



Summary of the PhD thesis



**Phosphate starvation as a trigger of stress-induced proline biosynthesis in  
*Arabidopsis thaliana***

**Dávid Aleksza**

Supervisors:

Dr. László Szabados-scientific advisor

Dr. Gábor V. Horváth-senior research associate

University of Szeged  
Faculty of Science and Informatics  
Doctoral School of Biology

Biological Research Center of the Hungarian Academy of Science  
Institute of Plant Biology

**Szeged  
2018**

## Introduction

Plants as sessile organisms had to develop different protective mechanisms to cope with different environmental stresses. These mechanisms including the alteration of the plants architecture, changes in gene expression and metabolic pathways both serves to avoid or reduce the effects of damages. One of the most common stress induced metabolit in plants is the proline, it accumulates in high amount in case of low water potential caused by salt salinity and/or drought. Several studies showed its accumulation in case of either heavy metal and pathogen stress. Information on the effect of nutrients and particular nutrient starvation on proline metabolism is, however, scarce. Various protective functions attributed to proline, it can act as osmoprotectant, can stabilize cellular structures and enzymes and scavenging reactive oxygen species (ROS), and plays role in the maintenance of the redox equilibrium in adverse conditions. Proline participates in the regulation of plant development either, especially in flowering, pollen embryo and leaf development. The level of free proline depends on the balance of the synthesis and degradation. The synthesis of proline can happen by the glutamate and the ornithine derived pathways, but the former is the most relevant and the importance of the latter is being questioned. The key and rate limiting component of the glutamate pathway is the pyrroline 5 carboxylate synthase enzyme which reduces glutamate to reach glutamate-semialdehyde. In *Arabidopsis thaliana* the P5CS enzyme exists in two isoforms, the so called stress-inducible P5CS1 and the P5CS2 with “housekeeping” functions. The *P5CS1* induction is depend on light and responds to ROS signals, hyperosmotic stress and can be regulated by abscisic acid (ABA)-dependent and independent signals. While *P5CS2* can be activated by incompatible plant-pathogen interactions associated with hypersensitive response. Beside ABA, P5CS1 can be induced by several transcription factors since the promoter sequence of P5CS1 contains motifs that can be binding sites for basic Leu zipper (bZIP), myeloblastosis (MYB), c-Myc (MYC), APETALA2/Ethylene-responsive element binding protein (AP2/EREBP), C2H2 Zn finger protein (C2H2\_Zn) type transcription factors. Phosphorus is an essential constituent of biomolecules, such as phospholipids, nucleic acids, and ATP, and is important for reversible protein modification. Soluble phosphate is limited in many soils due to insoluble complex formation with different metals or by

microbial consumption converting an inorganic phosphate into an organic one, which is not available to plants. Phosphate deficiency affects 70% of cultivated land and seriously reduces crop yields, turning phosphate fertilization one of the essential elements of modern agriculture. Phosphate deficiency generates a complex stress in plants, reduces shoot growth and root elongation, but enhances formation of lateral roots and root hairs, which facilitates phosphate acquisition. Regulation of phosphate homeostasis requires complex signaling network coordinating uptake, transport, and metabolism of this essential nutrient. Genome-wide transcript profiling allowed the identification of large sets of phosphate regulated genes and defined the most important regulons responding to phosphate deprivation in shoots and roots. The MYB-type PHOSPHATE STARVATION RESPONSE1 (PHR1) and PHR-LIKE1 (PHL1) factors are the most important transcriptional regulators, which control the expression of target genes and define metabolic and developmental responses to phosphate deficiency. PHR1 was shown to be essential for adaptation to light stress and to maintain photosynthesis during Pi starvation

### Aim of the work

- 1, By yeast one-hybrid assay we tried to find some potential, yet unknown interactor partners of the *P5CS1* gene which can have an effect on the gene expression.
- 2, From the scope of the newly found interactor we tried to analyze the changes in the growth and metabolism of the wild type Arabidopsis
- 3, We tried to reveal the underlying molecular mechanisms and interactions
- 4, We tested the interaction on mutant lines
- 5, With a newly characterised interactor we tried to extend our knowledge of stress induced proline metabolism.

## Materials and methods

### Plant material:

- the Columbia-0 (wild type) served as control
- proline metabolism mutant lines: *p5cs1-1*, *pdh1-4*, *pdh2-2*
- phosphate starvation transcription factor mutant lines: *phr1*, *phl1*, *phr1phl1*
- abscisic acid biosynthesis and signaling mutant lines: *aba2-3*, *abi4-1*, *abi5-1*

### Methods:

- DNA and RNA isolation
- end-point and real time PCR
- yeast and bacterial cloning
- protein purification
- Y1H assay
- Electrophoretic Mobility Shift Assay
- chromatine immunoprecipitation
- polyacrylamide gel electrophoresis
- western blot
- proline, chlorophyll content, hydrogen-peroxide and lipid peroxidation measurements
- fluorescent microscopy

## Results and Discussion

In the yeast one-hybrid assay, besides a few interesting candidates we got colonies containing the sequence of PHR1 and PHL1 –the two main regulators of phosphate starvation response from two independent libraries. The existence of the interaction between the P5CS1 and PHR1 and PHL1 transcription factors had to be proven with independent assays. In Electrophoretic mobility shift assay with bacterially expressed PHR1 and PHL1 proteins we proved that *in vitro* the protein can bind to a binding site (P1BS) containing *P5CS1* fragment in a concentration dependent manner. We proved the interaction *in planta* with Chromatine Immunoprecipitation using PHR1:HA

containing mutant Arabidopsis line. These two results clearly proved, that the two important transcription factor of phosphate starvation interact with the known stress induced *P5CS1* and may have influence on its expression. By the following experiments we followed the effects of phosphate starvation stress on the proline biosynthesis. We germinated wild type Arabidopsis thaliana plants on normal, 2,5 mM phosphate containing media, than transferred to normal (Pi+), to phosphate lacking (Pi-) and 10mM phosphate containing media. After 4 days the free proline level started to elevate in the plants growing on Pi- plates, and after 14 days it proved to be 4 times higher than the others. The prolin level showed no difference between the plants growing on 2,5 or 10 mM phosphate containing media. In gene expression level, the *P5CS1* showed 3-5 fold induction, and the *PDH2* showed 4-6 fold induction in case of phosphate starved samples compared to the controls. The expression levels of the other proline metabolism genes (*P5CS2*, *P5CR*, *P5CDH*, *PDH1*) were not or just slightly changed. We could study the temporal and spatial changes of the P5CS1 protein in P5CS1-GFP tagged plants. Plants growing in Pi+ media expressed P5CS1-GFP only in the root tip, and the signal was very weak. In case on Pi- grown plants the strengthening of the signal could be seen upon phosphate starvation. As the inhibition of the main root growth was more visible, most of the signal came from the tips of the newly formed lateral roots, and from the root branchings. The typical symptoms of nutrition deficiency on the root system we observed in the wild type plants first: the growth of the main root was inhibited, and increased lateral root formation could be seen too. In shoot level we could observed significant decrease of the rosette size and anthocianin accumulation in the starved plants compared to the controls. As a secondary stress the phosphate starvation caused the production of reactive oxygen species and it could be detected through the elevated H<sub>2</sub>O<sub>2</sub> level, and lipid peroxidation. For further experiments we could obtain *phr1*, *phl1* and *phr1phl1* mutants from the Paz-Ares lab (Madrid). At first we compared the proline accumulation of these mutants with the col0 plants, and found, that in Pi+ media they produce almost equal amount free proline but in case on Pi- media the increase of the proline level was about the half of the wild type. It showed that proline accumulation is inhibited when the main regulators of phosphate starvation response are lacking. Salt and ABA treatment could enhance the prolin level in *phr1*,

*phl1* and *col0* lines in equal amount, but the increase was significantly lower in the *phrphl1* mutant. Our results suggest that PHR1 and PHL1 transcription factors are important for proline accumulation both in case of phosphate starvation and in smaller compass in case of salt stress, and ABA treatment. We tested the expression changes of proline metabolism genes in phosphate starvation mutants and found that following the phosphate starvation the induction of the *P5CS1* was only slight in *phr1* and *phrphl1* plants and it was reduced in *phl1*. The transcript level of *PDH2* in these mutants were similar to wild type in the leaves both of the mutants but were reduced in the root. The expression of other proline metabolism genes wasn't changed. While *PHR1* and *PHL1* genes are not induced in phosphate-starved plants, the encoded transcription factors are necessary for the activation of *P5CS1*, *PDH2* and other genes to avoid the starvation caused damages. If we take a look to the rosette size, we found that on Pi+ media the Columbia and the phosphate starvation mutants grew the same sized rosettes. On Pi- media the rosettes of the wild type plants reduced by nearly 50% but the *phr1* and *phrphl1* lines were significantly smaller. Rosette growth of *aba2-3* mutant was, however, much less reduced by phosphate starvation than *col0*, *phr1* and *phl1* lines, as it was only 10% smaller in standard medium. The phosphate starvation stimulates ROS accumulation, oxidative damage and cell death and these appears as bleaching and necrosis of the leaves. During the starvation the Columbia plants however were smaller, did not showed any sign of bleaching while in case of *phrphl1* and *aba2-3* lines most of the leaves started to bleach. These results suggests that ABA plays role in the restriction of rosette growth in phosphate limited environment. It is well known, that ABA can play role signaling processes in salt and osmotic stress. To examine whether proline accumulation is regulated by ABA-dependent signals during phosphate starvation, proline content and transcript levels of proline metabolic genes were tested in several ABA mutants. The *aba2-3* line, in which ABA biosynthesis is blocked and in *abi4-1* and *abi5-1* mutants, in which key ABA signaling pathways are deficient. In wild-type and in *abi5-1* mutant, the phosphate starvation increased the proline content 3 to 4 times, it was only slightly enhanced in *aba2-3* and in *abi4-1* lines. When compared to wild-type plants, Pi starvation dependent activation of *P5CS1* gene was reduced by half in *aba2-3* and in *abi4-1* mutants, while it was less affected in

shoots and more repressed in roots of *abi5-1*. Expression of *PDH2* was not really concerned in shoots of these mutants, however in roots of *abi4-1* and *abi5-1* it was higher and lower than wild type, respectively. The key gene of ABA biosynthesis is the drought-induced *nine-cis-epoxycarotenoid dioxygenase 3 (NCED3)*, which encodes the rate-limiting 9-cis-epoxycarotenoid dioxygenase enzyme. During phosphate starvation the expression of *NCED3* was induced, suggesting that ABA biosynthesis is enhanced in such conditions. Our results suggest that proline accumulation and *P5CS1* induction during phosphate starvation is at least in part controlled by ABA dependent signals. The role of proline metabolism in phosphate starvation was subsequently tested by monitoring growth of the *p5cs1-1*, *pdh1-4*, and *pdh2-2* mutants in phosphate containing and lacking media. Proline accumulation in the *p5cs1-1* mutant was completely annuled in phosphate-depleted conditions, suggesting that the *P5CS1* gene encodes the rate-limiting enzyme of proline biosynthesis under such conditions too. The concentration of free proline in the *pdh1-4* mutant was similar to the wild-type plants, while it was 30% higher in the *pdh2-2* mutant under phosphate starvation. Shoot growth of these mutants was similar to wild type in both standard and phosphate limiting conditions. Root growth of the mutants was similar to wild-type plants on  $Pi^+$  medium, while in  $Pi^-$ , *p5cs1-1* mutant roots were slightly but significantly shorter than Columbia. In the *p5cs1-1* line the transcriptional response of other proline metabolic genes to phosphate starvation was similar to the wild type, while in *pdh1-4* and *pdh2-2* mutants, transcript levels of *PDH2* and *PDH1* genes were diminished, respectively. Exogenously supplied proline (1 mM and 10 mM) decreased rosette and root growth of wild type and *phr1phl1* double mutants on  $Pi^+$  medium. In the absence of phosphate, size of *phr1phl1* plants was smaller than the wild-type and was not affected significantly by proline. These results suggest that enhanced proline biosynthesis is necessary to maintain root elongation during phosphate starvation but has no effect on rosette growth, while growth defects cannot be alleviated by externally supplied proline.

## Summary

Our studies revealed that free proline content is increased in Arabidopsis plants during phosphate starvation. Both PHR1 and PHL1 transcription factors were identified in our Y1H screen as an interactor of *P5CS1*, and the sequence element (P1BS) responsible for their binding was identified in the first intron. Sequence-specific binding of both PHR1 and PHL1 proteins to this motif could be demonstrated by EMSA, and in vivo binding of PHR1 was confirmed by ChIP assays. Proline accumulation in phosphate starved plants is driven by enhanced expression of *P5CS1*, encoding the key enzyme in the proline biosynthetic pathway. Besides *P5CS1*, one of the proline catabolic genes, *PDH2*, was induced by phosphate starvation. Elevated *P5CS1* and *PDH2* expression suggests that enhanced proline turnover might take place in phosphate-starved plants. Our results suggests that abscisic acid dependent signals activate the proline biosynthetic pathway not only during dehydration but also during phosphate insufficiency.

## Acknowledgements

This work was supported by OTKA-NN110962, OTKA-K7623, OTKA-K109719, GINOP 2.3.2-15-2016-00001, and NTP-EFÖ-P-15-0157 grants.

Publications related to the doctoral process:

**Aleksza D**, Horváth GV, Sándor G, Szabados L. Proline accumulation is regulated by transcription factors associated with phosphate starvation. *Plant Physiol.* 2017 Sep;175(1):555-567. doi: 10.1104/pp.17.00791. Epub 2017 Aug 1. IF:6,456

Olah Z, Bush AI, **Aleksza D**, Galik B, Ivitz E, Macsai L, Janka Z, Karman Z, Kalman J, Datki Z. Novel in vivo experimental viability assays with high sensitivity and throughput using a bdelloid rotifer. *Ecotoxicol Environ Saf.* 2017 Oct;144:115-122. doi: 10.1016/j.ecoenv.2017.06.005. Epub 2017 Jun 9. IF:3,743

**MTMT identifier: 10060588**

Further publications:

Conference presentation:

Aleksza D, Kovács H, Szabados L, Horváth GV: Regulation of protective proline synthesis in *Arabidopsis thaliana*, *Advances in plant breeding & biotechnology techniques* 27–29 April, 2014 Mosonmagyaróvár, Hungary

Posters:

Nagy, Z., **Aleksza, D.** and Pécsváradi, A.: Aluminium activates the chloroplastic glutamine synthetase in wheat. ISIRR 2010. 11th International Symposium Interdisciplinary Regional Research. Szeged, Hungary. 13-15. October 2010. SB-21, p.: 9.

**Aleksza D.**, Turóczy Z, Szabados L and Horváth GV: Enzyme deactivation by reactive carbonyls: in vitro protection by proline 2012. MTA SZBK Straub-napok

**Aleksza D.**, Kovács H, Szabados L and Horváth GV: Identification of AtP5CS1 promoter binding transcription factors by yeast one-hybrid system (2015.) MTA-SZBK Straub-days

**Aleksza D**, Kovács H, Szabados L and Horváth GV. Connection of phosphate starvation response regulation and proline metabolism in *Arabidopsis thaliana*. Signalling in plant development. 20 – 24 September 2015. Brno

**Aleksza D**, Kovács H, Horváth GV and Szabados L. Connection of phosphate starvation response regulation and proline metabolism in *Arabidopsis thaliana* FESPB Congress 2016. June 26-30 Prague