Final Report of the OTKA research grant No. K-81765.

Title: Transcription regulation and stress tolerance in plants: analysis of novel Arabidopsis transcription factors controlling salt and drought tolerance.

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Introduction

In our research program we planned the characterization of several transcription factors which were previously identified with our COS overexpressing system or through screening our T-DNA insertion mutant collection. The COS system was designed to identify regulatory genes on base of their regulated overexpression in transgenic plants (Papdi et al., 2008, Plant Physiol 147: 528-542), while the insertion mutagenesis program produced loss-of function mutants (Szabados, 2002, Plant J 32: 233-242). Several COS lines were originally selected for further analysis in this project including those which overexpressed the RBP7-type subunit of the Polymerase II complex, the heat shock factor A4A (HSFA4A), the zinc finger protein 3 (ZFP3) or the ERF/AP2 type factor Related to AP2 12 (RAP2.12). Preliminary data suggested, that these factors control responses to drought and salt tolerance, including signals, which are mediated by abscisic acid (ABA). Besides the transcription factors, genetic screens lead to the identification of other regulatory genes which influence stress responses, including the pentatricopeptide repeat 40 (PPR40) and the CDPK-related kinase 5 (CRK5). Characterization of such regulatory genes have been performed in the present research program.

Results

Gene cloning, verification of phenotypes.

The COS gene identification system allows easy cloning of the identified genes. It consist of a cDNA library cloned in a plant expression vector using the Gateway cloning system. PCR amplification of the inserted cDNA is therefore straightforward with PCR primers ER8A and ER8B, annealing to the flanking 5' promoter and 3' termination regions, respectively. Amplified PCR fragments carried the conserved gateway recombination sites, facilitating easy cloning of the cDNA clones into pDONR222 entry vector. Determination of the sequence of the inserts and subsequent homology search in the Arabidopsis database (TAIR, http://www.arabidopsis.org) leads to easy identification of the corresponding Arabidopsis gene. The COS gene identification system have been described in detail in Rigó et al., (2012). Verification of the phenotype of the selected COS lines was done by segregation analysis of progenies of T2 generation plants and rigorous testing of estradiol-dependence of the observed phenotype. The cDNAs were subsequently cloned into the estradiol-inducible pER8GW expression vector or the constitutive pB2GW7 vector and introduced into wild type Arabidopsis plants. Generation of new transgenic lines with the gene constructs allowed us to compare the phenotype of the original COS line and independent lines overexpressing the cloned cDNA. Phenotypic

characterization was extended to loss-of function mutants using T-DNA insertion lines in which the insertion inactivated the investigated genes.

Previously we have identified several tagged mutants in our T-DNA insertion mutagenesis program, which displayed differences in stress tolerance. The *ppr40-1* mutant was hypersensitive to stress, while the *crk5-1* mutant showed difference in root growth, hairy root abundance and geotropism. The corresponding genes were cloned, and characterized. With all these genetic tools we could compare gain-of function and loss-of function phenotypes allowing the functional characterization of the identified genes. While verification of phenotypes associated to the RAP2.12, ZFP3 and HSFA4A transcription factors, PPR40 gene and the CRK5 kinase was success, reproducibility of results obtained with the RBP7-related factor was not very good. Therefore we concentrated on the characterization of the listed factors.

Characterization of the zinc finger protein ZFP3

Seed germination is controlled by environmental signals, including light and endogenous phytohormones. Abscisic acid (ABA) inhibits, whereas gibberellin promotes germination and early seedling development, respectively. To identify novel regulators of ABA signaling, COS-transformed seeds were germinated in the presence of 2.5μM ABA and estradiol. Screening for ABA insensitive germination lead to the identification of several COS lines, including A44, which could germinate in the presence of even 10μM ABA. The cDNA insert in this line encoded ZFP3, member of a larger zinc finger protein family.

Subsequent characterization of A44 line showed, that ZFP3, a nuclear C2H2 zinc finger protein acts as a negative regulator of ABA- suppressed germination (Figure 1). Regulated overexpression of ZFP3 and the closely related ZFP1, ZFP4, ZFP6 and ZFP7 zinc finger factors conferred ABA insensitivity to seed germination in the presence of estradiol. In germination tests we showed, that ZFP3 overexpression increased ABA insensitivity of *abi2-1, abi4-101* and *abi5-1* mutants, suggesting that ZFP3 modulates ABA signals through independent regulatory pathways.

To analyse phenotype of loss-of function mutants, T-DNA insertion lines were identified in public databases. Single *zfp3* or *zfp4* insertion mutants displayed only slight difference in ABA sensitivity, while the *zfp3 zfp4* double mutant had enhanced ABA susceptibility.

Zinc finger proteins are often transcriptional regulators and ZFP3 was found to be nuclear (Figure 2). To identify target genes controlled by ZFP3, whole genome transcript profiling was performed with wild type and ZFP3 overexpressing seedlings, using RNAseq technology. Reduced expression of a number of ABA-induced genes, such as *RAB18* and transcription factor *ABI4* in ZFP3ox seedlings suggests that ZFP3 can suppress ABA-induced transcription. Differences in transcript abundance of selected target genes was verified by quantitative RT-PCR.

Identification of putative DNA binding sites of the ZFP3 factor was attempted by chromatin immune precipitation coupled with genome-wide deep sequencing (ChIP-seq) technology. Epitope-tagged ZFP3 was overexpressed in Arabidopsis and chromatin precipitation with epitope-recognizing antibodies was performed from isolated nuclei. Deep sequencing of the precipitated DNA was performed. Bioinformatic analysis however failed to identify DNA sequences, which could be overrepresented in ZFP3 ChIP samples. Further experiments are therefore needed to

reveal whether ZFP3 really binds nuclear DNA, or is involved in other molecular regulatory mechanisms.

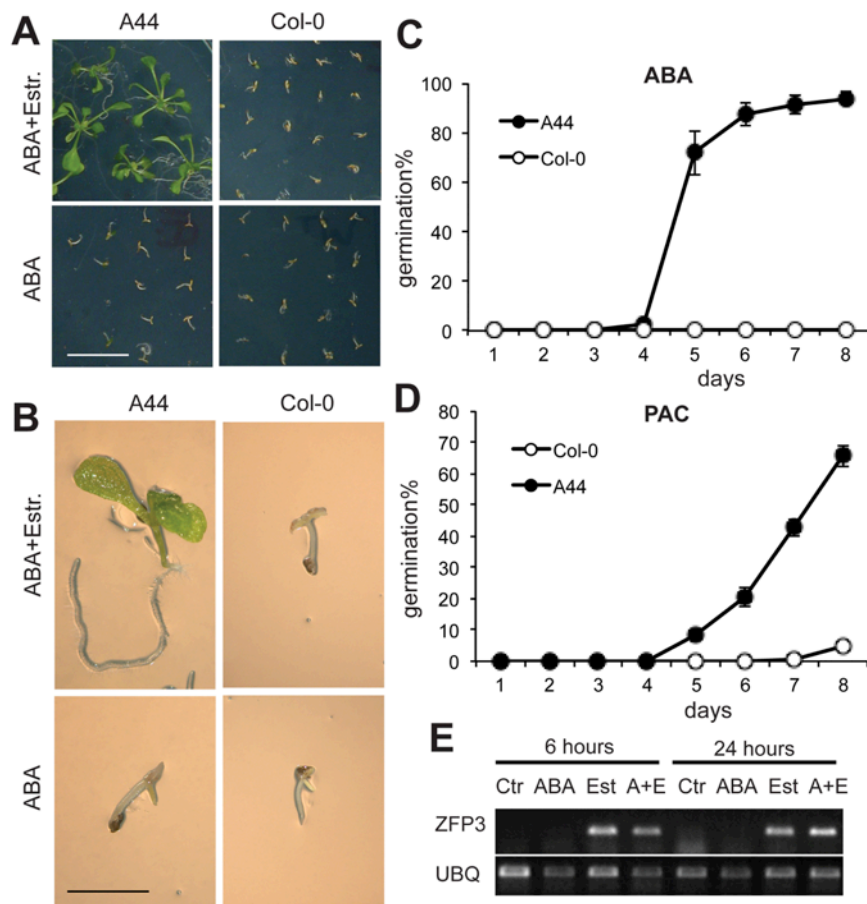

Figure 1. ABA insensitive germination of the COS line A44. A) Germination and growth of Col-0 wild type and A44 seedlings on media supplemented by 3μM ABA in the presence or absence of 4mM estradiol. Image shows 2 weeks-old seedlings, bar indicates 10mm. B) 6 days-old Col-0 and A44 seedlings germinating on media containing 5μM ABA with or without 4μM estradiol. Bar indicates 2mm. C) Time course of germination in the presence of 5μM ABA and 4μM estradiol. D) Germination of Col-0 wild type and A44 seeds on medium containing 40μM paclobutrazol and 4μM estradiol. E) Expression of *ZFP3* gene in A44 plants. *ZFP3* transcript was detected by RT-PCR in 2 weeks-old plantlets treated with ABA and/or estradiol. Abbreviations: Ctr: non-treated control, ABA: 20μM ABA, Est: 4μM estradiol, A+E: 20μM ABA and 4μM estradiol. Reference gene: UBQ10 (*AT4G05320*). Standard error bars are shown from 4 repeats.

We have initiated studies to identify proteins or protein complexes, which interact with ZFP3. Epitope-tagged ZFP3 was overexpressed in Arabidopsis and purification of the ZFP3-associated proteins was attempted by pull-down technology. Subsequent identification of putative interacting proteins was performed by mass spectrometry. Preliminary results suggest that ZFP3 might interact with different chromatin remodelling factors. Verification of the results via yeast two hybrid system and bimolecular fluorescence complementation is in progress.

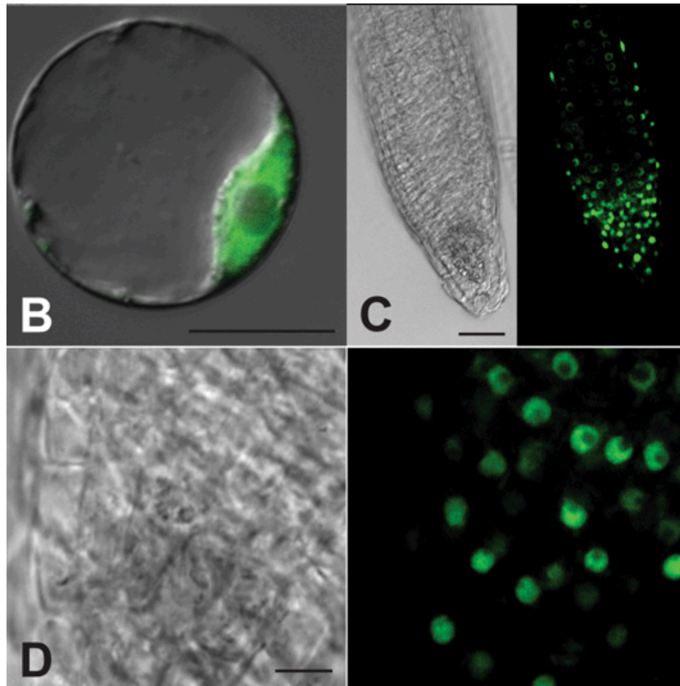

Figure 2. Intracellular localization of the ZFP3 protein. B) Nuclear localization of ZFP3-eGFP fusion protein in protoplasts. Bars indicate 20μm. C, D) ZFP3-eGFP is localized in nuclei of root cells of transgenic plants. Bar indicates 50μm.

Analysis of transgenic plants with constitutive overexpression of ZFP3 revealed multiple phenotypic alterations, such as semidwarf growth habit and defects in fertility. These data suggest, that ZFP3 is not only regulate germination and ABA signalling in early seedling development, but has a more general function controlling plant growth in several developmental stages.

Short hypocotyl phenotype was observed in seedlings overexpressing ZFP3 and the closely related ZFP1, ZFP4, ZFP6 and ZFP7 zinc finger factors. Hypocotyl shortening was particularly sensitive to red but not to far-red or blue light (Figure 3). Visible light perception is mainly mediated by photoreceptors including phytochromes absorbing red/far-red light and phototropins and cryptochromes sensing blue light. Analysis of genetic interactions with phytochrome mutants showed that ZFP3 is implicated in red light signaling independent of PhyB and suggest a role for ZFP3 in the amplification of red light signals from the PhyC, PhyD or PhyE photoreceptors. Analysis of genetic interactions with phytochrome mutants therefore indicates that ZFP3 enhances red light signalling by photoreceptors other than phyA.

To investigate light responses of *abi2*, *abi4* and *abi5* ABA signalling mutants and to test if they are influenced by ZFP3, hypocotyl elongation of *abi*/ZFP3ox lines was measured in different light regimes. We found that *abi5-1* prevents ZFP3-promoted hypocotyl shortening suggesting that *ABI5* regulates this photomorphogenic response downstream of ZFP3 and is essential for the light-dependent function of ZFP3.

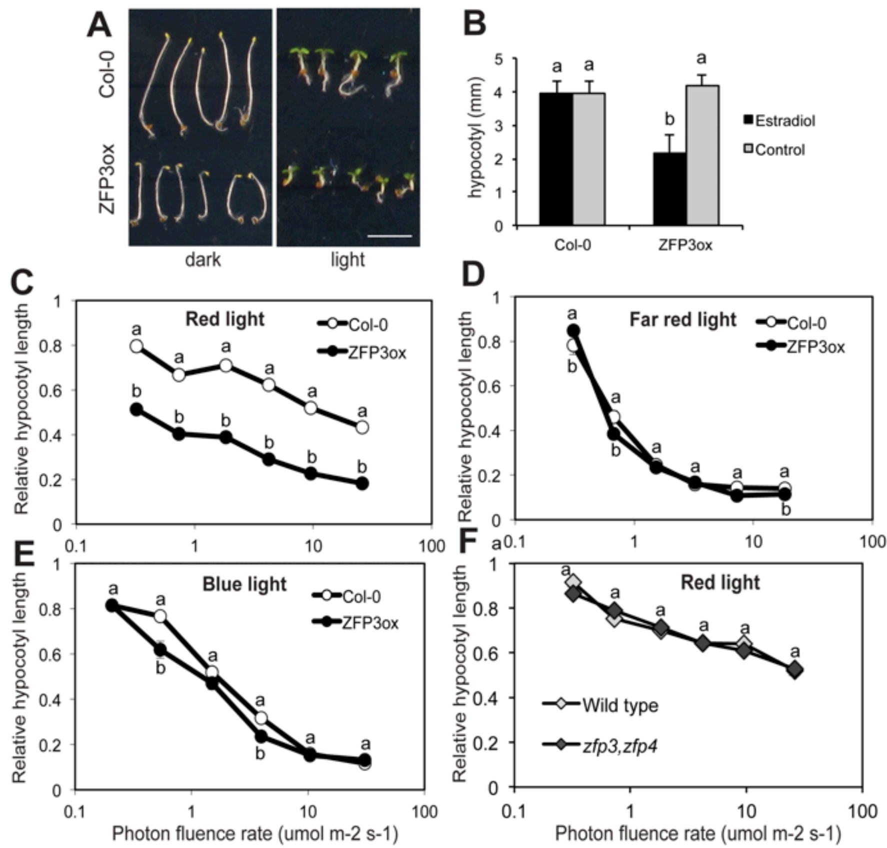

Figure 3. ZFP3 modulates light-controlled hypocotyl elongation. A) 3-days-old Col-0 wild type and ZFP3 overexpressing seedlings grown in the dark and light. Seeds were germinated in the presence of 4μM estradiol. Bar indicates 5mm. B) Change of hypocotyl lengths in white light in the absence or presence of estradiol. C-E) Relative hypocotyl lengths of wild type and ZFP3ox seedlings grown under different fluence rates of monochromatic red (C), far red (D) and blue (E) light. F) Relative hypocotyl lengths of wild type and *zfp3 zfp4* double mutant germinated under different intensities of red light.

Our study showed that ZFP3, together with other closely related ZFP factors, is a negative regulator of ABA signaling during germination and early seedling development. Upon overexpression of ZFP3, seeds can germinate in the presence of inhibitory concentrations of ABA. Moreover, ZFP3 strenghten red light signals perceived by photoreceptors other than phyA, leading to reduced hypocotyl elongation of germinating seedlings. ABI5 seem to be epistatic to ZFP3 in control of red light-dependent repression of hypocotyl elongation (Figure 4).

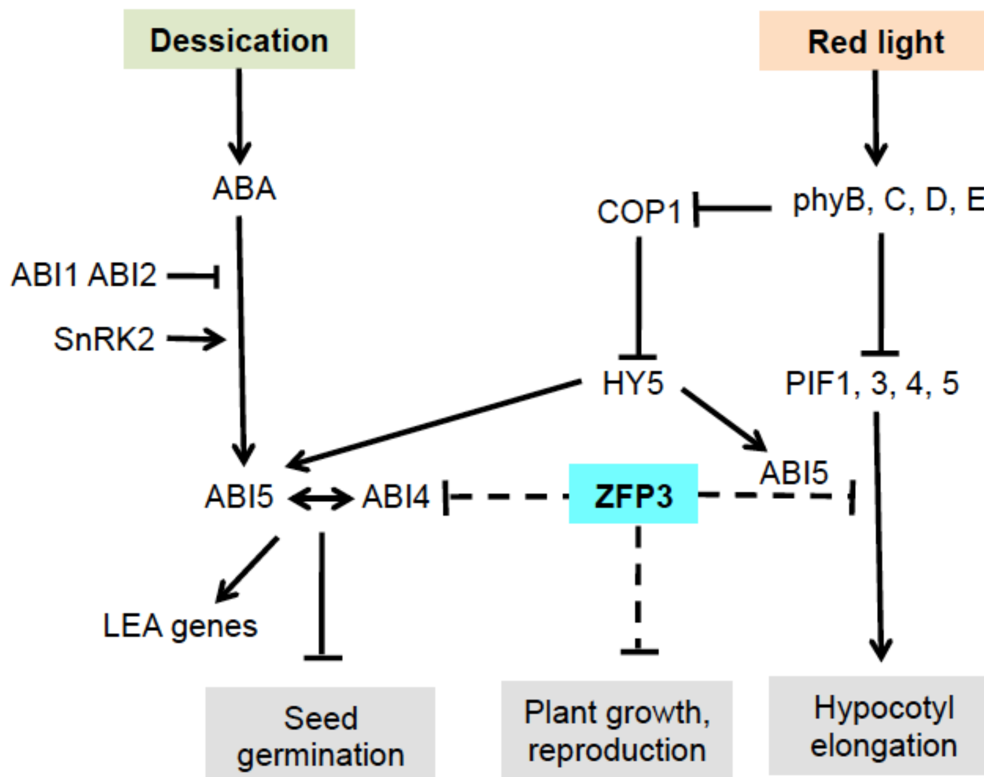

Figure 4. Model of ZFP3 action in seed germination and plant development. ABA is sensed by the receptor complexes containing PP4C-type protein phosphatases ABI1 abd ABI2 which function as negative regulators of ABA signals. Transcription factors ABI4, ABI5 are positive components of ABA signaling. Red light is perceived by Phytochromes phyB, C, D, E and light signals are controlled by transcription factors PIF1, 3, 4, 5. ZFP3 is negative regulator of ABA signals, promotes red light-dependent reduction of hypocotyl elongation and reduces plant growth and fertility. Transcription of ABI4 was repressed by ZFP3, while ABI5 seem to be epistatic to ZFP3 in reduction of red light-dependent hypocotyl elongation.

Publication. This study have been published in the paper of Joseph et al., (2014). Results were divulgated in several scientific congresses (see publication list).

Characterization of HSFA4A

To identify novel regulators of stress responses in plant cell level, we have combined the COS cDNA library transformation system with a cell selection. Large-scale Agrobacterium-mediated transformation of the COS library was adapted to in vitro conditions. The Arabidopsis wild type established cell suspension culture “Kata” was used for transformation experiment. Fast growing cell cultures were infected with the COS library, and after 2 days co-incubation, cells were subcultured in liquid culture medium containing claforan (to eliminate Agrobacteria) and hygromycin (to select for transformed cells). After 4 weeks of hygromycin selection, the transformed cell culture was plated on selective medium containing salt (150-175mM NaCl), claforan and estradiol (to activate the introduced gene constructs) (Figure 5).

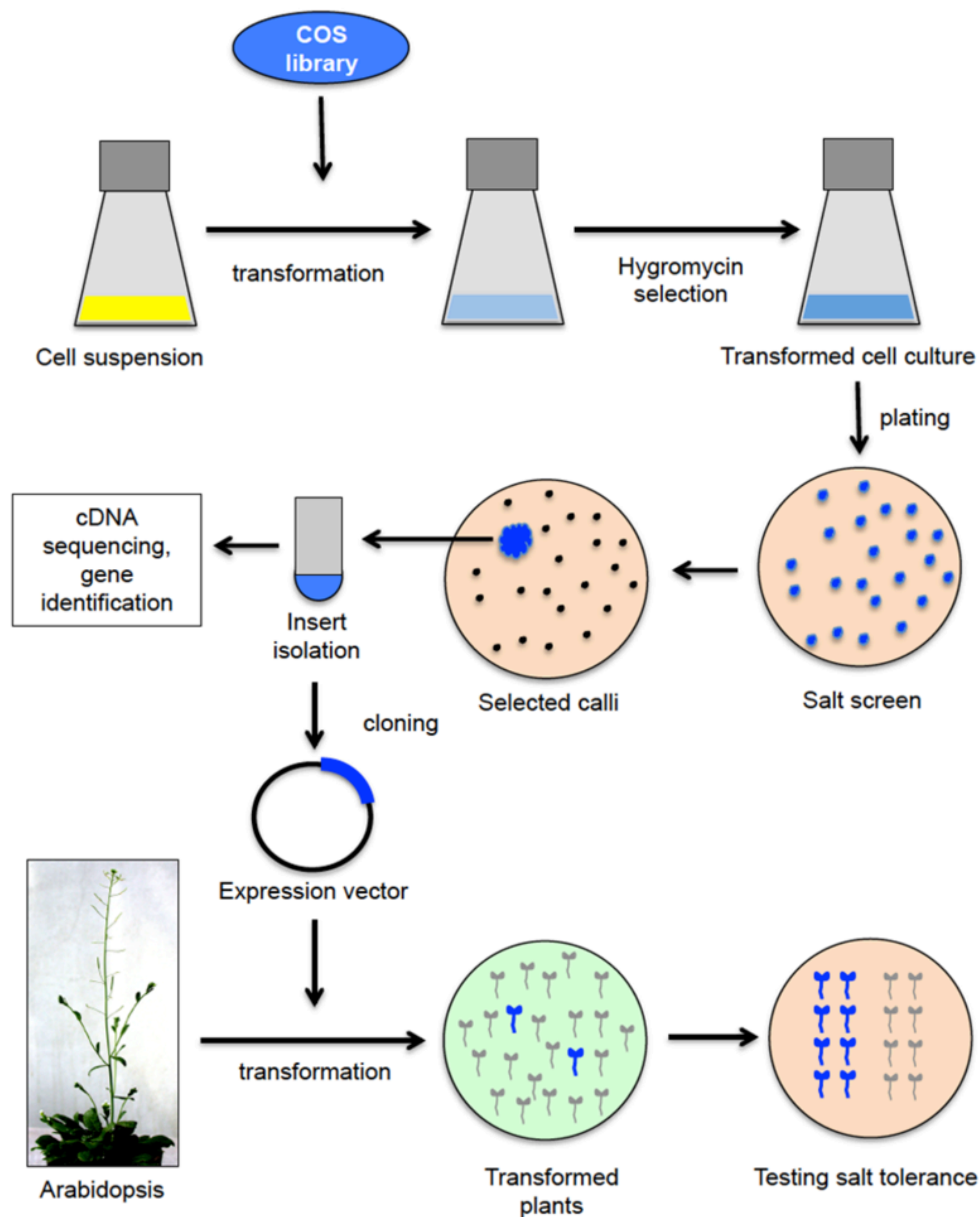

Figure 5. Schematic illustration of COS screening and cloning strategy.
Arabidopsis cell suspension was transformed with the COS cDNA library. Salt tolerant calli were selected in the presence of 5µM estradiol and used for DNA isolation and identification of cDNAs carried by the COS expression vector T-DNA insertions. The identified HSFA4A cDNA was cloned into a plant expression vector pER8GW, and transformed into Arabidopsis to verify its capacity to improve stress tolerance in plants.

Cell colonies, able to grow on selective conditions (Figure 6) were subsequently used for gene identification by PCR amplification of the inserted cDNA and determination of their nucleotide sequence. Amplified cDNA were cloned in transformation vectors and the new gene constructs were used for transformation of cell cultures or Arabidopsis plants to verify the observed phenotype of the selected calli (Figure 5). The developed and optimized method has been described in Pérez-Salamó et al., (2014b).

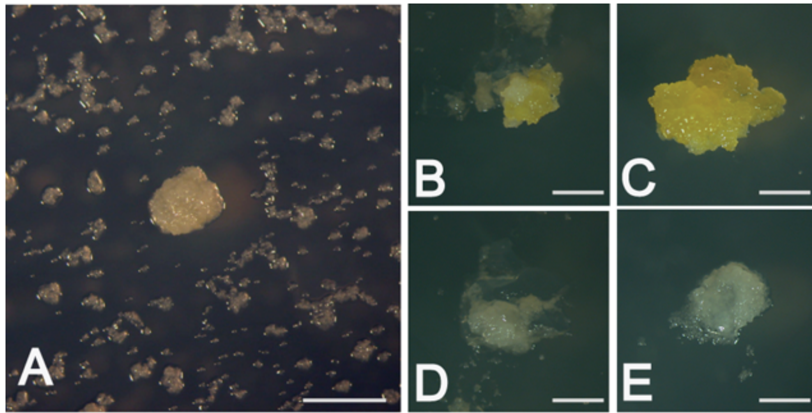

Figure 6. Identification of salt tolerant COS-transformed Arabidopsis calli. **(A)** Screening of COS-transformed cell culture on MS medium containing 175 mM NaCl and 5µM estradiol. **(B, C)** Growth of transformed line no. 1 on high salt medium without (B) and with estradiol (C). **(D, E)** Wild type Arabidopsis calli on high salt medium in the absence (D) or presence (E) of estradiol.

One of the cDNAs identified by our newly developed cell selection screen encoded the full length clone of the Arabidopsis heat shock factor A4A (HSFA4A), which was not characterized before. Heat shock factors (HSFs) are transcriptional regulators, which mediate the activation of large set of genes induced by high temperature or other stress conditions. HSFs are important regulators of cellular stress responses in plants and animals. HSFs recognize the heat stress elements (HSEs), conserved motifs in promoters of heat-induced targets, such as heat-shock protein (HSP) genes. The yeast and Drosophila genomes encode a single HSF, whereas mammals have four HSF genes. Plants possess diverse families of HSFs that are encoded by 21 genes in Arabidopsis, and 52 loci in soybean. Remarkable differences in their transcriptional regulation indicate that plant HSFs underwent considerable functional diversification.

In our studies we showed that estradiol-dependent induction of HSFA4A in transgenic Arabidopsis plants confers enhanced tolerance not only to salt but also to osmotic and oxidative agents (Figure 7). Inactivation of HSFA4A by T-DNA insertions results in hypersensitivity to salt stress in Arabidopsis. Estradiol-induction of HSFA4A in transgenic plants decreases, while the knockout *hsfa4a* mutation elevates hydrogen peroxide accumulation and lipid peroxidation, suggesting that this transcription factor is an important regulator of responses to oxidative stress.

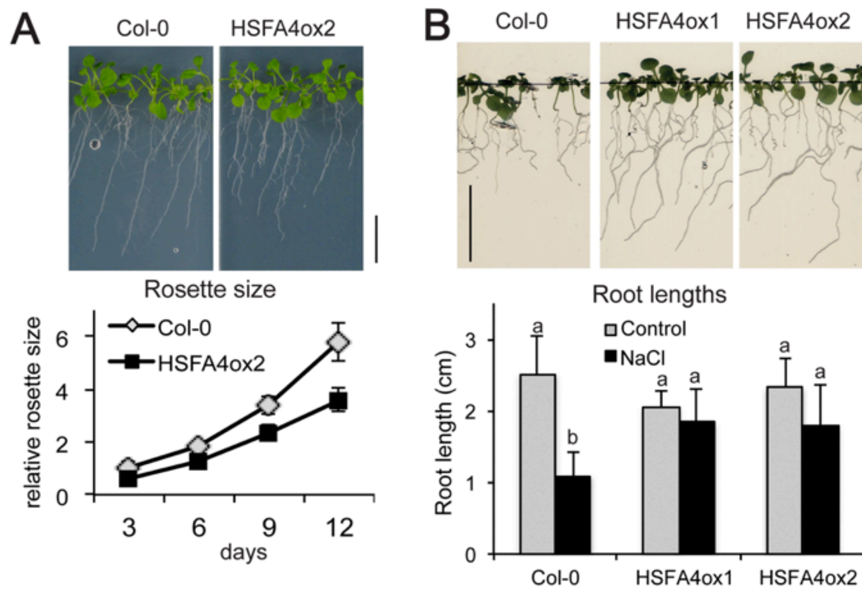

Figure 7. HSFA4A overexpression confers stress tolerance to Arabidopsis. **(A)** Growth of Col-0 wild type and HSFA4A overexpressing (HSFA4ox2) plants on 0,5MS media with estradiol (control media). Graph shows growth of relative rosette sizes. **(B)** Root growth of Col-0, HSFA4ox1 and HSFA4ox2 plants on high salt medium (100 mM NaCl, 12 days) supplemented with estradiol. Graph shows average root lengths after 12 days of growth.

To identify target genes of HSFA4A, transcript profiles of HSFA4A overexpressing plants were compared to those of wild type using the recently developed RNA-seq technology. Whole genome transcript profiling showed, that overexpression of HSFA4A alters the transcription of a large set of genes regulated by oxidative stress and included genes involved in ROS detoxification, chaperone activity, stress adaptation or transcription factors implicated in abiotic or biotic stress responses.

HSFs in yeast and animal cells were previously shown to form trimers during heat stress. We have tested the ability of HSFA4A to form multimers in heterologous and homologous cell systems. In yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays HSFA4A showed homomeric interaction, confirming the analogy with animal and yeast HSFs. Furthermore we showed that multimerisation is reduced by alanine replacement of three conserved cysteine residues, suggesting that these amino acids are important for the stabilization of HSFA4A trimmers during stress conditions.

Activity of different classes of stress-induced transcription factors were previously shown to be regulated by post-translational modifications, including phosphorylation. Moreover, MAP kinases were previously shown to phosphorylate some of the HSFs in several organisms. In order to test whether HSFA4A is phosphorylated by Arabidopsis MAP kinases, a series of phosphorylation and interaction studies were performed. Our results clearly showed that HSFA4A interacts with and phosphorylated by mitogen-activated protein kinases MPK3 and MPK6 in yeast and plant cells. Mass spectrometry revealed that MPK3 and MPK6 phosphorylate HSFA4A *in vitro* on three distinct sites, Ser309 being the major phosphorylation site (Figure 8).

Figure 8. Identification of HSFA4A phosphorylation sites. **(A)** Identification of HSFA4A phosphorylation site by mass spectrometry. CID spectrum of m/z: 481.247 (2+) representing the phosphorylated [304-311] sequence of HSFA4A (Uniprot ID:. O49403). Fragment ions b_3 (unmodified) and y_5 and y_6 (phosphorylated) unambiguously prove that the site of modification is Ser-6, i.e. Ser-309 of HSFA4A. “-P” denotes the 98 Da neutral loss of the phosphate from the corresponding fragment ions. Observed peptide backbone cleavages are indicated in the sequence. **(B)** Amino acid sequence of HSFA4A with MPK3 phosphorylation sites boxed. **(C)** Phosphorylation of wild type and mutant HSFA4A protein by MPK3 and MPK6. 5xmutant: all identified Ser and Thr residues changed to Ala. 4xmutant: all Ser and Thr changed to Ala, except the indicated one. MyelinBP was used in positive control reactions. Upper panel shows phosphorylation reaction, lower panel shows coomassie-stained gels indicating equal loading.

To study functional relevance of HSFA4A phosphorylation, activation of a test gene construct, composed of promoter of the HSFA4A target HSP17.6A gene and firefly luciferase reporter was tested in a transient expression system. Our data clearly confirmed that activation of the MPK3 and MPK6 MAPK pathway leads to the transcriptional activation of the heat-shock protein gene *HSP17.6A*. In agreement that mutation of Ser309 to alanine strongly diminished phosphorylation of HSFA4A, it also strongly reduced the transcriptional activation of *HSP17.6A*. These data suggest that HSFA4A is a substrate of the MPK3/6 signalling and it regulates stress responses in Arabidopsis.

Publications: Results of this research have been published in the following papers: Pérez-Salamó et al., 2014a, Pérez-Salamó et al., 2014b.

Characterization of RAP2.12

In order to identify regulatory genes which control a particular signaling pathway, a reporter gene activation system was adapted to our conditions. A stress-induced reporter gene construct (*ADH1 promoter::enhanced luciferase*, pADH1-LUC+) was expressed in Arabidopsis, which was shown to respond to a number of environmental stimuli and ABA. Transformation of the reporter line with the COS cDNA library and subsequent screening for enhanced luciferase aktivity lead to the identification of the ERF-VII transcription factor RAP2.12 as activator of the p*ADH1-LUC+* reporter (Papdi et al., 2008, Plant Physiol 147: 528-542). Further characterization of the RAP2.12 and related genes was performed in this research project.

We showed that estradiol induced overexpression of RAP2.12 and its close homologs RAP2.2 and RAP2.3 can enhance the ABA-dependent activation of *ADH1* transcription (Figure 9) and elevate the transcription of hypoxia marker genes such as *ADH1, SUS1, PDC1 and SUS4* in normoxic conditions. These data suggest, that members of this subgroup of ERF factors are positive regulators of hypoxia stress response. RAP2.12 could activate the pADH1-LUC+ and pADH1-GUS reporter gene constructs, suggesting that it regulates transcription activity through interacting with the ADH1 promoter. Analysis of deletion series and point mutants of the ADH1 promoter could delimit RAP2.12-mediated ADH1 activation between -384bp to -510bp fragment of the promoter. Promoter binding studies by gel retardation assays were however not succesfull.

RAP2.12, RAP2.2 and RAP2.3 overexpressing lines showed enhanced tolerance to anoxia, osmotic and oxidative stresses and hypersensitivity to ABA, while insertion mutants of these genes were more sensitive to submergence. While stress responses of single knockouts were indistinguishable from wild type, the double mutant *rap2.12-2 rap2.3-1* was hypersensitive to submergence as well as osmotic stress. Our results not only confirm recent results, that RAP2.12 is an essential component of low oxygen stress, but suggest that this and the two other transcription factors are controllers of stress responses in a much broader way.

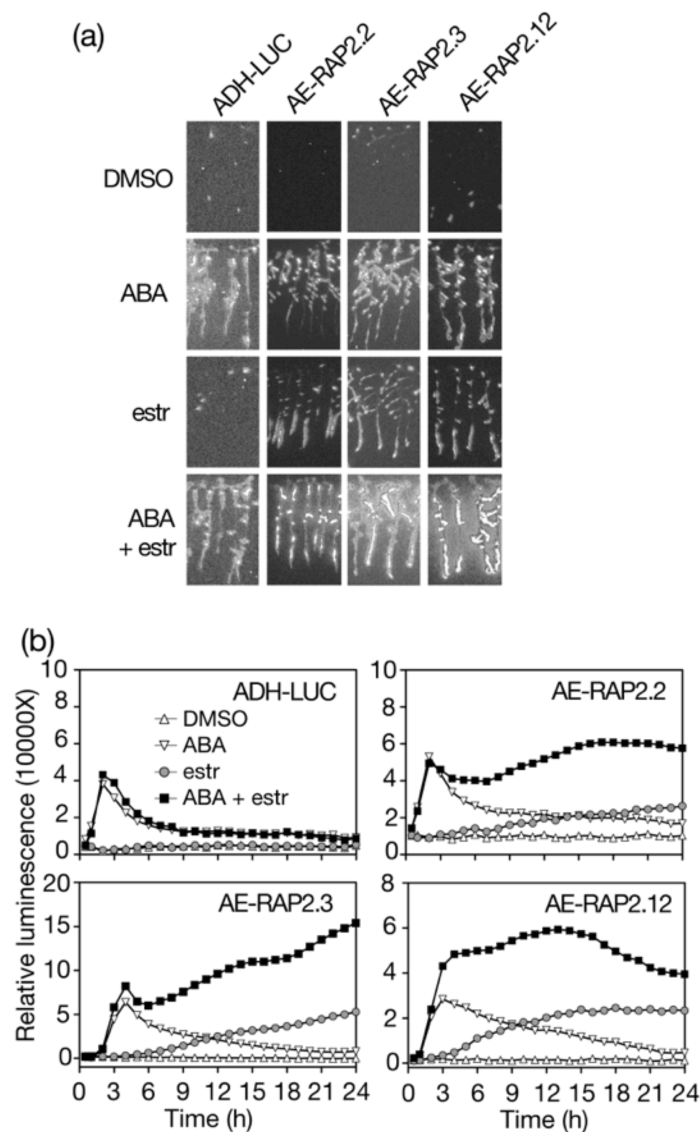

Figure 9. Bioluminescence detection of RAP2.2, RAP2.3 and RAP2.12 overexpressing lines to monitor *ADH1-LUC* activity. A) Images of ADH-LUC, AE-RAP2.2, AE-RAP2.3 and AE-RAP2.12 plants, showing different levels of LUC activities after 9 hours of 0.04 % DMSO solvent as control, 30µM ABA and/or 5µM estradiol (estr) treatments. B) Kinetic analysis of quantified bioluminescence values based on the intensity of light emissions detected in each hour for 24 hours.

Oxygen-dependent degradation of RAP2.12 was previously shown to be mediated by the N-end rule pathway (NERP) (Gibbs *et al.* 2011, *Nature*, 479, 415-418. Licausi *et al.* 2011, *Nature*, 479, 419-422.). Our results showed that RAP2.12 turnover is also regulated by an alternative proteolytic pathway, which is mediated by the E3 ubiquitin ligase SEVEN IN ABSENTIA of *Arabidopsis thaliana* 2 (SINAT2). We showed that SINAT2 ubiquitinates RAP2.12, which labels it for proteasomic degradation (Figure 10). To test the functional importance of SINAT2-mediated proteolysis, the SINAT1 and SINAT2 genes were silenced simultaneously by artificial micro RNA (amiRNA) technology. Silencing of *SINAT1/2* genes lead to enhanced RAP2.12 abundance, independent of the presence or absence of its N-terminal degron. Our results suggest that abundance of RAP2.12 (and possibly other

members of the ERF-VII transcription factors family) are controlled by multiple ubiquitin/26S proteasome dependent degradation pathways.

Our results confirm that the RAP2-type ERF-VII transcription factors have multiple, partially overlapping functions in diverse hormonal and stress signalling pathways. Intracellular abundance of the RAP2.12 and possibly other ERF-VII transcription factors seems to be controlled by at least two proteolytic pathways. The N-end rule pathway of targeted proteolysis connects RAP2.12 to the perception of gaseous (oxygen and NO) signals, while the SINAT1/2-mediated ubiquitination and proteolysis functions independently of the NERP pathway and can regulate protein abundance during different (stress) conditions when the NERP pathway is inactive. Further studies are required to reveal the complexity of these interactions and determine their functional relevance.

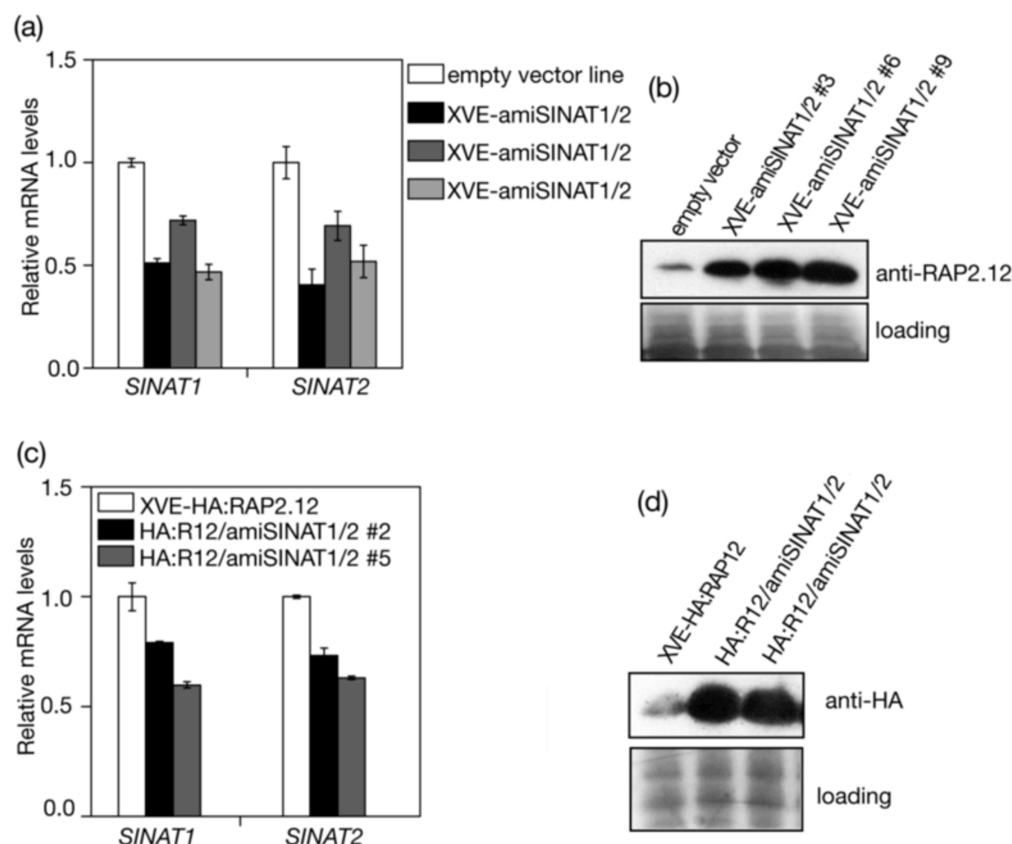

Figure 10. Silencing of *SINAT1* and *2* results in higher abundance of the native RAP2.12 and its N-terminal modified version HA:RAP2.12 *in planta*. a) XVE-amiSINAT1/2 lines were generated to silence *SINAT1* and *2* expressions in estradiol-dependent fashion. Three XVE-amiSINAT1/2 lines show reduced *SINAT1* and *2* transcript levels 48 hours after 20μM estradiol treatment of 10 days old plants. b) Silencing *SINAT1* and *2* increases the accumulation of RAP2.12. c) HA:R12/amiSINAT1/2 lines were generated by transforming XVE-HA:RAP2.12 with the constitutively expressed amiSINAT1/2. The HA:R12/amiSINAT1/2 #2 and #5 lines show repression in the transcription of both *SINAT1* and *2*. d) Comparison of HA:RAP2.12 abundance in XVE-HA:RAP2.12 and HA:R12/amiSINAT1/2 #2 and #5 lines after 5μM estradiol treatment.

Results of these studies are being published in a scientific paper (Papdi et al., 2014).

Characterization of PPR40 and CRK5

Previous mutant screens in our laboratory have identified several mutants characterized by altered stress response or growth patterns (Szabados et al., 2002, Plant J. 32:233-242, Zsigmond et al., 2008, Plant Physiol. 146:1721-1737). Characterization of the identified mutants and corresponding genes was continued within the frame of in the present research program.

PPR40 can enhance salt tolerance.

Mitochondrial respiration is sensitive to environmental conditions and can be influenced by a number of abiotic stresses. In an earlier report we described the Arabidopsis mitochondrial pentatricopeptide repeat domain protein PPR40, and showed that the stress hypersensitive *ppr40-1* mutant is compromised in mitochondrial electron transport (Zsigmond et al., 2008, Plant Physiol. 146:1721-1737).

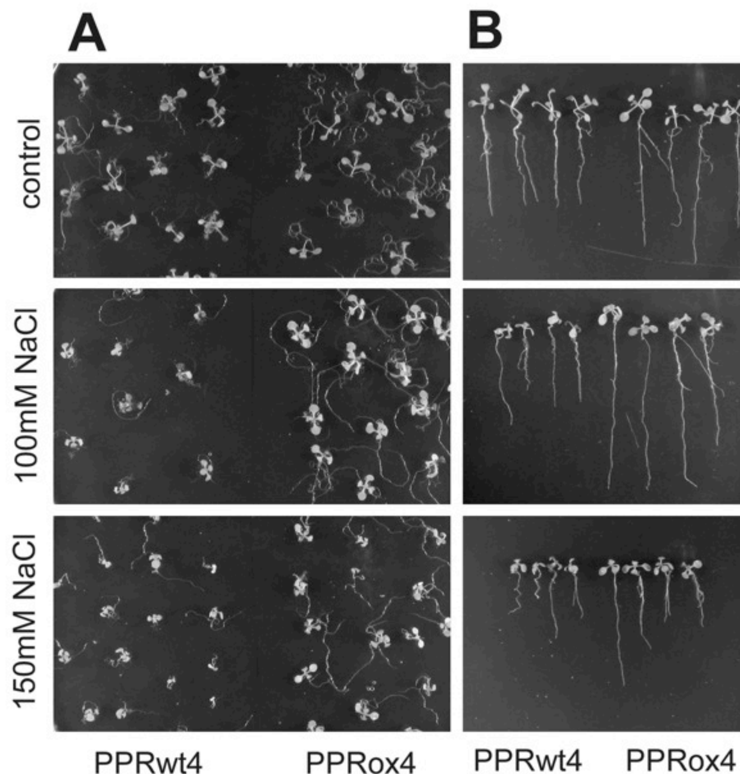

Figure 11. Salt tolerance of *PPR40* overexpressing transgenic lines and their respective control plants. T3 generation homozygous transgenics (PPRox4) and wild type lines (PPRwt4) are progenies of the same, independent transgenic plants. A) 2 weeks-old transgenic plants of the PPRox4 and PPRwt4 lines, grown on 1/2MS medium supplemented by 0, 100 and 150mM NaCl. B) Root elongation of wild type and *PPR40* overexpressing plants on saline and control plates.

In this study we analysed the effect of PPR40 overexpression on germination and growth responses, oxidative damage and mitochondrial respiration during salt stress. Overexpression of the *PPR40* gene in Arabidopsis resulted in enhanced germination and superior plant growth in saline conditions (Figure 11). Various physiological characters were altered in the *PPR40* overexpressing lines. Respiration increased in *PPR40* overexpressing plants during salt stress. Reduced amount of hydrogen peroxide, diminished lipid peroxidation, lower ascorbate peroxidase and superoxide dismutase activity accompanied salt tolerance. Proline accumulation was enhanced in the *ppr40-1* mutant, but unaltered in the *PPR40* overexpressing plants. Our data suggest that PPR40 can diminish the generation of reactive oxygen species by stabilization the mitochondrial electron transport and protect plants by reducing oxidative damage during stress.

Results were published in the scientific paper Zsigmond et al., 2012.

CRK5 kinase controls root growth and geotropism.

The *crk5-1* T-DNA insertion mutant was identified due to its distorted root growth and abnormal geotropic response. CRK5 is a member of Arabidopsis Ca2+/calmodulin-dependent kinase-related kinase family. In our research program we showed that inactivation of CRK5 by T-DNA insertion inhibits primary root elongation and delays gravitropic bending of roots (Figure 12). Reduced activity of auxin-induced DR5-GFP reporter suggests that auxin is depleted from the *crk5* mutant root tips. Tip collapse was not observed in the mutant and the transcription of genes of auxin biosynthesis, AUX/LAX auxin influx and PIN efflux carriers was unaffected by the *crk5* mutation.

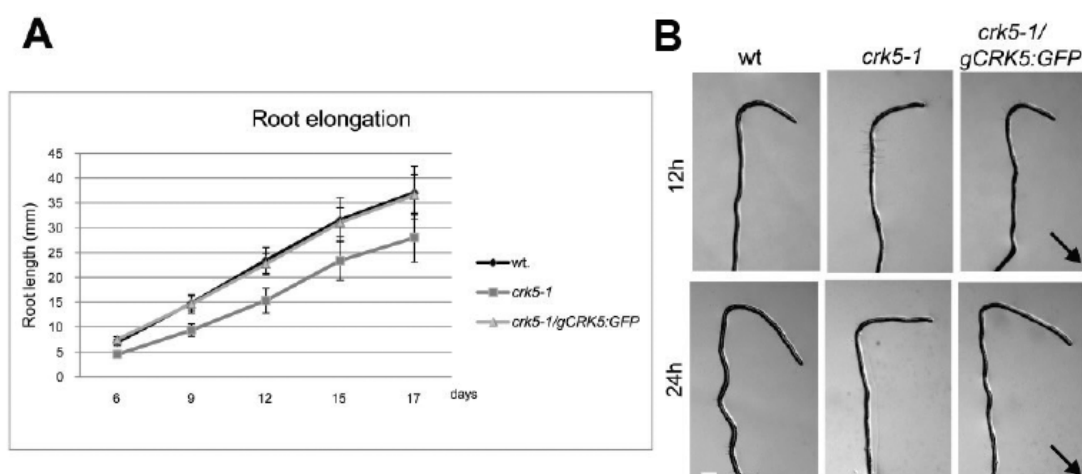

Figure 12. Root developmental and gravitropic defects caused by the *crk5-1* mutation.
A) The *crk5-1* mutant shows reduced elongation of primary roots. Root elongation rates of wild type, *crk5-1* mutant and complemented *crk5-1*/gCRK5-GFP seedlings are compared by measuring root lengths at different time points. **B)** Comparison of root gravitropic responses of 7 days old wild type (wt), *crk5-1* and a genetically complemented *crk5-1*/gCRK5-GFP) seedlings.

Whereas AUX1, PIN1, PIN3, PIN4 and PIN7 displayed normal localization, PIN2 was depleted from apical membranes of epidermal cells and showed basal to apolar and apical relocalization in the cortex of *crk5* root transition zone. These

results, together with an increase in the number of *crk5* lateral root primordia, suggested a facilitated auxin efflux through the cortex towards the elongation zone.

Localization of GFP-tagged CRK5 by laser scanning confocal microscopy showed that CRK5 is a plasma membrane-associated kinase, which forms U-shape patterns facing outer lateral walls of epidermis and cortex cells. Brefeldin inhibition of exocytosis stimulated CRK5 internalization into brefeldin-bodies.

Altered intracellular localization of PIN2 suggested that CRK5 might directly influence transport through post-translational modification of this auxin transporter (Figure 13). We found that CRK5 phosphorylates the hydrophilic loop of PIN2 *in vitro* and PIN2 shows accelerated accumulation in brefeldin-bodies in the *crk5* mutant. Delayed gravitropic response of the *crk5* mutant thus likely reflects defective phosphorylation of PIN2 and deceleration of its brefeldin-sensitive membrane recycling.

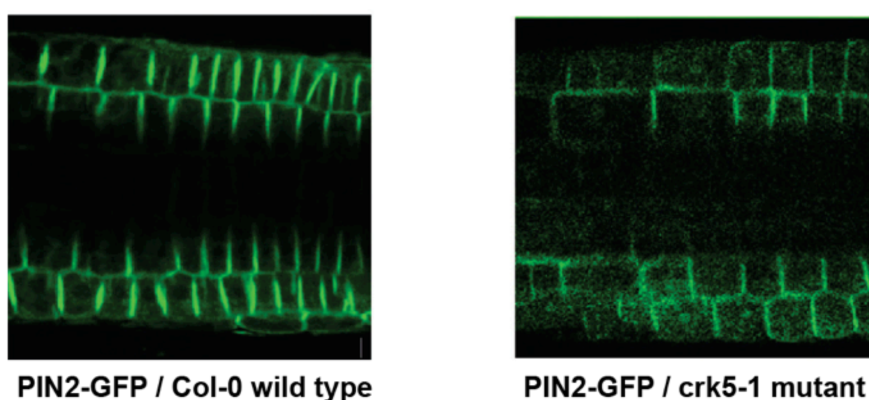

Figure 13. Comparison of Polar PIN2-GFP localization in the transition zones of wild type and *crk5-1* mutant roots before and after gravistimulation. Compared to wild type, PIN2-GFP is expressed at lower level in *crk5-1* and shows depletion from the apical membranes of epidermal cells and apolar to apical localization in cortex cells in the transition zones of vertically grown roots.

Publication of this study: Rigó et al., 2013.

Conclusions

In our research program we have characterized a number of regulatory genes, transcription factors which were identified previously in our laboratory. Using combination of genetic, molecular, cellular, physiological and biochemical techniques we could get complex information about the biological function of the characterized genes. We confirmed that most of the studied factors are significant regulators of responses to different environmental stresses. Some of them turned out to posses more complex regulatory function and have pleiotrop effect on plant growth and development (eg. ZFP3, HSFA4A), while others have more restricted function confined to certain developmental stage or tissue type (eg. CRK5, RAP2.12). Although the aim of the present proposal was not to create a biotechnological application, the information gained can serve as a basis to develop technologies and strategies which can serve as tools to improve salt and drought tolerance of important crops.

Publication list

Support of the OTKA grant was acknowledged in the following scientific papers.

1. Rigó G, Papdi Cs, Szabados L (2012) Transformation using Controlled cDNA Overexpression System. In: *Methods in Molecular Biology,* New York: Humana Press, 913: 277-290.

2. Zsigmond L, Szepesi Á, Tari I, Király A, Szabados L (2012) Overexpression of the mitochondrial *PPR40* gene improves salt tolerance in Arabidopsis. Plant Sci 182:87-93. IF: 2,945 PDF

3. Rigó G, Tietz O, Ayaydin F, Zsigmond L, Kovács H, Páy A, Salchert K, Szabados L, Palme K, Koncz Cs, Cséplö Á (2013) Inactivation of plasma-membrane localized CDPK-related kinase 5 decelerates PIN2 exocytosis and root gravitropic response. Plant Cell 25:1592-1608, IF: 10,224 PDF

4. 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 (2014a) 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, IF: 7,908 PDF

5. Joseph MP, Papdi C, Kozma-Bognar L, Nagy I, Lopez-Carbonell M, Koncz C, Szabados L (2014) The Arabidopsis Zinc Finger Protein 3 interferes with ABA and light signaling in seed germination and plant development. Plant Physiol 165(3):1203-1220, IF: 7,908 PDF

6. Fichman Y, Gerdes S, Kovács H, Szabados L, Zilberstein A, Csonka L, (2014) Evolution of Proline Biosynthesis: Enzymology, Bioinformatics, Genetics, and Transcriptional Regulation. Biological Reviews (in press). IF: 9,79

7. Szabados L (2014) A szárazság és sótűrés szabályozása a viragos növényekben. In.: A Növények Molekuláris Biológiájától a Zöld Biotechnológiáig. Ed.: Fehér A., Györgyey J. Akadémiai Kiadó, Budapest, pp. 192-210.

8. Perez-Salamó I, Boros B, Szabados L (2014b) Screening Stress Tolerance Traits in Arabidopsis Cell Cultures. In: *Methods in Molecular Biology,* New York: Humana Press. (in press).

9. Papdi CS, Pérez-Salamó I, Joseph MP, Giuntoli B, Perata P, Bögre L, Koncz Cs, Szabados L (2014) RAP2.12 confers low oxygen and osmotic stress tolerance with overlapping function with its homologs RAP2.2 and RAP2.3, and is directed to proteolysis by the E3 ubiquitin ligase SINAT2. (submitted to Plant J.)

Congress presentations with acknowledgement of the OTKA financial support

1. Salamó IP, Joseph MP, Papdi Cs, Szabados L (2011) Identification of Novel Regulatory Factors of Plant Stress Responses Using New Genetic Approaches. IX. Magyar Genetikai Kongresszus és XVI. Sejt- és Fejlődésbiológiai Napok, Siófok, 2011 március 27-29.

2. Salamó IP, Ábrahám E, Joseph MP, Papdi Cs, Koncz Cs, Szabados L (2011) Use of the Conditional Overexpressing System for the identification of novel stress regulatory genes. Plant Abiotic Stress: from Systems Biology to Sustainable Agriculture, Limmasol, Cyprus, 2011.nov.17-19.

3. Zsigmond L, Szepesi Á, Tari I, Rigó G, Király A, Szabados L, (2011) overexpression of the mitochondrial PPR40 gene improves salt tolerance in Arabidopsis. 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Budapest, 2011 July 5-8

4. Rigó G, Tietz O, Ayaydin F, Zsigmond L, Kovács H, Palme K, Szabados L, Koncz Cs, Cséplő Á (2011) Egy *Arabidopsis thaliana* protein kináz, CRK5, szerepe a gravitáció szabályozásában. A Magyar Növénybiológiai Társaság X. Kongresszusa, Szeged, 2011.08.31. – 09.02.,

5. Mary Prathiba Joseph, Csaba Papdi, Csaba Koncz, László Szabados (2012) A novel Zinc finger transcription factor family controls ABA, light and gibberelling signalling in Arabidopsis. Straub Napok, Szeged, 2012.

6. Joseph, MP, Papdi, Cs, Nagy I, Koncz Cs, Szabados L. (2012) A novel C_2H_2 zinc finger factor integrates ABA, light and gibberellin signalling in germinating Arabidopsis seedlings. 23rd. International Conference on Arabidopsis Research (ICAR), Vienna, Austria.

7. Papdi Cs, Salamó IP, Giuntoli B, Perata P, Szabados L. (2012) Functional characterization of the Arabidopsis transcription factor RAP2.12. 23rd. International Conference on Arabidopsis Research (ICAR), Vienna, Austria.

8. Salamó IP, Papdi Cs, Rigó G, Zsigmond L, Nagy I, Szabados L. (2012) The heat shock factor HSFA4A controls oxidative stress responses in Arabidopsis. 23rd. International Conference on Arabidopsis Research (ICAR), Vienna, Austria.

9. Imma Pérez Salamó, Csaba Papdi, Gábor Rigó, Laura Zsigmond, Belmiro Vilela, István Nagy, Balázs Horváth, Mónika Domoki, Csaba Koncz, László Szabados (2013) The Heat Shock Factor A4A regulates oxidative stress tolerance in Arabidopsis. Hungarian Molecular Life Sciences 2013, Siófok, 2013.04.5-7

10. Imma Pérez Salamó, Csaba Papdi, Gábor Rigó, Laura Zsigmond, Belmiro Vilela, Montserrat Pagés, István Nagy, Mónika Domoki, Zsuzsa Darula, Csaba Koncz, László Szabados (2013) Oxidative stress responses are regulated by Heat Shock Factor HSFA4A in Arabidopsis. 11th International

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11. Joseph MP, Papdi Cs, Kozma-Bognár L, Nagy I, López-Carbonell M, Koncz Cs, Szabados L. (2013) The Arabidopsis ZFP3 protein integrates ABA and light signaling. 7th EPSO, Porto Heli, Greece

12. Salamó IP, Papdi Cs, Rigó G, Zsigmond L, Vilela B, Pagés M, Nagy I, Horváth B, Domoki M, Darula Zs, Medzihradszky K, Koncz Cs, Szabados L (2013) The Heat Shock Factor A4A controls oxidative stress responses in Arabidopsis. 7th EPSO, Porto Heli, Greece

13. Szabados László, Rigó Gábor, Papdi Csaba, Zsigmond Laura, Imma Pérez Salamó, Kovács Hajnalka, Mary Prathiba Joseph, Cséplő Ágnes, Koncz Csaba (2014) A szárazság és sótűrés szabályozása a növényekben. Kolozsvári Biológus Napok, Kolozsvár, 2014 április 3-6.

14. Imma Pérez Salamó, Csaba Papdi, Gábor Rigó, Laura Zsigmond, István Nagy, Balázs Horváth, Zsuzsa Darula, Mónika Domoki, Csaba Koncz, László Szabados (2014) The Arabidopsis Heat Shock Factor HSFA4A controls salt tolerance and is regulated by the MAP kinases, MPK3 and MPK6. Plant Biology 2014, American Society of Plant Biologists, Portland, Oregon, USA,

15. Mary Prathiba Joseph, Csaba Papdi, László Kozma-Bognár, István Nagy, Marta López-Carbonell, Csaba Koncz, László Szabados (2014) The Arabidopsis Zinc Finger Protein 3 integrates ABA and light signaling in seed germination and plant development. Plant Biology 2014, American Society of Plant Biologists, Portland, Oregon, USA,