SUMMARY OF THE THESIS

THE EFFECT OF THE FUNGAL ELICITOR CHITOSAN ON GUARD CELL FUNCTION: THE CONNECTION BETWEEN STOMATAL MOVEMENT AND THE PHOTOSYNTHETIC ACTIVITY OF GUARD CELLS

Attila Ördög

Supevisor: Dr. Ferenc Horváth

Ph.D. School in Biology

University of Szeged

Faculty of Science and Informatics

Department of Plant Biology

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INTRODUCTION

Stomata control gas exchange and transpirational water loss by turgor-driven volume changes, but they also provide the main entrance for pathogens. Guard cells (GC) respond to the presence of microbes by narrowing stomatal pores following perception of microorganism-associated molecular patterns (MAMPs), such as chitosan, a deacylated derivative of a major fungal cell wall component chitin. Besides the inhibition of the light-induced stomatal opening chitosan also induces stomatal closure, an action with distinct signalling pathways and transporters. Stomatal opening and closure is related to the H^+-ATPase activity in the GC plasma membrane, as this activity affects the transport of osmotically active solutes, such as the passive movement of K^+ via different sets of potassium channels, the activity of Cl^-/H^+ symporters and anion channels. ATP for proton pumping is supplied mostly from mitochondrial respiration; however, partial inhibition by DCMU implied a role of GC photosynthetic electron transport in the ATP supply. In order to ascertain whether chitosan affects GC photosynthetic ATP production, the light-dependence of the photosynthetic electron transport rate of a single GC was assayed. *Vicia faba* epidermal peels were bathed in solutions containing different molecular weight chitosan and chlorophyll fluorescence parameters were determined by a Microscopy-PAM chlorophyll fluorometer (Walz, Germany). A possible role of hydrogen peroxide and nitric oxide molecules in chitosan signalization, as well as photosynthetic ATP production, was also investigated.

AIMS OF THE STUDY

The purpose of this work is to examine the effect of chitosan, the most common fungal elicitor affecting the function of guard cells. Since chitosan is impermeable to cell membranes, its effect can be elucidated only by the signal compounds of the activated intracellular signalling pathways. So our additional goal was to investigate the potential effect of certain members in this pathway.

The particular goals of this work are summarized by the following points:

1. Are *Vicia faba* guard cells able to recognize the chitosan molecule as MAMP? If so, does chitosan cause stomatal closure or inhibit the stomatal opening? If chitosan can influence the stomatal movements, will it raise the level of hydrogen peroxide or nitric oxide, the two major compounds of the chitosan-induced signalling cascade? We were also curious about in what compartment and how long after the chitosan treatment do these compounds accumulate?
2. Do mesophyll cells surrounding the guard cells influence the effect of chitosan on stomatal movements?
3. Does chitosan affect the photosynthetic activity of guard cells, thereby influencing the amount of the required NADPH and ATP for stomatal closing and opening?
4. EPR measurements on thylakoid membrane complexes have shown that NO inhibits electron transfer processes in PSII and target sites have also been indentified, such as the nonheme iron between the Q_A and Q_B binding sites. If we can detect chitosan-induced hydrogen peroxide and nitric oxide, we aim to investigate the effect of both molecules on the photosynthetic activity of guard cells, using external donor chemicals.

MATERIALS AND METHODS

Plant material: Leaves of hydroponically grown four-week-old broad bean (*Vicia faba L.*) plants.

Treatment: Prior to measurements, leaves were sprayed with experimental solutions containing 1 mM $C_2H_3NaO_2$, 10 mM MES, 10 mM KCl and 100 μM $CaCl_2$ (pH 6.15 with KOH) with 100 μg ml^{-1} chitosan (CHT solution) or without chitosan (control solution). Leaves were treated at 5:30 AM (inhibition of stomatal opening) or at 10:00 AM (induction of stomatal closure).

S-nitroso-gluthatione (GSNO) was used as NO donor molecule. The concentration of NO was measured aperometrically using a NO electrode (ISO-NOP, WPI, USA).

Stomatal aperture measurements: The width of stomatal apertures was measured on pictures taken from freshly peeled epidermal strips with the Image-Pro Plus 5.1 image analyser software. The experiments were repeated on three different days, each yielding the average of at least 90 aperture widths.

Epidermal strip bioassay: Prior to each experiment, the abaxial epidermis was peeled carefully from the third to fourth completely unfolded leaves and submerged in the experimental solution. The strips contained only small regions contaminated with mesophyll cells – mainly around the major veins – and these regions were excised with a razor blade. The strips were transferred and washed for 5 minutes in the hypoosmotic experimental solution in order to remove any remaining mesophyll cell debris and mesophyll chloroplasts by severe osmotic shock.

Chlorophyll *a* fluorescence measurements: Chlorophyll *a* fluorescence of 4 to 5 stomata from a mesophyll-free abaxial epidermis patch was monitored with a MYCROSCOPY-PAM chlorophyll fluorometer (Walz GmbH, Germany) mounted on an inverted epifluorescence microscope (Zeiss Axiovert 40, Zeiss GmbH, Germany). During rapid light curves the following photosynthetic parameters were obtained:

- Maximal quantum efficiency (F_v/F_m)
- The PSII efficiency factors (qP and qL)
- The effective quantum efficiency of PSII (Φ_{PSII})
- The maximal quantum efficiency of the light-adapted PS II photochemistry (F_v'/F_m')
- The apparent relative linear electron transport rate (ETR)

- The Stern-Volmer type non-photochemical quenching (NPQ)

Localization of the fluorescent probes using confocal microscopy: Localization of the fluorescent probes (AmplexRed – H_2O_2; DAF-FM DA – NO) in the abaxial epidermis of intact leaves were carried out using confocal laser scanning microscopy (Olympus FV1000 LSM, Olympus Life Science Europa GmbH, Hamburg, Germany).

Statistical analysis: Results are expressed as mean ± SD. In indicated cases, Student's t-test was used (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

RESULTS

It has been shown in many types of plants that chitosan not only inhibits light-induced stomatal opening but can also induce stomatal closure, an action with distinct signalling pathways and transporters. Our goals were to prove the effect of chitosan on *Vicia faba* guard cells. Our results can be summarized in the following points.

1. As a new result we confirmed that, in *Vicia faba*, chitosan does not induce stomatal closure but it does inhibit stomatal opening. Using specific fluorescent probes we have shown that chitosan enhances the level of hydrogen peroxide and nitric oxide already after one hour of treatment. Hydrogen peroxide accumulates mainly in the chloroplasts (probably in the stroma) and in the nuclei, while the level of nitric oxide increases in chloroplasts, in the cytoplasm and in cell walls around the stomatal pores.
2. Using abaxial epidermal peels we show that mesophyll cells do not play an exclusive role in chitosan induced stomatal movements.
3. Since stomatal opening and maintaining of the open state are energy dependent processes, the effect of chitosan on photosynthetic activity of guard cells has been investigated. As a new result we show that guard cells on leaves treated at dawn showed decreased ETR values, which remained low compared to the control during the whole day. The values of *q*P, *q*L, Φ_{PSII} and NPQ remained also very low. Interestingly, our results also indicate that treatment at 10:00 AM does not influence the photosynthetic activity of guard cells.

4. Since chitosan can act only through cell surface receptors, two compounds from the intracellular signalling pathway have been further investigated: we examined the effect of exogenously applied H_2O_2 (100μM) and NO (450 nM) on the photosynthetic activity of guard cells. H_2O_2 was not able to mimic the effect of chitosan. NO, on the other hand, was able to slow down the photosynthetic activity of guard cells in a similar way as chitosan: the NO treatment decreased the values of ETR, qP, qL, Φ_{PSII} and NPQ, which closely resembles the results of the chitosan treatment. The effect of NO has been shown to be reversible, using a high amount of bicarbonate. These results suggest that NO might act on the nonheme iron between the Q_A and Q_B binding sites.

   It is also very important to note, that chitosan can also induce stomatal closure, which in turn can decrease the amount of CO_2 inside the leaves. This may slow down the activity of the Calvin-Benson cycle, which also influences the linear electron transport processes.

In summary, data in this work provide a confirmation of the effect of chitosan on guard cells of *Vicia faba*. Chitosan, possibly through increasing the amount of NO inside the chloroplasts, can block the photosynthetic activity of guard cells leading to a decreased amount of ATP and NADPH, which may contribute to the induction of stomatal closure and to the inhibition of the stomatal opening.

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