM SA in the WT and *nahG* plants. (C) SA at 50 μM did not induce stomatal closure both in the WT and *nahG* plants.

Notes: Each bar represents averages of stomatal apertures from at least total 80 stomata. Stomatal apertures from four independent experiments ($n = 4$) are shown. Error bars represent SE. *, and ** indicate $p < 0.05$, $p < 0.01$, respectively.

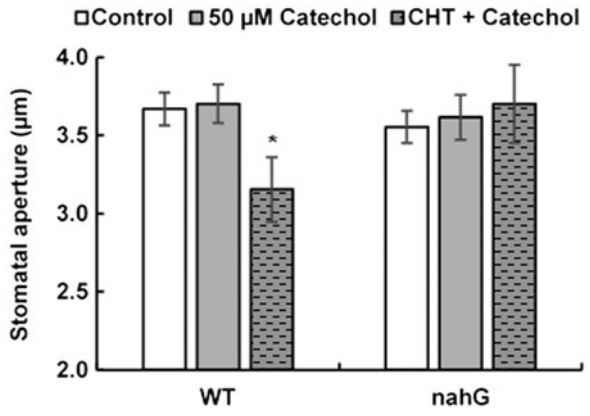

Fig. 2. Effects of catechol on stomatal apertures of wild-type (WT) and *nahG* plants. Catechol at 50 μM did not induce stomatal closure in the WT plant and *nahG* mutant. Co-treatment of CHT (50 μg/mL) and catechol (50 μM) induced stomatal closure in the WT plant but not in the *nahG* mutant.

Notes: Each bar represents averages of stomatal apertures from 60 stomata. Stomatal apertures from three independent experiments ($n = 3$) are shown. Error bars represent SE. * indicates $p < 0.05$.

the group. The χ^2 test was performed to assess the significance of differences between frequencies of $[Ca^{2+}]_{cyt}$ oscillations. Significant differences were considered at $p \leq 0.05$.

Results

CHT in presence of SA induced stomatal closure in the nahG but not in absence of SA

To investigate whether endogenous SA is required for CHT signaling in guard cells, we examined the effects of the application of CHT with or without SA on stomatal apertures of *nahG* mutants. We found that CHT (50 μg/mL) without SA did not induce stomatal closure (Fig. 1(A)) but co-treatment of CHT (50 μg/mL) and SA (50 μM) significantly induced stomatal closure in the *nahG* (Fig. 1(B)), suggesting endogenous SA is involved in CHT-induced stomatal closure. Note that SA at 50 μM did not induce stomatal closure in wild-type or *nahG* (Fig. 1(C)), indicating that 50 μM SA did not affect CHT-induced stomatal closure in wild-type where endogenous SA levels were higher than those in the *nahG*. Note that SA induces stomatal closure at 100 and 150 μM but not at 50 μM (data not shown) so that we employ 50 μM as the highest concentration not to induce stomatal closure.

In the *nahG* mutants, the enzyme salicylate hydroxylase catalyzes the conversion of SA to catechol.[23] Therefore, we tested the effects of catechol on the stomatal apertures of wild-type and *nahG* plants. We found that co-treatment of CHT and catechol (50 μM) induced stomatal closure in the wild-type plants but not in the *nahG* mutants (Fig. 2). Catechol did not induce stomatal closure in the wild-type plants (Fig. 2, Fig. S1). This result suggests that catechol has no effect on the SA-dependent CHT-induced stomatal closure in *nahG*.

CHT in presence of SA triggered ROS production in the nahG but not in absence of SA

Next, to test whether endogenous SA is necessary for CHT signaling in guard cells, we examined the effects of the application of CHT with or without SA on apoplastic ROS production in the *nahG* mutants. Histochemical staining using DAB (1 mg/mL) revealed that CHT did not induce ROS production in the *nahG* mutants in the absence of SA (Fig. 3(A)) but in the presence of SA (Fig. 3(B)), suggesting endogenous SA

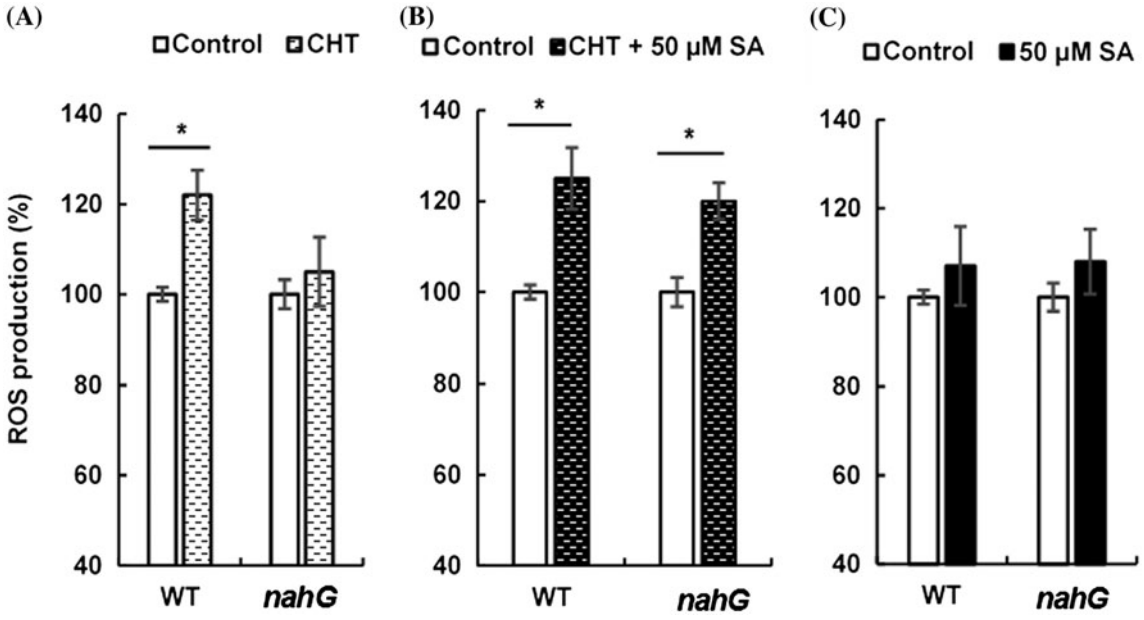

Fig. 3. CHT-elicited apoplastic ROS production in the wild-type (WT) and *nahG* plants. (A) CHT at 50 μg/mL induced apoplastic ROS production in the WT plant but not in the *nahG* mutant. (B) Apoplastic ROS production induced by co-treatment of 50 μg/mL CHT and 50 μM SA both in the WT and *nahG* plants. (C) SA at 50 μM did not induce apoplastic ROS production both in the WT and *nahG* plants.

Notes: Each bar represents averages of ROS production from 16 leaves. ROS production from four independent experiments ($n = 4$) is shown. The vertical scale shows the relative values of pixel intensity of the DAB brown color of H_2O_2 formation when the values of CHT or SA or CHT and SA treated leaves are normalized to control value taken as 100 for each experiment. Error bars represent SE. * indicates $p < 0.05$.

is required by CHT-triggered apoplastic ROS production. Note that 50 μM SA did not induce apoplastic ROS production in wild-type or *nahG* (Fig. 3(C)).

CHT elicited $[Ca^{2+}]_{cyt}$ oscillations in the nahG in the presence of SA but not in the absence of SA

To further investigate the involvement of endogenous SA in CHT signaling in guard cells, we tested the effects of the application of CHT in the presence or absence of SA on $[Ca^{2+}]_{cyt}$ oscillations in the *nahG* mutants. We found that CHT in the presence of SA elicited $[Ca^{2+}]_{cyt}$ oscillations in the 100% guard cells ($n = 30$ of 30 cells) of *nahG* mutant but in 28% guard cells in the absence of SA ($n = 10$ of 36 cells) (Fig. 4(E) and (F)), indicating the requirement of endogenous SA in CHT-elicited transient $[Ca^{2+}]_{cyt}$ increments in the *nahG* mutant.

Discussion

CHT induces stomatal closure in plants and thus plays a crucial role in adaptation to stress conditions. Apoplastic ROS production and $[Ca^{2+}]_{cyt}$ oscillation are the two important early events in CHT signaling in guard cells. Here, we revealed the involvement of endogenous SA in stomatal closure and also demonstrated the requirement of endogenous SA in CHT-induced apoplastic ROS production and CHT-elicited transient $[Ca^{2+}]_{cyt}$ increments in guard cells.

Endogenous SA is essential for both the production of apoplastic ROS and the stomatal closure induced by CHT

Induction of stomatal closure by CHT is the important phenotype of CHT response in plants. In this

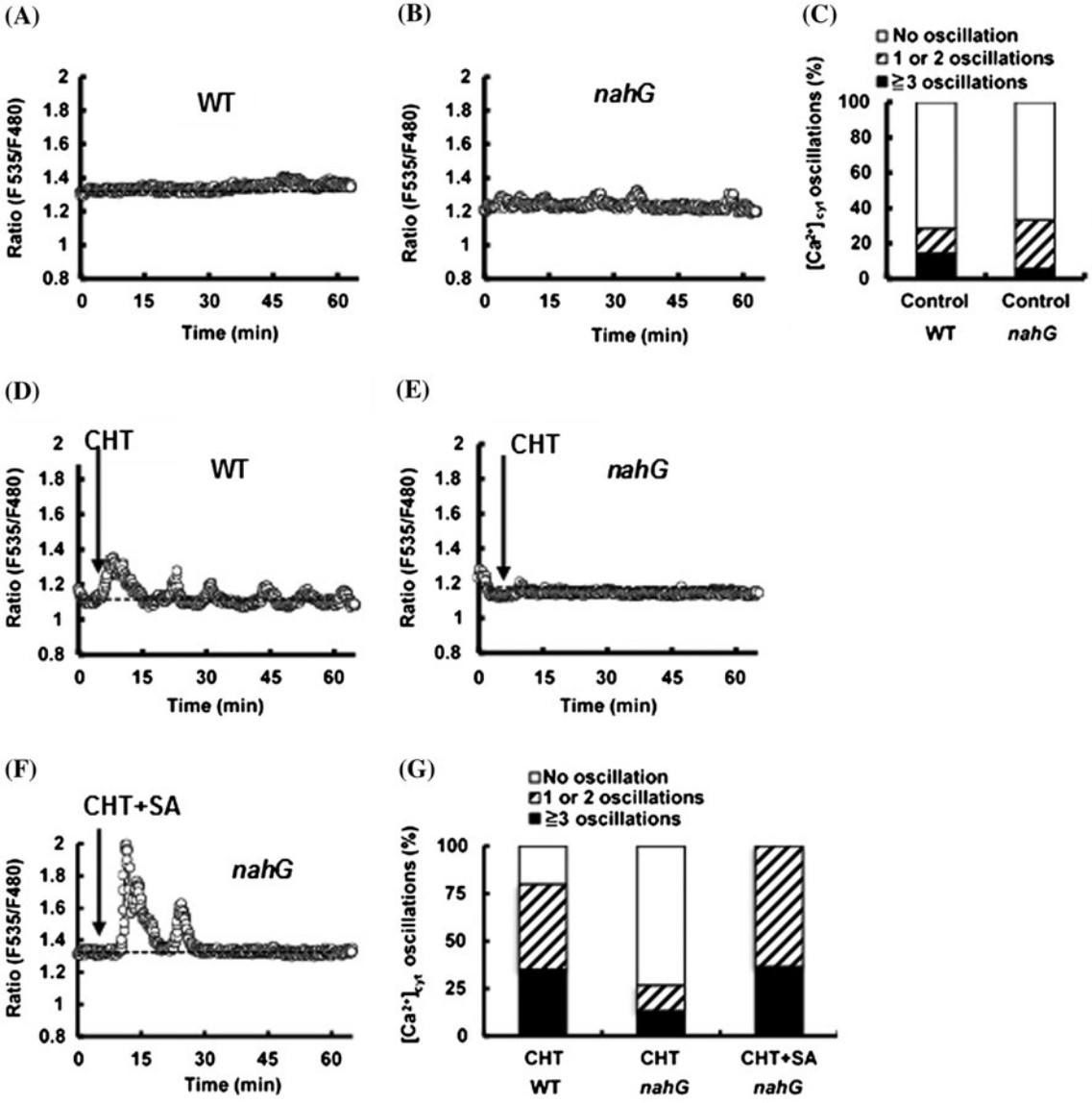

Fig. 4. CHT-induced $[Ca^{2+}]_{cyt}$ oscillations in the guard cells of wild-type (WT) and *nahG* plants. Representative trace of fluorescence emission ratios (535/480 nm) demonstrating $[Ca^{2+}]_{cyt}$ oscillations in A, untreated WT guard cells ($n = 8$ of 28 cells; 29%); B, untreated *nahG* guard cells ($n = 9$ of 28 guard cells; 32%); C, Stack column representation of untreated $[Ca^{2+}]_{cyt}$ oscillations (%) in WT guard cells ($n = 28$) and *nahG* guard cells ($n = 28$). Representative trace of fluorescence emission ratios (535/480 nm) showing $[Ca^{2+}]_{cyt}$ oscillations in D, CHT-treated WT guard cells ($n = 24$ of 30 cells; 80%); E, CHT-treated *nahG* guard cells ($n = 10$ of 36 cells; 28%); F, (50 uM SA + 50 μg/mL CHT)-treated *nahG* guard cells ($n = 30$ of 30 cells; 100%). G, Stack column representation of CHT-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in WT and *nahG* guard cells, and (CHT + SA)-induced $[Ca^{2+}]_{cyt}$ oscillations (%) in the *nahG* guard cells.

study, the promotion of stomatal closure in *nahG* in response to co-treatment of CHT and SA (Fig. 1(B)) indicates the involvement of endogenous SA in CHT signaling in guard cells. However, another possibility cannot be ruled out that CHT lowered the threshold concentration of SA to induce stomatal closure possibly via changing properties of SA receptors. Furthermore, the stomatal apertures of *nahG* without any treatment are variable [24–27] so that endogenous SA may collaborate with certain other determinant(s) to regulate stomatal apertures.

Apoplastic ROS production which acts as a second messenger is a crucial event in CHT signaling in guard cells.[5,6] Khokon et al.[12] reported that SA-induced ROS production in the extracellular spaces is moved into the guard cells. Allan and Fluhr[28] have shown that ROS migrates from one cell to another cell via the apoplastic route. Here, in the *nahG* mutants, the treatment of CHT did not trigger apoplastic ROS production (Fig. 3(A)) but co-treatment of CHT and SA triggered apoplastic ROS production (Fig. 3(B)), indicating the involvement of endogenous SA in CHT-triggered apoplastic ROS production. It was reported that ROS production in CHT signaling and SA signaling in guard cells is mediated by SHAM-sensitive peroxidases.[4–6,12,29] Hence, endogenous SA might be required for the activation of peroxidases.

Endogenous SA is required for the $[Ca^{2+}]_{cyt}$ oscillations in guard cell CHT signaling

Oscillation of $[Ca^{2+}]_{cyt}$ is an important event for the CHT signaling in guard cells and is attributed to Ca^{2+} influx from the extracellular space mediated by Ca^{2+} permeable channels.[11,30] Application of CHT elicits $[Ca^{2+}]_{cyt}$ oscillations, leading to stomatal closure in Arabidopsis.[6] Our data showed that CHT elicited transient $[Ca^{2+}]_{cyt}$ increments in the presence of SA but not in the absence of SA in the *nahG* mutants, which suggests that endogenous SA is essential for CHT signaling in guard cells. In Fig. 4 of this study, we did not investigate $[Ca^{2+}]_{cyt}$ oscillations in response to 50 μM SA because our research group already reported that exogenous SA did not elicit $[Ca^{2+}]_{cyt}$ oscillations in Arabidopsis guard cells.[12]

SA priming by endogenous SA is required for the CHT signaling in guard cells

Previous reports suggested the interactions among the hormone-induced signaling pathways in guard cells. It has been reported that endogenous ABA is required for the MeJA-induced stomatal closure[9] and that neither endogenous JA nor endogenous ABA is necessary for CHT-, YEL-, or SA-induced stomatal closure in Arabidopsis[10] In this study, we found that endogenous SA is required for CHT signaling in guard cells. Our findings with the previous reports suggested that CHT signaling in guard cells requires endogenous-SA priming but not priming by either endogenous ABA or endogenous JA.

Taken together, we illustrated CHT signaling in guard cells showing early signaling events which are primed by endogenous SA (Fig. 5). Briefly, CHT due to endogenous SA priming elicits both apoplastic ROS production and transient $[Ca^{2+}]_{cyt}$ elevation that activates downstream cascade leading to stomatal closure. On the contrary, in the absence of endogenous SA, CHT neither elicits apoplastic ROS production nor induces $[Ca^{2+}]_{cyt}$ oscillations and hence inactivates downstream signals.

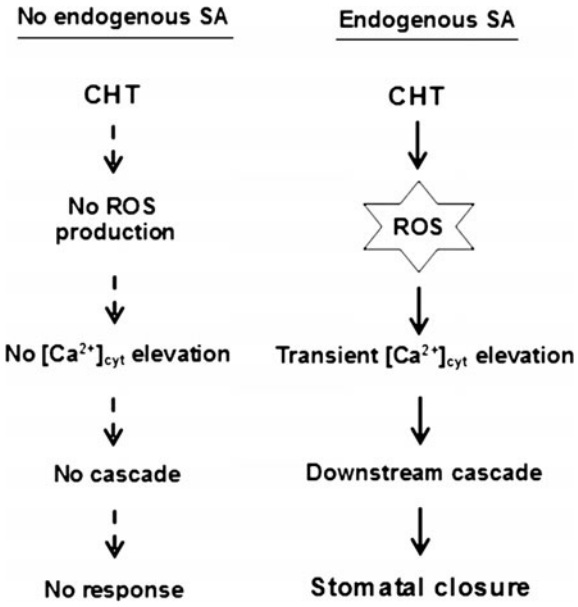

Fig. 5. A simplified illustration of early CHT signaling events in Arabidopsis guard cells due to endogenous SA priming. In the absence of endogenous SA, CHT neither elicits apoplastic ROS production nor induces transient free $[Ca^{2+}]_{cyt}$ increment and consequently, downstream signaling targets are inactive. In the presence of endogenous SA, CHT triggers both apoplastic ROS production and transient free $[Ca^{2+}]_{cyt}$ increment which activate downstream targets leading stomatal closure.

Author contributions

M. Y. P. and M. I. performed the experiments. T. N., S. M., and Y. N. assisted with the experiments and contributed to discussions. M. Y. P. and Y. M. wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental material

Supplemental material for this article can be accessed at http://dx.doi.org/10.1080/09168451.2017.1332979.

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