Final report (01.11.2016 – 31.01.2020.)

Project number, title: PD 121027, Redox homeostasis under hormonal- and stress treatments

1. Introduction

Continuous root growth and development which are essential for the survival during stress are sustained by the root apical meristem. Plant hormones control the root growth and development by balancing between cell division and differentiation and their interactions are crucial for the temporal and spatial coordination of root development (Lee et al. 2012). The function of the plant hormones and several transcription factors in formations of the root meristem have been investigated earlier, but the regulatory roles of reactive oxygen species (ROS) and antioxidants came to the front only in the recent years. In order to keep ROS levels tightly regulated, different non-enzymatic antioxidants and enzymatic systems have evolved in aerobic organisms (Munns and Tester 2008, Zhang et al. 2012). Non-enzymatic scavengers of ROS include ascorbic acid (AsA), glutathione (GSH), phenolic compounds, carotenoids, flavonoids and tocopherol. Enzymatic ROS scavengers include superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase-like, dehydroascrobate reductase (DHAR) and glutathione transferase (GST) enzymes (Bela et al. 2018).

Glutathione transferases belong to a very ancient protein superfamily that is thought to have evolved in response to the development of oxidative stress. Plant genomes contain dozens of *GST* genes and most of the proteins can be found in homodimer or heterodimer form, leading to enormous diversity within GST protein families (Csiszár et al. 2019). Plant GSTs are grouped into ten classes and among them, the most abundant tau (GSTU), phi (GSTF), DHAR and lambda isoenzymes are plant-specific. Due to their GSH conjugating activity, they play a crucial role in detoxification processes and through glutathione peroxidase or DHAR activities, they influence the redox state of GSH and AsA. The GSTU and GSTF classes of glutathione transferase enzymes may reduce the GSH pool using GSH as a co-substrate, thus influence numerous redox-dependent processes including hormonal and stress responses (Marrs et al. 1996, Cummins et al. 2011).

In its classic function as an antioxidant, GSH serves to remove ROS and hence limit the lifetime of the oxidative signal (Diaz-Vivancos et al. 2015). GSH can regulate the expression of genes through modulation of redox state of proteins and transcription factors, but this mechanism is poorly understood yet (Dietz 2014). Redox homeostasis is a fundamental cell property, in which regulation includes the control of ROS generation, sensing and readjustment of the cellular redox state. Thiol redox biochemistry is considered to have fundamental role in cellular processes, thus maintenance of a high GSH level and GSH/GSSG ratio are important to proper function of the cells and organs. Maintenance of a highly negative redox potential for glutathione (around -310 mV) is achieved through continuous reduction of glutathione disulfide by glutathione reductase (GR) (Aller et al. 2013). It is widely accepted that the ratio of reduced and oxidized glutathione (GSH:GSSG) is an effective marker of cellular redox homeostasis (Noctor et al. 2012, Rahantaniaina et al. 2013). From the concentration of GSH and GSSG, the glutathione half-cell reduction potential

($E_{GSSG/2GSH}$) can be calculated (Schafer and Buettner 2001). An alternative option for imaging of thiol redox potential is a redox sensitive green fluorescent protein (roGFP2) targeted to cytoplasm in *Arabidopsis thaliana* plants which allows us to monitor the redox state of selected parts using microscope (Meyer et al. 2007, Schwarzländer et al. 2008). In this project we applied the roGFP transgenic technique to detect the redox status of the plant *in vivo*. Our main aim was to compare the redox state and ROS levels in different zones of roots under control conditions and after applying stress- or hormonal treatments.

2. Responses of *Arabidopsis thaliana* mutants to stress treatments

2.1 Functional characterization of selected AtGST isoenzymes using salt stress treatments

2.1.1 The AtGSTF8 and AtGSTU19 enzymes are involved in the maintenance of the redox homeostasis of roots under control conditions and after NaCl treatment

We performed detailed analysis of the redox potential and ROS levels in longitudinal zones of 7-day-old roots of *Arabidopsis thaliana* L. Col-0 wild type and glutathione transferase (*Atsgtf8* and *Atgstu19*) T-DNA insertional mutants. Using redox-sensitive cytosolic green florescent protein (GRX1-roGFP2) the redox status of the meristematic, transition, and elongation zones (PM, TZ and EZ) was determined under control and salt stress (3-hour of 75 or 150 mM NaCl treatment) conditions (Horváth et al. 2019).

Using florescent dyes significantly higher superoxide radical ($O_2^{\bullet-}$) levels was detected in both *Atgst* mutants than in the Col-0 control. Salt treatment resulted in the highest $O_2^{\bullet-}$ increase in *Atgstf8* roots, while the amount of H_2O_2 elevated the most in the case of *Atgstu19*. Moreover, vitality decreased in *Atgstu19* roots more than in wild type under salt stress.

According to our results, the redox status of untreated *Atgstu19* roots was more oxidized, than that of the Col-0 or *Atgstf8*, and the size of the mutant's meristem proved to be shorter compared to the wild type. The redox potential showed the biggest differences in the proximal meristem of the roots. Treatment with 75 or 150 mM NaCl for 3 h resulted in more oxidized redox state generally in all studied zones of the roots of the investigated genotypes, but the highest redox potential values (most oxidized redox status) were detected in the transition zone of the *Atgstu19* mutant. In general, one can state that the relative redox potential changes in the mutant roots were lower than in the wild type likely due to their already more positive value under control conditions. Their increased salt sensitivity can be associated with this decreased redox potential response. It is supported by the observation that the highest sensitivity towards 75 mM NaCl was shown by the *Atgstu19* mutant exhibiting no redox potential change in response to this salt concentration.

Our data did not reveal direct correlation between the E_{GSH} values and the level of investigated ROS or between the E_{GSH} and cell vitality. However, in conclusion we can state that both proteins, AtGSTF8 and especially the AtGSTU19 might represent ROS-processing enzymes fine-tuning and maintaining redox homeostasis under plant development especially under stress conditions (Horváth et al. 2019).

2.1.2 Two-day long NaCl treatment altered the redox state and the gene expression of tau group AtGSTs in Arabidopsis seedlings

To reveal the particular role of tau group GSTs (AtGSTU19 and AtGSTU24) in salt stress responses, we investigated the effect of 150 mM NaCl on two-week-old *Arabidopsis thaliana* wild type (Col-0), *Atgstu19* and *Atgstu24* T-DNA insertion mutant plants. While the two isoenzymes show high amino acid identity and our earlier results indicated that both of them play positive role in salt stress responses (Horváth et al. 2015), the changes in total extractable GST activity in the two mutants showed different tendencies. *Atgstu19* seedlings had lower GST activity and vitality both under control conditions and after salt stress than the wild type, but the level of total ROS were similar to the Col-0 plants. The GST activity of the knockout *Atgstu24* mutant was even higher under control conditions compared to the Col-0 plants, while the ROS level and its vitality did not differ significantly from the wild type.

Analysis of the *AtGSTU* expression pattern revealed that the mutation in a single *AtGSTU* gene was accompanied by the up- and downregulation of several other *AtGSTUs*. Under control conditions, *AtGSTU1, AtGSTU5, AtGSTU21* and *AtGSTU22* were upregulated (2-6 times higher gene expressions than in Col-0) in both *Atgstu* mutants. Additionally, *AtGSTU9, AtGSTU10, AtGSTU23* and *AtGSTU27* were induced in the *Atgstu19* mutant, while in *Atgstu24, AtGSTU17* showed elevated expression compared to the wild type. However, some genes such as *AtGSTU2* and *AtGSTU3* in both mutants, *AtGSTU4* and *AtGSTU11* in *Atgstu19* and *AtGSTU14* in *Atgstu24* showed repression.

Salt stress induced the expression of *AtGSTU3-6, AtGSTU9, AtGSTU11* and *AtGSTU12* in all investigated lines after two days and *AtGSTU1* and *AtGSTU2* in Col-0 and *Atgstu19* plants. In *Atgstu19* mutants, *AtGSTU13* and *AtGSTU17* showed additional induction, furthermore the expression of the *AtGSTU5* gene was significantly higher than in Col-0 plants (Fig. 2). Along with Col-0 plants' repression of the *AtGSTU14* gene in *Atgstu19* and of the *AtGSTU28* gene in *Atgstu24*, *AtGSTU21* had lower expression only in *Atgstu24* mutants.

Besides the altered *AtGSTUs* expression pattern, elevated AsA and GSH levels, an altered GSH redox potential and increased DHAR and GR activities may contribute to compensation for the mutation of *Atgstu* genes under control conditions and in the case of salt stress. Moreover, maintained or even increased GSH/GSSG ratios could support the preservation of the redox potential of plant cells during salt stress in mutants, but it may also influence their response. On the one hand, these results indicate that highly similar GST isoenzymes have a unique role and regulation in plants. On the other hand, altered GSH and AsA levels as well as the *GSTU* gene expression pattern in *Atgstu* mutants suggest that the investigated isoenzymes influence redox homeostasis under control conditions and after salt treatment in *Arabidopsis* seedlings (Horváth et al. 2020).

2.2 Mannitol and methyl viologen treatments resulted ichanges in root tips of Atgr, Atgst and Atdhar mutants

We performed further experiments using mannitol and methyl viologen treatments for 1 hour and from our results we can highlight:

One hour of 300 mM mannitol treatment significantly elevated the redox potential in roots of Col-0 plants. The biggest changes in the redox status of wild type plants were detected in the EZ. The vitality decreased in the investigated zones by 50-70%. After mannitol treatment the highest differences in the E_{GSH} values were in the PM of *Atgstf8*, *Atgstu19* and *Atdhar2* mutants compared to Col-0, while *Atgr1* had more oxidized E_{GSH} in the EZ than the wild type. The $O_2^{\bullet-}$ level was elevated in the PM of all genotypes after applying the mannitol but in *dehydroascorbate*- and *glutathione reductase* mutants it was even higher than in Col-0 plants. The resorufin fluorescence indicated that the H_2O_2 was increased due to mannitol treatment especially in the TZ and EZ regions; the highest levels were detected in the roots of *Atgstf8*, *Atdhar1* and *Atdhar2*.

Interestingly one hour long, 1 μM methyl viologen treatment elevated only slightly the E_{GSH} values, $O_2^{\bullet-}$ and H_2O_2 levels of Col-0 plants. However vitality of root tips decreased by 20-50%. Mutant plants had similar changes in all investigated parameters, but in some cases the $O_2^{\bullet-}$ and/or H_2O_2 levels were even lower compared to the wild type.

3. Responses of *Arabidopsis thaliana* plants to hormonal and H_2O_2 treatments

3.1 Effect of abscisic acid and H_2O_2 treatments on ROS levels and redox states

One-hour-long application of abscisic acid (0.3, 1 and 3 μM ABA) increased the $O_2^{\bullet-}$ levels in all investigated regions of the root tip. For redox state detection of roGFP2-containing wild type *Arabidopsis* roots we applied 3 μM ABA treatments for 1 and 3 hours. We found that the changes in the redox potential were region specific in the root tips. 3 μM ABA increased the redox potential, which means a more oxidized state, in the PM and TZ of the root tips after 3-hour-long treatment.

In case of 3 hours of 1, 2.5 and 5 mM H_2O_2 treatments we detected elevated $O_2^{\bullet-}$ levels. At the same time 2.5 and 5 mM H_2O_2 treatments induced cell death in the root rips according to propidium iodide staining. 1 mM H_2O_2 treatment increased the E_{GSH} value after 1 h and further elevation in redox potential was detected after 3 h of treatment.

3.2 Role of the Arabidopsis thaliana glutathione transferases in response to salicylic acid treatment

The role of AtGSTF8 and AtGSTU19 in salicylic acid (SA) response using T-DNA mutants was studied in more detail. (In this case our preliminary results suggested the role of AtGSTF8 and AtGSTU19 in salicylic acid induced changes.) SA has not only a pro-oxidant, but also an antioxidant role in concert with GSH in the stress response (Herrera-Vásquez et al. 2015). Furthermore accumulating molecular genetic evidence suggests that GSH is also important in potentiating ROS signals in plants, particularly through interactions with plant stress hormones such as SA and jasmonic acid (Diaz-Vivancos et al. 2015).

We investigated the effect of 10 and 100 μM SA treatment after 1, 3 and 24 hours on seven-day-old *Arabidopsis thaliana* wild type (Col-0), *Atgstf8* and *Atgstu19* mutant plants. Under control conditions similar vitality was measured in all genotypes, although *Atgstu19* mutants had slightly lower FDA dependent fluorescence (by 10-30%) than Col-0. SA

treatment reduced the vitality of root tips in a concentration dependent manner. In Col-0 100 μM SA treatment decreased the vitality already after one hour of treatment, while the effect of 10 μM SA could be measured only after 24 hours, mostly in TZ and EZ. *Atgst* mutants had lower vitality after 3 and 24 hours of 100 μM SA treatment than the Col-0 and the 10 μM SA decreased the vitality already after 3 hours of treatment especially in EZ of mutant plants. A recovery of vitality could be observed after 24 hours of SA treatment in PM and TZ of all genotypes, but in EZ further decrease was detected.

The redox potential in SA treated Col-0 plants became more positive on a time dependent manner; after one hour mostly in the EZ-, after three hours in PM and EZ while following 24 hours of SA treatments in all investigated zones of the roots. The redox potential was more positive (more oxidized redox state) in mutants than in Col-0, but different changes could be observed than in wild type. In *Atgstf8* mutant the redox potential of glutathione decreased after one and 24 hours compared to its own control, while in *Atgstu19* the redox potential became more negative after three hours in TZ and EZ, and after 24 hours in PM and TZ. We did not find direct correlation between the vitality and redox potential of the investigated lines, although in the *Atgstu19* mutant, which had the highest redox potential under control condition, lower vitality was detected than in Col-0 or *Atgstf8* plants. The redox potentials of the *Atgstu19* mutant's PM and all zones of *Atgstf8* roots became more negative after 24 h of SA treatments compared to untreated ones, indicating the induction of response mechanisms in mutants.

Time- and concentration dependent changes were measured in H_2O_2 levels after SA treatment. One and 24 h of SA treatment elevated the H_2O_2 levels in Col-0, but 3 h of treatment increased the H_2O_2 level only in 10 μM SA treated plants. The H_2O_2 levels of mutants was higher after one hour of SA treatment, however after 3 h only in *Atgstf8* mutant treated with 100 μM SA could be measured higher H_2O_2 levels than in wild type.

We chose one hour of 10 μM and 100 μM SA treatments to investigate the expression patterns of the *AtGST* and other, stress inducible genes in *Atgstf8* and *Atgstu19* mutants by high throughput quantitative RT-PCR (HT-qPCR) since pronounced changes could be detected in the vitality and H_2O_2 levels at this time point (Fig. 1). From 22 investigated genes, 11 showed induction after SA treatment. Beside 5 *AtGSTs* (*AtGSTF8*, *AtGSTU7*, *AtGSTU19*, *AtGSTU24* and *AtGSTU25*), 3 oxidative stress marker (*AtOXI1*, *AtANAC032* and *AtAKR4C9*), 2 stress-associated (*AtGPXL6* and *AtWRKY46*) and 1 SA responsive (*AtWRKY38*) gene sequences were induced in all investigated genotypes after one hour of 100 μM SA treatment. The lower, 10 μM SA concentration induced the expression of the investigated WRKY transcription factors (*AtWRKY38* and At *WRKY46*) and that of the *AtGSTU7* gene. However, the expression of *AtGSTU7* elevated only in Col-0 and *Atgstf8*, while *AtGSTF8* in *Atgstu19* (Fig. 1).

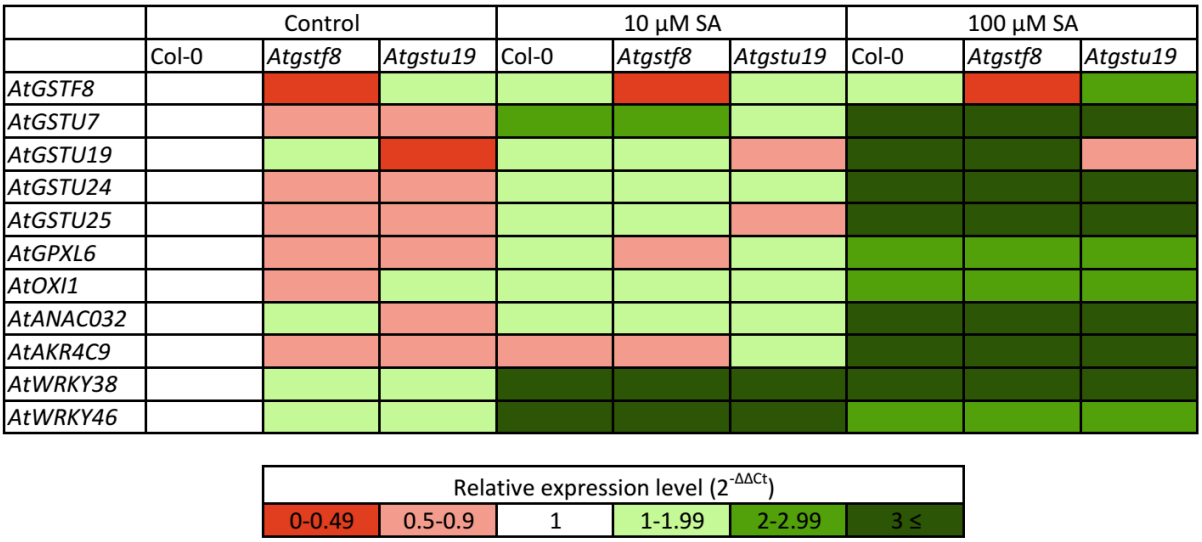

Figure 1. Heat map of 11 *A. thaliana* genes expression levels which showed induction after SA treatment. Relative transcript amounts of selected genes were determined by HT-qPCR under control conditions (Control) and 10 and 100 µM salicylic acid (SA) treatment in wild-type (Col-0) and *Atgstu* mutant (*Atgstf8* and *Atgstu19*) *Arabidopsis* plants. For this, the expression of investigated genes was normalized first by reference to the average transcript amount of Glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPDH2)* and MONENSIN SENSITIVITY1, a SAND family protein (*MON1*) genes, and second to the average transcript amount of each gene in wild-type control plants. The presented data are the average of two biological replicates. $2^{-\Delta\Delta Ct}$ data were presented as a heat map. Red colours show repression, while green colours show activation, according to the colour scale bar.

Our results suggest a complex regulation of SA response in the investigated genotypes. Mutation of *Atgstf8* and *Atgstu19* affected the redox status, vitality and H_2O_2 production of root tips after SA treatment, although in a different manner. The observed induction of gene expressions indicates that mutation of *Atgstf8* or *Atgstu19* affected only the expression of other *AtGSTs* after one hour SA treatment. These results verify overlapping and specific roles of AtGSTU19 and especially of AtGSTF8 isoenzymes in the fine tuning of the redox homeostasis in SA response of root tips. (Publication under preparation)

4. Conclusions

In this project we detected the redox status of *Arabidopsis* plants using the roGFP transgenic technique and by measuring GSH/GSSG and AsA/DHA levels. We compared the redox state and ROS levels in different zones of roots under control conditions and after applying several treatments. Summarizing our results we can highlight:

Mutation of *AtGSTF8* induced changes in the redox state and ROS levels under various treatments and especially after SA treatment the mutants had lower vitality than the wild type, indicating the role of AtGSTF8 in SA response.

Our results regarding *Atgstu19* and *Atgstu24* mutants, underline that highly similar GST isoenzymes have a unique role and regulation in plants. Mutation of AtGSTU genes with

similar function acted differently on the total extractable GST activity. GST activity elevated in the *Atgstu24* and decreased in the knockdown *Atgstu19* mutant where it did not increase after salt treatment, despite of the increased expression of *AtGSTU* genes. As far as we know, these results provide the first evidence that AtGSTU19 is irreplaceable in seedlings. Furthermore, the *Atgstu19* mutant had more oxidized redox status in all root zones throughout the experiments. Salt- or SA treatments resulted in elevated amounts of H_2O_2 levels and lower vitality in the case of *Atgstu19* compared to the wild type.

The investigated mutations (*Atgstf8*, *Atgstu19* and *Atgstu24*) triggered different responses in many aspects in *Arabidopsis* seedlings, but, according to the results we may assume that the redox-coupled changes are common elements of the mechanisms of GSTs. Altered redox state, GSH and AsA levels as well as the modified *AtGST* gene expression pattern in *Atgst* mutants suggest that the investigated AtGST isoenzymes influence redox homeostasis under control conditions and in response to various treatments in *Arabidopsis* seedlings.

5. Dissemination of the results:

Connecting to this project, five scientific papers were published and one is under preparation, and our results were presented in Hungarian and international conferences (2 lectures and 5 posters). Two BSc and one MSc student participated in the project during their research works.

Publications:

Horváth E, Bela K, Gallé, Á, Riyazuddin, R, Csomor, G, Csenki, D, Csiszár, J (2020) Compensation of mutation in *Arabidopsis glutathione transferase* (*AtGSTU*) genes under control or salt stress conditions. Int J Mol Sci 21: 2349.

Horváth E, Bela K, Holinka B, Riyazuddin R, Gallé Á, Hajnal Á, Hurton Á, Fehér A, Csiszár J (2019) The Arabidopsis glutathione transferases, AtGSTF8 and AtGSTU19 are involved in the maintenance of root redox homeostasis affecting meristem size and salt stress sensitivity. Plant Sci 283: 366-374.

Riyazuddin R, Bela K, Horváth E, Rigó G, Gallé Á, Szabados L, Fehér A, Csiszár J (2019) Overexpression of the *Arabidopsis* glutathione peroxidase-like 5 gene (*AtGPXL5*) resulted in altered plant development and redox status. Environ Exp Bot 167: 103849.

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Lectures:

Bela K, Riyazuddin R, Horváth E, Hajnal Á, Gallé Á, Bangash SAK, Csiszár J (2019) Sóstressz hatása lúdfű glutation-peroxidáz-szerű enzim mutáns gyökerek redox potenciáljára. Magyar Szabadgyök-Kutató Társaság X. Kongresszusa, 2019.08.29.-2019.08.30, Szeged, Magyarország. In: Poór P, Blázovics A (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók, p. 13.

Horváth E, Bela K, Kulman K, Gallé Á, Riyazuddin R, Csiszár J (2019) Szalicilsav-indukálta redox állapot változások *Arabidopsis Atgstf8* mutáns gyökerében. Magyar Szabadgyök-Kutató Társaság X. Kongresszusa, 2019.08.29.-2019.08.30, Szeged, Magyarország. In: Poór P, Blázovics A (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók, p. 14.

Posters:

Riyazuddin R, Bela K, Horváth E, Rigó G, Szabados L, Gallé Á, Fehér A, Csiszár J (2019) Overexpression of membrane localised *Arabidopsis* glutathione peroxidase (*AtGPXL5*) resulted in altered plant development and protection against salt stress. In: Pareek A, Gupta Kapuganti J, Foyer CH, Singla-Pareek SL (Eds) Sensing and signalling in plant stress response - Programme and Abstract Book, p. 49 Paper: P15.

Riyazuddin R, Bela K, Horváth E, Rigó G, Szabados L, Gallé Á, Fehér A, Csiszár J. (2019) Az Arabidopsis glutation-peroxidáz-szerű (*AtGPXL5*) gén túltermeltetése javítja a növények fejlődését és sóstressz elleni védekezését a redox potenciál javításával. Magyar Szabadgyök-Kutató Társaság X. Kongresszusa, Szeged, 2019.08.29.-2019.08.30, Szeged, Magyarország. In: Poór P, Blázovics A (szerk.) Magyar Szabadgyök-Kutató Társaság X. Kongresszusa: Program és összefoglalók, p. 51.

Horváth E, Bela K, Holinka B, Csiszár J (2018) Salt stress induced changes of reactive oxygen species and redox state in the roots of *Atgstu19* and *Atgstf8* mutants. In: Maciej T Grzesiak (Ed.) 11th International Conference on Plant Functioning Under Environmental Stress. Cracow, Poland, 2018.09.12-2018.09.15. p.120. (ISBN:978-83-86878-37-6)

Bela K, Riyazuddin R, Horváth E, Gallé Á, Hurton Á, Bangash SAK, Ayaydin F, Csiszár J (2018) Alteration of redox potential in the roots of *Arabidopsis glutathione peroxidase-like* mutants under salt stress. In: Maciej T Grzesiak (Ed.) 11th International Conference on Plant Functioning Under Environmental Stress. Cracow, Poland, 2018.09.12-2018.09.15. p. 58. (ISBN:978-83-86878-37-6)

Horváth E, Gallé Á, Bela K, Hurton Á, Holinka B, Riyazuddin, Csiszár J (2017) Can the mutation of *AtGSTU24* gene modify salt stress response? In: Györgyey János (ed.). XII. Congress of Hungarian Society for Plant Biology. Szeged, Hungary, 30/08/2017-01/09/2017. p. 49. (ISBN: 978-963-12-9736-2)

Defended dissertations connected to this project:

Holinka Botond (Biológus MSc, 2018) *Arabidopsis gstf8* és *gstu19 glutation transzferáz* inszerciós mutánsok sóstressz válaszának fluoreszcens mikroszkópos vizsgálata.

Fazekas Cintia (Biomérnöki BSc, 2020) *Arabidopsis glutation transzferáz* mutánsok (*Atgstf8* és *Atgstu19*) genotipizálása és stresszválaszuk vizsgálata.

Kulman Kitti (Biológia BSc, 2019) *Arabidopsis gstf8* és *gstu19* glutation transzferáz inszerciós mutánsok redox állapotának változása szalicilsav kezelés hatására.

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