

Ph.D. Thesis

**Investigation of plant glutathione peroxidase  
enzymes in *Arabidopsis thaliana***

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## **Introduction**

Due to the effects of climate changes, stress related research has a major focus in the field of plant physiology. Increasing number of extreme weather events cause severe losses in agricultural production. Plants are constantly exposed to the effects of the environmental factors, extreme changes some of these factors may be detrimental to them, cause stress. Due to environmental stresses, oxidative stress is also appear as a secondary stress. During this, the accumulation of reactive oxygen species (ROS) can easily overturn the reduction state of the plants, therefore damages the membranes, proteins and nucleic acids, thus impairing plants' growth and development. To avoid this, plants are protected by the complete antioxidant defence system.

Plant glutathione peroxidase-like enzymes (GPXL) are members of the antioxidant enzymes, and are able to reduce  $H_2O_2$  and organic hydroperoxides to water or the corresponding alcohols by using thioredoxin (TRX) (or glutathione (GSH)) as reducing substrates. GPXLs are similar to animal phospholipid hydroperoxide glutathione peroxidases (PHGPX), but they contain a cysteine in their catalytic site instead of selenocysteines. Because of the chemical properties of cysteine, the activity of these enzymes is lower compared to selenocysteine-containing enzymes. This raises the possibility that they may be involved in processes other than enzymatic antioxidant functions. In *Arabidopsis thaliana* 8 isoenzymes were identified in different subcellular particles, they are localized in the cytoplasm,

nucleus, plasma membrane, mitochondria, chloroplasts and Golgi. *Arabidopsis* is a commonly used model plant of physiological research, since its genetic background is known and many mutants are available. In our experiments *Atgpxl* T-DNA insertional mutant plants were used to investigate the role of AtGPXL proteins in stress responses. We compared the sensitivity of wild-type (Col-0) and mutant plants to abiotic stress through H<sub>2</sub>O<sub>2</sub> and malondialdehyde (MDA) contents, and we investigated some antioxidant enzyme activities and levels of non-enzymatic antioxidants in the plants. The effect of the lack of one AtGPXL on glutathione redox potential (*E<sub>GSH</sub>*) was studied using a redox sensor protein (roGFP2), expressed in selected *Atgpxl* mutant plants. Experiments of the biochemical properties of GPXL proteins were performed with purified recombinant enzymes.

## **Aims**

Glutathione peroxidases have been investigated in animals for nearly 60 years, but many issues remain unanswered in plants, as there are many differences between the two groups. *Arabidopsis glutathione peroxidase-like (AtGPXL)* genes can be activated by abiotic stress factors or hormones. Following the *in silico* examination of their promoter regions, several cis-regulating elements can be found that are associated with abiotic or biotic stress responses or linked to hormone signalling. Nevertheless, the function of GPXLs in stress responses, signalling processes, or regulation of development is not

yet fully understood. In our work, we aimed the investigation of the role of 8 GPXLs in Arabidopsis.

In the dissertation we were looking for the answers to the following questions:

- 1) Which reducing agents and substrates are preferred by each AtGPXL enzymes?
- 2) Which physiological parameters change in the roots and shoots of Arabidopsis plants in the absence of one AtGPXL under control conditions?
- 3) What is the role of AtGPXLs in stress responses? What changes can be observed in the physiological processes of wild-type and *Atgpxl* mutant plants under abiotic stress?
- 4) How does the level of 8 *AtGPXL* transcripts change in the shoots and roots of the plants during osmotic stress?
- 5) Can AtGPXLs have role in plant growth and development? How can they affect the redox state of plants? Can they participate in signalling processes?

## **Materials and methods**

*Arabidopsis thaliana* (L. Heynh, Columbia ecotype; Col-0) and glutathione peroxidase-like T DNA insertion mutant plants (*Atgpxl1-8*) were used for part of the experiments. To determine the redox potential of the roots of *Atgpxl2* and *Atgpxl3* mutants, the plants were crossed with Col-0 plants, expressed redox sensitive green

fluorescence protein (roGFP2). Plants were exposed to abiotic stress under different conditions (grown hydroponically, on medium, in soil) at different ages (5-,14-day-old seedlings, 6-,10-week-old plants). Abiotic stress was induced with different concentrations of NaCl, mannitol, polyethylene glycol and H<sub>2</sub>O<sub>2</sub>, moreover low and high temperature stress as well as drought stress were applied.

We determined rosette diameter, root length and the mass of the plants. Viability and the level of reactive oxygen species were detected by fluorescent microscopy. For gene expression assays RNA was isolated by LiCl-method, and after cDNA writing, quantitative RT-PCR was performed, using primers designed by Primer3 program. The H<sub>2</sub>O<sub>2</sub> and malondialdehyde contents, the antioxidant enzyme activities (thioredoxin peroxidase, glutathione peroxidase, glutathione transferase, guaiacol peroxidase, ascorbate peroxidase, catalase) and the amount of non-enzymatic antioxidants were determined spectrophotometrically. The half-cell redox potential was calculated from the specified glutathione contents. Glutathione dependent redox potential was calculated using roGFP2 protein, which fluorescent signal was detected on laser scanning confocal microscope.

For the study of biochemical properties of GPXL enzymes, recombinant proteins were produced in *Escherichia coli* BL21/Origami cells, and were purified on His-Trap column after extraction. Activity measurements and oxidation of purified roGFP2 were detected on plate reader.

Correlation analyses were performed using R program, for statistical analyses and evaluation of the results the SigmaPlot11.0 software was used.

## Summary

In our experiments recombinant enzymes were used to study the biochemical properties of AtGPXL enzymes. We investigated the degradation of various peroxides in the presence of a few selected reductive substrates. In addition, we tested the signalling ability of AtGPXL proteins using recombinant roGFP2. For the assessment of the role of AtGPXL proteins in stress responses, *Atgpxl* insertion mutant plants were used. We compared the abiotic stress response of wild-type (Col-0) and mutant plants grown in different conditions. Furthermore, the roGFP2 redox sensor has been expressed in selected *Atgpxl* mutant plants to study the effect of the lack of one AtGPXL isoenzyme on the regulation of glutathione redox potential ( $E_{GSH}$ ). Based on our results, we have made the following observations:

1. All the studied recombinant AtGPXL2, AtGPXL3 and AtGPXL8 enzymes are capable to convert  $H_2O_2$  and organic hydroperoxides *in vitro*. All these selected recombinant enzymes favour the TRX reducing substrate against GSH, and also distinguish between TRXs, in the presence of TRXh2 and TRXh3 the measured peroxidase activities were much higher than with TRXh9. Determination of the enzyme kinetic

parameters ( $K_M$ ,  $v_{max}$ ) of AtGPXL8 resulted in similar values to that already published.

2. In control conditions, H<sub>2</sub>O<sub>2</sub> or MDA contents of mutant plants were often higher than that of the wild type. In the hydroponically grown *Atgpxl5*, -6, -7, and -8 plants increased H<sub>2</sub>O<sub>2</sub>, in the *Atgpxl2* mutant elevated MDA contents were detected. The activity of the antioxidant system changed, mostly in the shoots of mutants the TPOX activity decreased and the APX increased, while in the roots the level of non-enzymatic antioxidants was higher. It has been supposed, that the plants this way tried to compensate the lack of AtGPXLs.
3. Investigating the effects of abiotic stresses on *Atgpxl* mutants in different ages and various growth conditions came out that some mutant plants were more sensitive compared to the wild type. The content of H<sub>2</sub>O<sub>2</sub> significantly increased in *Atgpxl2* mutants after osmotic stress, while in the shoots of *Atgpxl3* seedlings salt and osmotic stresses caused elevated total ROS content. The hydroponically grown mutant plants are tend to tolerate osmotic stress by increasing the amount of non-enzymatic antioxidants. Our correlation analysis also highlights the different stress response of shoots or roots.
4. AtGPXL gene expressions were changed by salt and osmotic stresses, differently due to various treatments and in organs. 100 mM NaCl treatment caused the induction of *AtGPXL6* and -8 in the shoots, and *AtGPXL1*, -3 and -8 in the roots. By

contrast, after the iso-osmotic PEG treatment, *AtGPXL4* and -8 in the shoots and *AtGPXL1* and -4 in the roots were activated. These results suggest that the given AtGPXLs may play an important role in the osmotic stress response.

5. In our experiments, the  $E_{hc}$  values, calculated from the glutathione contents, in the mutant plants have often been changed, which refers to their role in regulation of the redox state. By other approaches, the  $E_{GSH}$  values, determined by using roGFP2 sensor protein, were also different in the roots of the *Atgpxl2* and *Atgpxl3* mutants compared to the wild-type, usually the root cap and meristematic zones of the mutant roots were more oxidized. The result confirms their function in regulating the redox state, through which they can also influence on growth and development. In addition, testing the interaction of recombinant AtGPXLs with roGFP2 revealed, that they were capable of transmitting the oxidation signal *in vitro*, so might also participate in signalling processes *in vivo* through interactions with other proteins.

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## List of publications

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### The 2 mandatory publications for the doctoral procedure

**Bela K**, Riyazuddin R, Horváth E, Hurton Á, Gallé Á, Takács Z, Zsigmond L, Szabados L, Tari I, Csiszár J (2018) Comprehensive analysis of antioxidant mechanisms in *Arabidopsis* glutathione peroxidase-like mutants under salt- and osmotic stress reveals organ-specific significance of the AtGPXL's activities. *Environmental and Experimental Botany*, 150: 127-140. **IF: 4.369 (2017)**

Horváth E, Brunner Sz, **Bela K**, Papdi Cs, Szabados L, Tari I, Csiszár J (2015) Exogenous salicylic acid-triggered changes in the glutathione transferases and peroxidases are key factors in the successful salt stress acclimation of *Arabidopsis thaliana*. *Functional Plant Biology*, 42(12): 1129-1140. **IF: 2.491**

### Peer-reviewed publications

**Bela K**, Riyazuddin R, Horváth E, Hurton Á, Gallé Á, Takács Z, Zsigmond L, Szabados L, Tari I, Csiszár J (2018) Comprehensive analysis of antioxidant mechanisms in *Arabidopsis* glutathione peroxidase-like mutants under salt- and osmotic stress reveals organ-specific significance of the AtGPXL's activities. *Environmental and Experimental Botany*, 150: 127-140. **IF: 4.369 (2017)**

Poór P, Takács Z, **Bela K**, Czékus Z, Szalai G, Tari I (2017) Prolonged dark period modulates the oxidative burst and enzymatic antioxidant systems in the leaves of salicylic acid-treated tomato. *Journal of Plant Physiology*, 213: 216-226. **IF: 3.121**

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**IF: 2.756**

**Cumulative IF: 22.486**

## *Other scientific publications*

### *Book chapters*

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- Bela K**, Horváth E, Kovács H, Brunner Sz, Csiszár J (2014) Investigation of the role of *Arabidopsis thaliana* glutathione peroxidases in drought, chilling and heat stress responses using insertion mutants. Tavasz Sél Konferencia 21-23. March 2014, Debrecen, Hungary.
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- Horváth E, Gallé Á, **Bela K**, Hurton Á, Holinka B, Riyazuddin, Csiszár J (2017) Can the mutation of *AtGSTU24* gene modify salt stress response? A Magyar Növénybiológiai Társaság XII. Kongresszusa. 30. August – 1. September 2017, Szeged, Hungary.
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- using redox sensitive GFP2. FIBOK 2016, 21-22. March 2016, Gödöllő, Hungary.
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- Takács Z, Poór P, **Bela K**, Szepesi Á, Tari I (2015) Comparison of exogenous salicylic acid-induced polyamine catabolism in the presence of light or in darkness in tomato leaf. 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field, 24-26. June 2015, Palazzo della Gran Guardia, Verona, Italy.
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- Horváth E, Brunner Sz, **Bela K**, Csenki D, Papdi Cs, Szabados L, Tari I, Csiszár J (2013) The influence of salicylic acid pre-treatments on the salt stress response of *Arabidopsis thaliana*. Oxidative stress and cell death in plants: Mechanisms and Implications; 26-28 June 2013, Florence, Italy.

## Conflict of interest

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

**Bela K**, Riyazuddin R, Horváth E, Hurton Á, Gallé Á, Takács Z, Zsigmond L, Szabados L, Tari I, Csiszár J (2018) Comprehensive analysis of antioxidant mechanisms in *Arabidopsis* glutathione peroxidase-like mutants under salt- and osmotic stress reveals organ-specific significance of the AtGPXL's activities. *Environmental and Experimental Botany*, 150: 127-140.

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