Functional characterization of the Arabidopsis heat shock factor A4A, identified by a novel genetic screen

Summary of PhD thesis

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INTRODUCTION

Plants, as sessile organisms are constantly subjected to external stimuli, which determine their growth, development and survival. The environmental conditions frequently fluctuate beyond optimal parameters, becoming a source of stress for the plants. To cope with the unfavourable environment, plants have developed several strategies, which involve different biochemical and physiological adjustments. Mechanisms of stress acclimation and tolerance are controlled by complex signalling pathways that regulate gene expression. Our knowledge of stress perception is limited, downstream components of the signalling cascade are activated by specific receptors that are mostly unknown. The signal is transmitted by secondary messenger molecules such as phospholipids, cyclic nucleotides, hormones, Ca^{2+} and reactive oxygen species (ROS) and activates enzymes involved in post-translational modifications, i. g. protein phosphorylation by CIPKs, CDPKs and MAP kinases. Phosphorylation regulates the activity of several stress response proteins, including transcription factors. In general, mechanisms of stress tolerance are based on the maintenance of homeostasis, ROS detoxification and direct protection of cellular components.

A substantial amount of information on regulation of tolerance to abiotic stress conditions and stress signalling pathways has been obtained using *Arabidopsis thaliana* as a model plant. Publication of the genome sequence of Arabidopsis, together with development of genetic tools has been essential to dissect molecular mechanisms behind stress responses. Particularly, genetic screens have proven to be very useful tools to identify stress-related genes.

AIMS

General aim of this project was the identification and characterization of novel regulatory genes in Arabidopsis thaliana, which are regulators of plant stress responses.

- Using the previously available Conditional cDNA Overexpression System (COS) establishment of a novel genetic system which allows the identification of previously unknown regulatory gene on cell level.
- Isolation of transformed cell lines which show estradiol-dependent stress tolerance.
- Identification of one or more Arabidopsis genes from the established cell lines which are responsible for the tolerance phenotype.
- Characterization of the biological and molecular function of at least one identified gene.
    - Generating Arabidopsis lines overexpressing the identified cDNA and testing their tolerance to stress conditions (salt, osmotic, oxidative sress). Physiological characterization of the stress tolerant lines.
    - Characterization of Arabidopsis mutant lines, in which the identified gene is inactivated.
    - Characterization of biochemical and molecular function of the encoded protein, using systems biology technologies.
    - Better description of signalling pathway regulating stress responses.

METHODS

- Transformation and screening of Arabidopsis cell cultures.
- Generation of Arabidopsis transgenic plants by *Agrobacterium*-mediated transformation.
- Characterization of an Arabidopsis T-DNA insertion mutant.
- Analysis of abiotic stress tolerance *in vitro* (salinity, osmotic, oxidative stress).
- Transient protein expression in Arabidopsis protoplasts.
- Molecular biology techniques:
  - DNA manipulation, cloning, site-directed mutagenesis.
  - RNA isolation, cDNA synthesis. Gene expression studies by quantitative and semi-quantitative PCR.
  - Recombinant protein extraction and purification, immun precipitation of tagged proteins, western blot.
  - Protein *in vitro* phosphorylation, in-gel kinase assay.
  - *In vivo* visualization of reporter constructs (GFP, YFP) by confocal microscopy, luciferase enzyme activity, histochemical staining (GUS).
  - *In vivo* protein-protein interaction techniques (yeast two-hybrid, Bimolecular Fluorescence Complementation).

RESULTS

1. An Arabidopsis random cDNA library under the control of an estradiol induced promoter was expressed in Arabidopsis cell suspension, and 1.2 million transformed cells were screened for enhanced salt tolerance. Four cell suspension-derived microcolonies were identified with salt tolerant growth that was dependent of estradiol.

2. cDNA inserts were isolated from salt tolerant cell cultures and their identity was determined. One of the colonies carried the full-length cDNA of the heat shock transcription factor HSFA4A, this gene was chosen for further functional characterization.

3. The conditional salt-tolerance phenotype was confirmed by re-transforming the estradiol-induced ER8-HSFA4A into Arabidopsis cell suspension. Thereafter, ectopic expression of HSFA4A in Arabidopsis plants provided tolerance to salt, paraquat, H_2O_2, anoxia, $CdCl_2$ and mannitol, all these stressors converge in oxidative stress.

4. Physiological parameters of HSFA4A overexpressing lines was characterized. During salt stress, HSFA4A overexpression resulted in lower lipid peroxidation rate and reduced H_2O_2 content, indicating a decreased effect of the salt-induced oxidative damage. These results suggested that HSFA4A contributes to Arabidopsis oxidative stress tolerance.

5. Transcriptional regulation of HSFA4A was characterized. Corresponding with public available transcript profiling, gene expression analysis showed that *HSFA4A* expression can be induced by several stress conditions. After H_2O_2 treatment, rapid induction of *HSFA4A* was detected and high levels of HSFA4A mRNA was sustained for several days.

6. To find target genes of HSFA4A, a transcript profiling was performed, comparing HSFA4A overexpressing plants with wild type. A set of stress response genes was identified as transcriptional targets of HSFA4A: *ZAT6, ZAT12, HSP17.6A, ATL31, CRK13, WRKY30* and *CTP1*. Quantitative RT-PCR was employed to validate expression patterns of selected HSFA4A-regulated genes.

7. Characterization of *hsfa4a* T-DNA insertion mutant revealed that the inactivation of *HSFA4A* causes salt hypersensitive growth, higher lipid peroxidation and increased H_2O_2 accumulation during salt stress. However, genetic complementation of *hsfa4a* could restore the wild type phenotype. Salt-treated *hsfa4a* plants showed reduced levels of *ZAT6, ZAT12, WRKY30* and *CTP1* comparing to wild type.

8. A model previously proposed that in plants, that ROS accumulation results in multimer formation of heat shock factors, which can be connected to their function. Performing yeast two-hybrid assay and Bimolecular Fluorescence Complementation (BIFC), it was demonstrated that HSFA4A is able to create homodimers in living cells. During salt stress, nuclear accumulation of HSFA4A homodimers was observed suggesting enhanced multimerisation in stress conditions. Multimer formation occurs between cysteine residues of two or more HSFA4A proteins. Substitution of 3 highly conserved cysteine residues to alanine greatly reduced HSFA4A homodimerization in yeast cells and in Arabidopsis protoplasts.

9. Using yeast two-hybrid assay and BIFC in tobacco leaves, we showed that HSFA4A interacts with two mitogen-activated kinases MPK3 and MPK6. Using *in-gel* and *in vitro* kinase assays, phosphorylation of HSFA4A by MPK3 and

MPK6 was demonstrated. Phosphorylation sites of HSFA4A were identified by mass spectrometry. Site-directed mutagenesis followed by an *in vitro* kinase assay revealed Ser309 as the major phosphorylation site.

10. Importance of MPK3 and MPK/-mediated phosphorylation of HSFA4A function was demonstrated by trans-activation of the pHSP17.6A-LUC reporter construct. Luciferase activity was significantly reduced when the Ser309Ala mutant HSFA4A was employed when compared to the wild type HSFA4A in transient expression.

Our results demonstrated that HSFA4A is an important player in a stress regulatory pathway mediated by reactive oxygens and MPK3 and MPK6 kinase signalling cascades .

PUBLICATION LIST

This PhD thesis is based on the article:

Perez-Salamo I, Papdi Cs, Rigo G, Zsigmond L, Vilela B, Lumbreras V, Nagy I, Horvath B, Domoki M, Darula Z, Medzihradszky K, Bogre L, Koncz Cs, Szabados L (2014) The Heat Shock Factor A4A Confers Salt Tolerance and Is Regulated by Oxidative Stress and the Mitogen-Activated Protein Kinases MPK3 and MPK6. Plant Physiol 165: 319-334, MTMT: 2570455, IF: 6.555

Other publications related to this thesis :

Papdi Cs, Joseph MP, **Pérez-Salamó I**, Vidal S, Szabados, L (2009) Genetic technologies for the identification of plant genes controlling environmental stress responses. Funct Plant Biol 36:696-720., MTMT: 1920786, IF: 2.471

Papdi Cs, Leung, J, Joseph MP, **Pérez-Salamó I**, Szabados L (2010) Genetic screens to identify plant stress genes. In: *Methods in Molecular Biology,* vol. 639. New York: Humana Press. 639: 121-139, MTMT: 1921541

Other publications :

Ruibal C, **Pérez-Salamó I**, Carballo V, Castro A, Bentancor M, Borsani O, Szabados L, Vidal S (2012) Differential contribution of individual dehydrin genes from Physcomitrella patens to salt and osmotic stress tolerance. Plant Sci 190:89-102. MTMT: 2014259, IF: 2.922

Ábrahám E, **Pérez-Salamó I**, Koncz C, Szabados L (2011) Identification of Arabidopsis and Thellungiella genes involved in salt tolerance by novel genetic system. Acta Botanica Szegediensis 55:53-57, MTMT: 1922012

DECLARATION OF CORRESPONDING AUTHOR

As corresponding author I confirm that Imma Pérez-Salamó had the major contribution to the publication listed below, as she performed the experiments. I give my consent to Imma Pérez-Salamó to use the results of this publication in her Ph.D. thesis. The published data have not been and will not been used in another Ph.D. thesis.

Perez-Salamo I, Papdi C, Rigo G, Zsigmond L, Vilela B, Lumbreras V, Nagy I, Horvath B, Domoki M, Darula Z, Medzihradszky K, Bogre L, Koncz C, Szabados L (2014) The Heat Shock Factor A4A Confers Salt Tolerance and Is Regulated by Oxidative Stress and the Mitogen-Activated Protein Kinases MPK3 and MPK6. Plant Physiol 165: 319-334

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