**Note**

**Chitosan-Induced Stomatal Closure Accompanied by Peroxidase-Mediated Reactive Oxygen Species Production in Arabidopsis**

Md. Atiqr Rahman Khokon,† Misugi Uraji,† Shintaro Munemasa,† Eiji Okuma,† Yoshimasa Nakamura,† Izumi C. Mori,‡ and Yoshiyuki Murata†,§

†Graduate School of Natural Science and Technology, Division of Bioscience, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan
‡Institute of Plant Science and Resources, Okayama University, Kurashiki, Okayama 710-0046, Japan

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To confirm the involvement of peroxidases, chitosan-induced ROS accumulation in guard cells was measured

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Chitosan induced stomatal closure in wild type-plants and NADPH oxidase knock-out mutants (atrbohD atrbohF), and reactive oxygen species (ROS) production in wild-type guard cells. Closure and production were completely abolished by catalase and a peroxidase inhibitor. These results indicate that chitosan induces ROS production mediated by peroxidase, resulting in stomatal closure.

Key words: chitosan; peroxidase; reactive oxygen species; stomatal closure; Arabidopsis

Plants regulate transpiration and gas exchange through stomata and limit the penetration of certain pathogens through the stomata, because some pathogens can enter through stomata. 1 Chitosan is an antifungal compounds that induces stomatal closure.2–4

Chitosan-induced stomatal closure is accompanied by ROS production as a second messenger in Commelina communis and Pismum sativum.2,4 In P. sativum, chitosan-induced ROS production is mediated by NADPH oxidase-like abscisic acid (ABA) and methyl jasmonate (MeJA),4–7 but it is unknown what enzyme ROS production is mediated in other plants, including Arabidopsis.

Chitosan-induced stomatal closure as well as ABA-MeJA-induced stomatal closure is accompanied by nitric oxide (NO) production.4,8,9 It has been suggested that NO functions as a second messenger downstream of ROS production in chitosan signaling in guard cells,10 but the involvement of NO in ABA signaling is still controversial, since Lozano-Juste and Leon have reported that the Arabidopsis nia1 nia2 noa1-2 triple mutant, which is deficient endogenous NO production, is hypersensitive to ABA.10

In this study, to elucidate chitosan signaling during chitosan-induced stomatal closure in Arabidopsis, ROS production and NO production in guard cells were investigated in genetic and pharmacological experiments.

The Arabidopsis (Arabidopsis thaliana) ecotype Columbia was grown in a growth chamber (22°C, 80μmol·m⁻²·s⁻¹ under a 16-h-light/8-h-dark regime). Stomatal apertures were measured as described previously.5–7 To measure ROS and NO production in guard cells, rosette leaves were blended and incubated under light for 2h. Then H₂DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) (Sigma, St. Louis, MO) and DAF-2DA (4,5-diaminofluorescein-2 diacetate) (Sigma), were added after the application of chitosan, as described previously.5–7 Note that inhibitors were added 30 min prior to chitosan application. The significance of differences between the mean values for stomatal apertures and ROS and NO production were assessed by Student’s t-test or one-way ANOVA with Dunnett’s test. Differences at p < 0.05 were considered significant.

We investigated chitosan-induced stomatal closure in wild-type plants (Fig. 1A). The application of 10μg/ml and of 50μg/ml of chitosan reduced stomatal apertures by 13% and 20% in wild-type plants, respectively, similarly to previous studies on tomato, C. communis, Arabidopsis, and P. sativum.2–4 Chitosan-induced stomatal closure was inhibited by catalase (CAT) in tomato.2 Salicylic acid induced stomatal closure via ROS production mediated by peroxidase, inhibited by a peroxidase inhibitor, salicylhydroxamic acid (SHAM), in Vicia faba.11 ABA- and MeJA-induced stomatal closure was inhibited by an NADPH oxidase inhibitor, diphenylene idonium chloride (DPI).3 Chitosan-induced stomatal closure was completely inhibited by 100 units/ml of CAT (p < 10⁻³) and 2 mM SHAM (p < 10⁻³), and was partially inhibited by 20 μM DPI (p < 0.009) (Fig. 1B). This indicates that ROS production mediated by peroxidase is involved mainly in chitosan-induced stomatal closure, which is inconsistent with a previous study using P. sativum, which found that ROS production mediated by NADPH oxidase is involved mainly in chitosan-induced stomatal closure.4

To clarify the involvement of NADPH oxidase in chitosan-induced stomatal closure, stomatal closure in NADPH oxidase knock-out mutant (atrbohD atrbohF) plants were observed (Fig. 1C). The application of 10μg/ml and of 50μg/ml of chitosan caused 11% and 20% reductions in aperture width, respectively, like the wild type (Fig. 1A).
using H$_2$DCF-DA (Fig. 2A). The application of 50 µg/ml of chitosan induced ROS production ($p < 0.004$). The chitosan-induced ROS accumulation was completely abolished by 100 units/ml of CAT ($p < 0.04$) and by 2 mM SHAM ($p < 0.04$), but not by 20 µM DPI ($p < 0.04$). This result suggests that chitosan induces ROS accumulation via activation of SHAM-sensitive peroxidases, leading stomatal closure, similarly to salicylic acid-induced stomatal closure in *Vicia faba*.11) Srivastava *et al.* have reported that chitosan-induced ROS production stomatal closure was drastically inhibited by DPI in *Pisum sativum*, suggesting the involvement of NADPH oxidases.15) Our results (Fig. 1) do not exclude the possibility of the partial involvement of other NADPH oxidases in chitosan-induced stomatal closure in *Arabidopsis*, but taken together, SHAM-sensitive peroxidase contributes mainly to chitosan-induced ROS production, leading to stomatal closure.

ROS production was not affected by an NO specific scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) ($p = 0.25$) (Fig. 2B). Chitosan also induced NO production, which was completely abolished by SHAM ($p < 0.05$) (Fig. 2C). These results indicate that chitosan-induced stomatal closure requires NO production downstream of ROS production in *Arabidopsis*, which is equivalent to chitosan-induced stomatal closure in *P. sativum*.4)

In conclusion, chitosan induced ROS production mediated by SHAM-sensitive peroxidase accompanied by NO production in guard cells, and then resulted in stomatal closure in *Arabidopsis*.

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**References**