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Abstract of PhD thesis

Role of *Arabidopsis* Heat Shock Factor A4A in combined salt and heat stress responses

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Introduction

In nature, simultaneously acting environmental effects has deleterious impact on plant growth, development and reproduction. During evolution, plants developed some complex mechanisms, which can help the adaptation and survival in extreme conditions. To cope with biotic and abiotic stresses, plants can activate several stress-induced signaling pathways and develop the most suitable stress response for acclimatization. These signaling pathways consists of many components, like plant hormones, receptors, kinases, transcription factors and even reactive oxygen species formed during stress. Heat shock factors (HSFs), as members of such signaling mechanisms, are involved in stress response establishment, including heat, salt, drought and heavy metal stresses. In active form HSFs are localized in nucleus, where they can induce the expression of stress-related genes, trough recognition of and binding to heat shock elements (HSEs) found in the promoters of target genes. Their role in individual stresses are relatively well studied, but their function in stress combination is not known.

Earlier, we showed that *Arabidopsis* heat shock factor A4A (HSFA4A) regulates responses to salt and oxidative stresses, and is phosphorylated by MAP kinases MPK3 and MPK6, while the dominant phosphorylation site was the Ser309 residue.

In this work, I continued the characterization of HSFA4A to elucidate its role in combined stress and stress signaling pathway.

Aims

Continue the characterization of HSFA4A transcription factor to understand its role in stress response.

- Study of *HSFA4A* gene expression in *Arabidopsis* plants exposed to different stress condition.
- Generate transgenic *Arabidopsis* plants expressing the YFP-tagged HSFA4A gene construct, driven by own promoter.
  - Test the effect of different stresses on protein level changes in transgenic plants.
  - Observe changes in intracellular localization of HSFA4A-YFP protein.
  - Test the promoter binding of HSFA4A using ChIP technic.
- Test the phosphorylation of HSFA4A by MPK4 kinase, as well *in vitro* and *in vivo* identification of phosphorylation sites.
- Create phosphorylation mutants version of HSFA4A (mimic and lack of phosphorylation).
  - Test the role of phosphorylation on multimerization using BiFC technic.
  - Effect of wild type and mutant HSFA4A overexpression on survival and oxidative damages in salt, heat and combined stresses.
- Describe a more accurate model concerning the role of HSFA4A in stress signaling pathway.

Material and methods

Plant material and growth condition

In all experiment *Arabidopsis thaliana* Col-0 was used, and all the transgenic plants (pHSFA4A::HSFA4A-YFP, pER8-HSFA4A-S309D and pER8-HSFA4A) had the same background. In experiments the growth conditions were the following: *in vitro*, sterile ½ MS culture medium, temperature 22°C, 8h light/16h dark cycle and 100 μE m^{-2} sec^{-1} light intensity (control condition).

The stress treatments were the following: (1) salt stress: 100 mM or 150 mM NaCl, temperature 22°C; (2) heat stress: temperature in light 37°C, temperature in dark 30°C; (3) combined stress: combination of salt and heat stresses. In all experiment for stress treatments we used liquid ½ MS medium, 8h light/16h dark cycle and 100 μE m^{-2} sec^{-1} light intensity.

Stress tolerance testing

To test the stress tolerance, we examined the survival and lipid peroxidation of wild type and overexpressing plants. In survival test we placed the 10 days old seedlings on stress treatments for 2 or 4 days, and after 10 days regeneration period we counted the survived plants. The survived plants we divided in two categories: healthy and damaged. To test the oxidative damage of control and stress treated plants we use TBARS method. For lipid peroxidation experiments we treated the plants for two days.

Other methods

To examine the expression of *HSFA4A* gene, we isolated RNA from control and stress treated plants, and after cDNA synthesis we performed qPCR.

To test the changes in protein level we performed SDS-PAGE and Western blot analysis on stress treated transgenic plants expressing the YFP-tagged HSFA4A protein.

The intracellular localization of HSFA4A-YFP protein was determined with confocal laser scanning microscopy. In experiments we focused on specific cells in roots of control and salt treated plants.

To test the multimerization of HSFA4A we used non-denaturing PAGE, Western blot and Bimolecular Fluorescence Complementation (BiFC).

The promoter binding of HSFA4A was tested with Chromatin Immunoprecipitation (ChIP) on promoter of *ZAT12*, *WRKY30* and *HSP17.6A* genes.

HSFA4A phosphorylation by MPK4 was tested *in vitro*, while the identification of phosphorylation sites was performed with mass spectrometry *in vitro* and *in vivo*.

Results

We showed that *HSFA4A* is activated not only by heat and salt, but also by combined heat and salt stress, and changes in protein abundance is correlating with endogenous gene induction. Microscopic studies, confirmed a fast accumulation of YFP-tagged HSFA4A in root cell nuclei during salt stress, while in case of longer stress the fluorescence signal was higher in

both cytoplasm and nucleus. Considering these results, we concluded, that HSFA4A is part of early and late stress responses in stress dependent manner.

Heat shock factors are subjects of different posttranslational modifications, like phosphorylation and sumoylation, which are necessary for proper regulation and function. We showed that, beside MPK3/6, HSFA4A is phosphorylated by MPK4 kinase and the dominant phosphorylation site is the Ser309 for all these kinases. *In vivo* phosphorylation assays revealed that the same amino acid residues are phosphorylated *in vitro* and *in vivo*. We also identified phosphorylation sites, which can be targets of other kinase families. Our results suggest, that HSFA4A has a complex regulation, involving MAP kinases and other kinase families too.

To accomplish its transcription activation role, HSFs has to form homotrimers, therefore the capability of multimerization is essential. To test the effect of phosphorylation on HSFA4A dimerization we used bimolecular fluorescence complementation (BiFC). For this purpose, we transformed positive and negative phosphorylation mutant and wild type version of HSFA4A in *Arabidopsis* protoplast cells and fluorescence was monitored by fluorescence microscopic studies. To generate the phosphorylation mutants, we changed the Ser309 amino acid to alanin (lack of phosphorylation, negative phosphorylation mutant) or aspartate (mimic of phosphorylation, positive phosphorylation mutant). Phosphorylation seems to have positive effect on dimerization, but is not essential. Beside the role of phosphorylation, we showed that redox status of the cells also affects multimerization and/or interactions with other proteins *in vitro*. Oxidative stress may therefore influence the HSFA4A function as suggested before (Péres-Salamó et al., 2014).

Overexpression of wild type and phosphorylation mimicking mutant version of *HSFA4A* in *Arabidopsis* plants, has favorable effect on plant

survival during salt, heat and combined stresses and can reduce lipid peroxidation in these conditions. In transgenic plants all versions of *HSFA4A* could enhance the stress tolerance by reducing the oxidative damages. Ser309 phosphorylation has no or only minimal effect on stress tolerance.

Pérez-Salamó et al., (2014) showed that overexpression of HSFA4A can influence the expression of a range of stress-induced genes. Here, we showed that the amount of HSFA4A protein increases in nuclei during stress. Considering that HSFs has transcriptional activity and the above mentioned results, we checked the promoter binding of HSFA4A, using chromatin immunoprecipitation (ChIP) assay. We revealed that HSFA4A can recognize and bind HSEs on promoter of *ZAT12* and *WRKY30* transcription factors and *HSP17.6A* small heat shock protein during salt, heat and combined stress. Both transcription factor and the heat shock protein are involved in stress response, and seems that HSFA4A directly regulates their function.

All together, we can say that HSFA4A transcription factor regulates multiple stress signaling pathways. HSFA4A connects stress-derived ROS signals, mediated by particular MAP kinases and promotes transcription of a set of target genes, including other classes of transcription factors or proteins with protective functions.

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List of publication

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The PhD thesis is based on the article:

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Other publications

Baba AI, **Andrási N**, Valkai I, Gorcsa T, Koczka L, Darula Z, Medzihradszky KF, Szabados L, Fehér A, Rigó G, Cséplő Á. (2019). AtCRK5 Protein Kinase Exhibits a Regulatory Role in Hypocotyl Hook Development during Skotomorphogenesis. *International Journal of Molecular Science 20, 3432*. IF: 4.183

Baba AI, Rigó G, **Andrási N**, Tietz O, Palme K, Szabados L, Cséplő Á. (2019). Striving Towards Abiotic Stresses: Role of the Plant CDPK Superfamily Members. In: Palocz-Andresen M, Szalay D, Gosztom A, Sípos L, Taligás T. (eds) *International Climate Protection. Springer, Cham.*

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Conferences

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Andrási N, Rigó G, Cséplő Á, Pérez-Salamó I, Szabados L. (2016). The role of MAP kinase-mediated phosphorylation in intramolecular interactions of the *Arabidopsis* heat shock factor A4A. *17. Kolozsvári Biológus Napok/17th Biology Days, April 8-9. Cluj-Napoca, Romania*.

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Factor A4A in combined salt and heat stress responses. *20. Kolozsvári Biológus Napok/20th Biology Days, April 12-13. Cluj-Napoca, Romania*.

Andrási N, Rigó G, Cséplő Á, Zsigmond L, Pérez-Salamó I, Baba AI, Klement É, Pettkó-Szandtner A, Siddiqui S, Szabados L. (2019). The Heat Shock Factor A4A regulates responses to combined salt and heat stresses. *Hungarian Molecular Life Science 2019, March 29-31. Eger, Hungary*.

Conflict of interest

I myself as corresponding or first author of the following publications declare that authors have no conflict of interest and Norbert Andrási Ph.D candidate had a great contribution to the published results. Results discussed in his thesis are regarded as outcomes of his own scientific work.

Andrási N, Rigó G, Zsigmond L, Pérez-Salamó I, Papdi C, Klement E, Pettkó-Szandtner A, Baba AI, Ayaydin F, Dasari R, Cséplő Á, Szabados L. (2019). The mitogen-activated protein kinase 4-phosphorylated heat shock factor A4A regulates responses to combined salt and heat stresses. *Journal of Experimental Botany erz217.*

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Baba AI, **Andrási N**, Valkai I, Gorcsa T, Koczka L, Darula Z, Medzihradszky KF, Szabados L, Fehér A, Rigó G, Cséplő Á. (2019). AtCRK5 Protein Kinase Exhibits a Regulatory Role in Hypocotyl Hook Development during Skotomorphogenesis. *International Journal of Molecular Science 20, 3432.*

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