Note

MAP Kinases, MPK9 and MPK12, Regulate Chitosan-Induced Stomatal Closure

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Chitosan (CHT)-induced stomatal closure was inhibited by an MAPKK inhibitor, PD98059, and was impaired in mpk9 mpk12 but not in mpk9 or mpk12. CHT induced the production of reactive oxygen species, cytosolic alkalization, and cytosolic Ca$^{2+}$ oscillation in mpk9 mpk12. These results suggest that MPK9 and MPK12 are involved in CHT-induced stomatal closure.

Key words: chitosan; cytosolic alkalization; cytosolic Ca$^{2+}$; reactive oxygen species; stomatal closure

Chitosan (CHT) is an antifungal compound that induces stomatal closure in several plant species. CHT-induced stomatal closure such as ABA- and MeJA-induced stomatal closure is accompanied by ROS production and [Ca$^{2+}$]$_{cyt}$ oscillation in guard cells. In Pisum sativum, CHT-induced ROS production is mediated by NADPH oxidases, whereas CHT-induced ROS production is mediated by SHAM-sensitive peroxydases in Arabidopsis thaliana.

Mitogen-activated protein kinases (MAPKs) are major components of cellular signal transduction pathways mediating various biotic and abiotic stress responses. We have found that (i) two members of the MAPK family, MPK9 and MPK12, are preferentially expressed in guard cells, (ii) MPK9 and MPK12 are involved in ABA-induced stomatal closure, and (iii) MPK9 and MPK12 function as positive regulators downstream of ROS production and [Ca$^{2+}$]$_{cyt}$ oscillation in the ABA signaling pathway. To our knowledge, it remains to be clarified whether MPK9 and MPK12 are involved in CHT-induced stomatal closure in Arabidopsis.

In this study, we examined stomatal closure, ROS production, cytosolic alkalization, and [Ca$^{2+}$]$_{cyt}$ oscillations in mpk9, mpk12, and mpk9 mpk12 mutants in response to CHT.

Arabidopsis wild type, Columbia-erecta (Col-er), mpk9 (Columbia accession, MPK9, At3g18040), mpk12 (Columbia accession, MPK12, At2g46070), and mpk9 mpk12 mutants were grown in a growth chamber as previously described. To obtain Yellow Cameleon 3.6 (YC3.6)-expressing plants, wild-type and mpk9 mpk12 double mutant plants were crossed with a Columbia-0 plant that had been transformed with YC3.6. Stomatal apertures were measured as previously described. An MAPK kinase (MAPKK) inhibitor was added 30 min prior to CHT application.

ROS production in guard cells was analyzed using H$_2$DCF-DA (2',7'-dichlorodihydrofluorescein diacetate), as previously described. Cytosolic pH elevation in the guard cells was analyzed using BCECF-AM (2',7'-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester), as previously described. [Ca$^{2+}$]$_{cyt}$ oscillation in the guard cells was analyzed using YC3.6, as previously described. The significance of differences between mean values of stomatal aperture, ROS production, and alkalization were assessed by Student’s t-test. The significant differences between mean values of frequency of [Ca$^{2+}$]$_{cyt}$ oscillations was assessed by χ$^2$ test. Differences were considered significant for p values < 0.05.

CHT induced stomatal closure in a concentration-dependent manner in the wild type (Fig. 1A), in agreement with our previous results. Treatment of rosette leaves with 100 μM PD98059 significantly inhibited the stomatal closure induced by CHT at 10 μg/mL and at 50 μg/mL (Fig. 1A). PD98059 alone did not affect stomatal apertures in the absence of CHT (Fig. 1A). These results suggest that an MAPK cascade plays a role in CHT-induced stomatal closure.

CHT at 10 μg/mL and 50 μg/mL induced stomatal closure in the mpk9 and mpk12 single mutants, but CHT at up to 50 μg/mL did not induce stomatal closure in the mpk9 mpk12 double mutant (Fig. 1B), suggesting that MPK9 and MPK12 positively regulate CHT-induced stomatal closure.

CHT at 50 μg/mL induced ROS production in wild-type guard cells, in agreement with our previous
results, and also induced ROS production in guard cells of the mpk9, mpk12, and mpk9 mpk12 mutants (Fig. 2A). CHT at 50 μg/mL induced cytosolic alkalization in guard cells of the wild-type, mpk9, mpk12, and mpk9 mpk12 mutant plants (Fig. 2B). Together, these results suggest that neither MPK9 nor MPK12 is involved in CHT-induced ROS production or cytosolic alkalization.

The application of 50 μg/mL of CHT induced one or more Ca^{2+} oscillations in 81% of the wild-type guard cells (n = 21 of 26 cells) and in 77% of the mpk9 mpk12 mutant guard cells (n = 20 of 26 cells) over 60 min of monitoring (Fig. 3). There was no significant difference in frequency of Ca^{2+} oscillations between the wild type and the mpk9 mpk12 double mutant, suggesting that neither MPK9 nor MPK12 is involved in CHT-induced [Ca^{2+}]_{cyt} oscillations in guard cells. ABA and MeJA also induced [Ca^{2+}]_{cyt} oscillation in guard cells, but there was no significant difference between CHT-induced and ABA- and MeJA-induced [Ca^{2+}]_{cyt} oscil-
Nevertheless, further study is needed to clarify the details of the \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillation induced by CHT.

MPK12 kinase activity is enhanced by \(H_2O_2\), and MPK8 activation requires direct binding of calmodulins in a \(\text{Ca}^{2+}\)-dependent manner in Arabidopsis,\(^8,12\) and a 46-kDa MAPK is activated by the ROS involved in ABA-induced antioxidant defense in \(Zea mays\).\(^3,5\) These results suggest that MAPKs function downstream of ROS production and \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation, consistently with our results in this study.

In this study, \(mpk9\) \(mpk12\) were markedly insensitive to CHT in stomatal movement, whereas \(mpk9\) and \(mpk12\) displayed CHT-responsive stomatal movement (Fig. 1B). In contrast, upstream cellular events such as CHT-induced ROS production, alkalization, and \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillations were not affected in the \(mpk9\), \(mpk12\), or the \(mpk9\) \(mpk12\) mutants (Figs. 2 and 3). These results suggest that MPK9 and MPK12 redundantly act downstream of these cellular events in guard-cell CHT signaling.

Our previous study indicated that MPK9 and MPK12 function downstream of ROS and \(\text{Ca}^{2+}\) oscillations in guard-cell ABA signaling.\(^8,9\) Together, MPK9 and MPK12 appear to be common signaling components between the CHT signaling pathway and the ABA signaling pathway.

In conclusion, MPK9 and MPK12 functioned downstream of ROS production, cytosolic alkalization, and \([\text{Ca}^{2+}]_{\text{cyt}}\) oscillation in CHT signaling in Arabidopsis guard cells.

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**References**