Final report on research work of OTKA K 105956 proposal entitled Redox homeostasis under salt and osmotic stress

1. Introduction

Redox reactions are fundamental metabolic processes through which cells convert and distribute the energy that is necessary for maintenance the growth. Cellular redox homeostasis is an essential buffering mechanism that prevents excessive reduction or oxidation. Reactive oxygen and nitrogen species (ROS, RNS), ROS-producing enzymes, antioxidants and their oxidation/reduction states all contribute to the redox homeostasis of plants. There is a close interplay among the individual redox active molecules, and the status of each of them can influence the plant metabolism and environmental responses. Key players of the redox processes are the ascorbate (ASC) and glutathione (GSH) which are central components of the highly complex plant antioxidative system (Foyer and Noctor 2011). Their common feature that (i) they are abundant and are present in plants in mmolar (0.5-10 mM) concentrations; (ii) specific enzymes couple them to peroxide metabolism; (iii) their oxidized forms are relatively stable; and (iv) recycling of these forms is to the reduced compounds by high-capacity enzyme-based systems that depend on the key electron carriers, NAD(P)H (Noctor and Foyer 1998a). It was suggested that NAD(P)H serves best in the organisation and the control over energy production pathways, ascorbate is the redox molecule which primarily regulates development and glutathione is involved in plant development, but is mainly important for stress defence and signaling (Potters et al. 2010). Redox regulation is thought to be a general and sensitive mechanism to perceive even small changes in environmental conditions, and orchestrate adequate, graded responses (De Tullio 2010). Changes in the concentration and in the reduction potential of the glutathione (reduced form: GSH, oxidized form: glutathione disulfide, GSSG) and other redox couples make possible the fine regulation of the cellular redox environment and consequently the growth and development of the plants.

The redox potential of the glutathione couple depends on the ratio of $[GSH]^2/[GSSG]$. From the concentration of GSH and GSSG the half-cell reduction potential ($E_{GSSG/2GSH}$) can be calculated. This parameter was suggested to be a general marker of the overall intracellular "redox environment". The cytosolic glutathione concentration can be determined directly using *in situ* fluorescent methods following GST-catalyzed conjugation of GSH to monochlorobimane (MCB) to give a fluorescent glutathione-bimane (GSB) adduct. Although bimane labelling gives an indication of the total GSH concentration, it cannot report the redox state of the GSH pool (Noctor and Foyer 1998b).

An alternative option for imaging of thiol redox potential in independent compartments has emerged with the introduction of redox sensitive green fluorescent protein (roGFP) (Hanson et al. 2004, Jiang et al. 2006, Meyer et al. 2007, Schwarzländer et al. 2008, 2009). Engineering of two surface-exposed cysteines into the GFP allows reversible disulfide formation. The thiol-disulfide status of the roGFP can be equilibrated with that of glutathione in the cells. The change in GFP-derived fluorescence in transgenic plants depends on the redox potential of the glutathione buffer based on specific interaction with glutaredoxins

(GRXs) (Meyer et al. 2007). The roGFP can be targeted selectively to the cytosol, mitochondria and to the plastids of Arabidopsis, but transient expression was achived also in the cytosol, mitochondria and ER of tobacco cells (Schwarzländer et al. 2008). The structure-dependent shift in the protonation status of the chromophore is suitable for ratiometric analysis. A fluorescence image can be obtained by confocal microscope and the ratio of I_{405}/I_{488} may provide information about the relative redox status of cells. The change in GFP-derived fluorescence in transgenic plants depends on the redox potential of the glutathione buffer (E_{GSH}) based on specific interaction with glutaredoxins. Determination of the fluorescent intensity of the fully reduced and fully oxidized form of the probe enables quantitative monitoring of E_{GSH} without disturbing the cell (Meyer et al. 2007, Schwarzländer et al. 2008). Different roGFPs (roGFP1-4, roGFP-iX) have been used as ratiometric redox sensor (Dooly et al. 2004, Cannon and Remington 2005, Lohman and Remington 2008, Gutscher et al. 2008, Meyer and Dick 2010, Aller et al. 2013). In this project we focussed on the possible application of this new transgenic technique to detect the redox status of the plant *in vivo* and use it in the investigations of plant stress physiology.

2. Applying the roGFP technology

2.1. Using roGFP1 as a redox sensor

First of all we have tested roGFP1 for employing it as a biosensor in *Arabidopsis thaliana* plants to monitor changes of the redox state. Crossings were performed between roGFP1 expressing plants targeted to cytoplasm (c-roGFP1) and several mutants (*gr1, dhar1, ppr40-1*). We have checked the fluorescence of roGFP1-harboring *Arabidopsis* either by fluorescent microscope or plate reader. Because this method results in relative values, the total oxidized or reduced state of plant organs were achieved by treatment with 100 mM H_2O_2 and 200 mM DTT (dithiothreitol). We established that the confocal fluorescence microscope is more suitable for investigation of young seedlings and roots of plants, while the fluorescent plate reader method is better for the determination of total fluorescence intensity of plant leafs or leaf discs. For calculation of redox potential of plants we have applied the equations published by Schwarzländer et al. (2008). Changes in redox state were monitored after treatment of *Arabidopsis* plants by H_2O_2, salicylic acid (SA) and salt stress. These parameters were detected on 4-8 weeks old plants grown in hydroponic culture. This fluorescent redox probe was successfully used for investigation the redox homeostasis in salicylic acid (SA) induced priming in *Arabidopsis* and the role of glutathione reductase 1 (GR1) enzyme in the stabilization of the redox state during acclimatization (Table 1, Csiszár et al. submitted publication. See more details about priming later).

Table 1 E'_{roGFP1} during the SA pre-treatment. Data are means ± SD. Columns with different letters are significantly different at P < 0.05, determined by Duncan's test.

		1 week	2 weeks	3 weeks
Control	**roGFP1**	-294.503±12.12^{d}	-283.60±10.75^{c}	-270.56±12.79^{bc}
	***gr1* x roGFP1**	-296.74±9.7^{d}	-269.06±13.68^{bc}	-261.56±9.74^{b}
10^{-7} M SA	**roGFP1**	-294.89±8.45^{d}	-275.48±8.2^{c}	-263.31±5.93^{b}
	***gr1* x roGFP1**	-295.24±7.70^{d}	-270.26±9.97^{bc}	-263.33±8.09^{b}
10^{-5} M SA	**roGFP1**	-296.37±6.03^{d}	-255.904±8.2^{ab}	-269.70±6.40^{bc}
	***gr1* x roGFP1**	-289.74±8.39^{d}	-246.79±10.67^{a}	-269.01±9.45^{b}

However, the intensity of the fluorescent signal and changes were sometimes relative small in the case of roGFP1. Moreover, introduction roGFP1 targeted to mitochondria (mit-roGFP1) into *ppr40-1* mutants revealed serious problems at *in vivo* detection of the real-time redox status of the moving organelles by confocal fluorescent microscopy (unpublished results). Although we originally planned to use roGFP1 variants wih different localization to follow the redox status of the organelles, our early results governed us to try other fluorescent probes.

2.2. Employing roGFP2 as a redox sensor

According to data in literature, the roGFP2 probe matches the most commonly used excitation laser wavelengths (405 nm and 488 nm) much better than roGFP1, and thus exhibits a much larger dynamic range when used in confocal microscopy applications (Meyer and Dick, 2010). The roGFP2 has bigger differences in fluorescence between the total oxidised and reduced state, and the fluorescent signals are brighter than that of roGFP1. A new construction was developed, in which the roGFP2 is fused with glutaredoxin (GRX) to enforce the interaction between the glutathione and the redox probe (Meyer and Dick, 2010, Lukyanov et al. 2013). Beside continuing the work with roGFP1, the GRX-roGFP2 construction have been got. We checked also these plants in order to employ roGFP2 as a biosensor. It was concluded that the roGFP2 is more suitable for detecting the redox status of plant cells. Monitoring the roGFP2 fluorescence in seedlings proved to be a useful and reliable method to determine the I_{405}/I_{488} ratio and the redox potential of Arabidopsis seedlings (Figs. 1, 2). The redox homeostasis of the entire plant is influenced by salt stress, the changes in roots can be followed more easily (Figs. 1, 2).

We also have got the roGFP2-harboring *cad2-1* (*cadmium-sensitive mutant2-1*, impaired in γ-glutamyl-cysteine synthetase, γ-ECS or *GSH1* gene), which have lower GSH levels comparing to the wild type Col-0. The effect of the isoosmotic NaCl, mannitol and sorbitol treatments on redox status of 6-day-old seedling was compared (Table 2, Bela et al., unpublished results). The redox potential of selected area was calculated according to the equation published by Schwarzländer et al. (2008) and ihe more oxidized state after the treatments was detected.

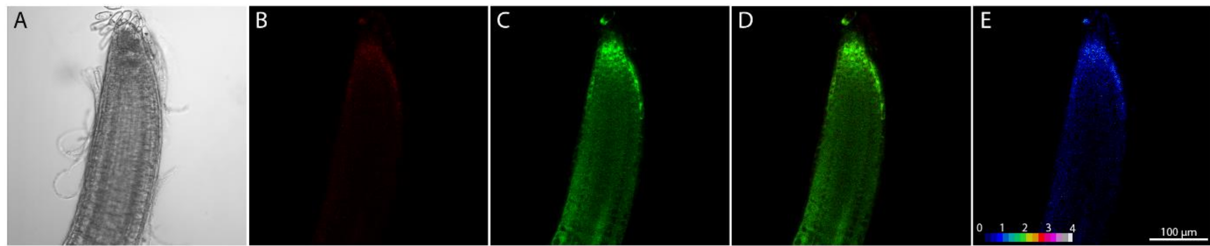


Fig 1. Ratiometric analysis of roGFP2-containing, untreated 6-day-old *Arabidopsis* root tip by confocal laser scanning microscope. A: Bright field image; B: Fluorescence intensity at 405 nm; C: Fluorescence intensity at 488 nm; D: Merge of I_{405} and I_{488}; E: Ratio of I_{405} and I_{488}.

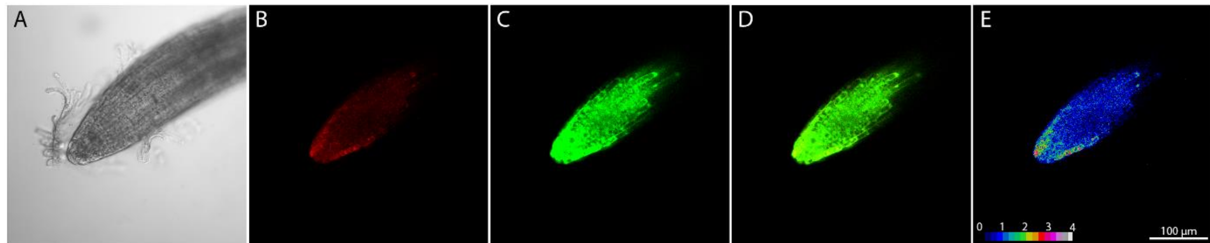


Fig 2. Ratiometric analysis of roGFP2-containing, 150 mM NaCl-treated 6-day-old Arabidopsis root tip by confocal laser scanning microscope. A: Bright field image; B: Fluorescence intensity at 405 nm; C: Fluorescence intensity at 488 nm; D: Merge of I_{405} and I_{488}; E: Ratio of I_{405} and I_{488}.

Table 2 E'_{roGFP2} (mV) of 6-day-old Arabidopsis Col-0 and *cad2-1* mutant seedlings treated with isoosmotic stresses for 2 hours.

	Cotyledons		**Roots**	
	Col-0	*cad2-1*	Col-0	*cad2-1*
Control	-311.577±14.57	-275.826±23.53	-294.522±7.28	-304.1±15.37
150 mM NaCl	-284.478±27.31	-270.871±20.54	-277.289±11.66	-254.275±9.96
300 mM mannitol	-283.571±12.27	-287.339±8.54	-294.512±16.21	-278.543±5.72
300 mM sorbitol	-282.931±20.07	-289.363±16.4	-299.419±6.31	-266.819±8.72

Several crossings were performed to introduce the *c-roGFP2-GRX1* into Arabidopsis mutants where the T-DNA insertion affected the GSH-related glutation reductase (GR), dehydroascorbate reductase (DHAR), glutathione transferase (GST) and glutathione peroxidase (GPX) genes.

3. Estimation the role of ROS and antioxidants during priming

Induction of defense mechanisms that empowers the stress tolerance may be triggered not only endogenously but also exogenously. Certain natural or synthetic compounds applied at

the range of μM concentrations prior to stress events can lead to enhanced stress tolerance and can be effectively used as priming agents. Plant priming (also known as hardening) improves tolerance of plants to adverse conditions and thus has great importance in plant stress physiology and crop stress management. Chemical priming agents can be reactive oxygen, -nitrogen and -sulfur species and their donors, melatonin, polyamins, proline or the plant hormone salicylic acid (Savvides et al. 2016). SA is a plant hormone with well-known effect on ROS-accumulation and influence on redox status of cells through regulation of ROS generating enzymes. ROS triggered changes have central role in GSH/GSSG and thiol-disulphide exchanges of non-expressor of PR protein 1 (NPR1) and in regulation the expression of pathogenesis-related (PR) proteins (Mou et al. 2003).

3.1. GSTs have a specific role in salt stress acclimation of tomato plants

Previously we have found that priming of tomato plants with SA was able to mitigate salt stress injury in a concentration dependent manner. Adding SA to hydroponic cultures of tomatoes initially can cause rather drastic changes in ROS formation and operation of antioxidant mechanisms. Pre-treatment of tomato plants with 10^{-4} M SA increased the efficiency of enzymatic and non-enzymatic antioxidant systems and provided protection against 100 mM NaCl stress in a hydroponic culture system (Szepesiet al. 2008; Szepesi et al. 2009; Gémes et al. 2011). We have shown that glutathione transferases (GSTs) are important in SA-induced acclimation to high salinity in tomato (Csiszár et al. 2014).

GSTs are multifunctional proteins induced by diverse environmental stimuli and were proposed to contribute to protection against various stress conditions that promote oxidative stress (Marrs 1996). Some members of the multigene GST enzyme family have GSH-dependent thiol transferase activity and participate in the recycling of antioxidants (ascorbate, flavonoids, quinones), while other isoenzymes, due to their S-transferase activity, are involved in the detoxification mechanisms using GSH as co-substrate. A significant portion of GST isoenzymes also has glutathione peroxidase activity and can convert lipid peroxides and other peroxides to less harmful compounds (Csiszár et al. 2016). We have created a phylogenic tree of the *Solanum lycopersicum* GST proteins and divided into eight classes based on homology to known *Arabidopsis* GSTs (Csiszár et al. 2014). The involvement of selected *SlGSTs* from the tau, lambda, phi, theta, zeta and dehydroascorbate reductase (DHAR) groups was studied in salt stress response of tomato primed SA or in un-primed plants by real-time qPCR. We concluded that GSTs are important participants in adaptation to changes in environmental signals. The altered expression levels of *SlGST*s and the increased or repressed GST enzyme activities with diverse functions may be the part of the stress response, fine-tuning of ascorbate and glutathione homeostasis and redox status (Csiszár et al. 2014).

3.2. Glutathione peroxidases and GSTs are important in the successful salt stress acclimation of Arabidopsis plants, but the stabilization of redox homeostasis may be the key element of the salicylic acid-induced priming

To estimate the development of the primed state in *Arabidopsis thaliana,* the plants were treated by 10^{-8} – 10^{-5} M SA for 2 weeks, followed by 100 mM NaCl exposure for one week. Analysis of several redox-related compounds at weekly intervals revealed that SA treatment

increased the level of ascorbate in the roots, while the amount of GSH and the activity of GR was elevated both in roots and shoots (Csiszár et al, submitted manuscript). The two-week-long pre-treatments with 10^{-6} and 10^{-5} M SA alleviated the salinity-induced H_2O_2 and the lipid peroxidation derivative malondialdehyde (MDA) accumulation and increased superoxide dismutase (SOD), guaiacol peroxidase (POD), GST, glutathione peroxidase (GPOX) and GR activities (Horváth et al. 2015b).

GR reduces GSSG to GSH and maintains it principally in reduced state. The role of GR in priming and maintaining the redox homeostasis was investigated with *gr1* insertional mutants expressing the cytoplasmic redox sensitive green fluorescent protein (c-roGFP1). The redox status of the *gr*1 mutant proved to be more oxidized comparing to wild type plants, which was more pronounced after salt stress. SA-mediated priming transitionally shifted the redox potential of the cytoplasm toward a more oxidized status. Increase in GSH levels can contribute to preserve the redox potential of SA-treated plants, in particular after salt stress, even in the *gr1* mutant plants (Csiszár et al, submitted manuscript).

Most antioxidative systems are constitutively present, but also show marked increases in activity in response to stress. Glutathione was reported to have pivotal role in oxidative stress defence, and depletion of the cytosolic GSH pool is associated with large changes in the abundance of transcripts encoding proteins that are involved in oxidative defence (Foyer and Noctor 2011). Mining of the proteome data for GSH-associated genes showed that disruption of the pathway for the synthesis and degradation of glutathione in the *Atggt1* knockout leaves was associated with the induction of genes encoding four GSTs in the phi class (GSTF2, GSTF6, GSTF9, and GSTF10), a GSH peroxidase (GPX1), and glyoxylase II (Tolin et al. 2013). The important role of the a high GSH/GSSG ratio, maintained by increased GSH synthesis and/or GSSG reduction, in the efficient protection of plants against abiotic stress-induced accumulation of ROS was indicated at several plant species (Szalai et al. 2009).

The plant-specific tau (GSTU) and phi (GSTF) classes of GSTs have important roles in protection against cytotoxic and xenobiotic compounds (Dixon et al. 2002). They are the two largest GST classes in Arabidopsis comprising of 28 and 13 members, respectively (Dixon and Edwards 2010). Both, GSTU and GSTF classes have members with high glutathione-conjugating (GST) and glutathione-dependent peroxidase (GPOX) activities (Dixon et al. 2009) and are known to be essential in alleviating oxidative damages (Roxas et al. 2000). According to our results, enhanced expression of *AtGSTU19* and *AtGSTU24* may be responsible for the induced GST and GPOX activities, which may play an important role in the acclimation (Horváth et al. 2015b).

4. Functional characterization of selected AtGST isoenzymes using salt- and osmotic stress treatments

Although the important functions of GSTs in stress tolerance is known for several decades, very little is known about the role of individual GSTs because of their possible functional redundancy. To more detailed investigations of the role of Arabidopsis GST isoenzymes in salt- and osmotic stress responses and in determination the redox homeostasis, experiments

were performed using *gstu19*, *gstu24, gstf9* and *dhar1* insertional mutants. (In these cases earlier results suggested the role of the corresponding citoplasmatic proteins in salt stress tolerance.) Several growth parameters, ROS and GSH levels, GSH/GSSG ratio and the activity of some antioxidant enzymes (including GSH-related enzymes) was determined under 25-150 mM NaCl and/or 50-300 mM mannitol-induced osmotic stresses on seedlings grown in Petri dishes.

In our earlier experiments, overexpression of *AtGSTF9* conferred dominant, estradiol-dependent salt tolerance to transgenic *Arabidopsis* (Papdi et al. 2008). Some of our results connected the role of AtGSTF9 in the salt- and salicylic acid response have got in the frame of current project was already published (Horváth et al. 2015a). *Atgstf9* mutants accumulated more ASC and GSH and had decreased glutathione peroxidase (GPOX) activity under control conditions. We found that the *Atgstf9* mutants had altered redox homeostasis under control and stress conditions, in which elevated ASC and GSH levels and modified GST and GPOX activities may play significant role. The half-cell potential values calculated from the concentration of GSH and GSSG indicate that this GST isoenzyme has an important role in the salt stress response (Table 3, Horváth et al. 2015a).

Table 3 Half-cell reduction potential of the GSH/GSSG redox couple ($E_{GSSG/2GSH}$) in *Arabidopsis* seedlings following 48 h of NaCl treatment

	$E_{GSSG/2GSH}$ (mV)	
	Col-0	*Atgstf9*
Control	-267.9	-259.5
50 mM NaCl	-239.7	-246.9
150 mM NaCl	-254.8	-265.3

Summarizing our other results we can highlight:

- While the viability, level of H_2O_2 or the lipid peroxidation marker MDA showed usually similar values in the wild type and mutant seedlings under control conditions, the of ASC and GSH levels were increased in all the investigated GST mutants. Changes in the concentration and in the reduction potential of the GSH/GSSG and other redox couples make possible the fine regulation of the cellular redox environment and consequently the growth of the plants.

- The viability of 2-week-old seedlings decreased after the 48-h 150 mM NaCl treatment, and this reduction was more pronounced in the *Atgs*t mutants. The ROS level decreased in roots and increased in cotyledons of wild type Arabidopsis seedlings due to stress treatment, however in *Atgstu24* and *Atgstf9* mutants no ROS accumulation was observed. The *Atgstu19* line had the highest H_2O_2 level and the lowest GST activity during the experiments, while the *Atgstu24* mutants had even elevated GST activities compared to the wild type plants. The lack of the AtGSTU19 gene expression decreased the GST activity more pronounced than the mutation of AtGSTU24 or AtGSTF9, indicating its central role in salt stress response. Interestingly, the GST activity of *Atgstu24* mutants and GPOX activity of *Atgstu19* plants

were even increased due to salt and especially mannitol treatments. However, according to several physiological parameters the function(s) even of the AtGSTU24 isoenzyme was not totally complemented. Expression pattern of all the AtGSTUs is evaluated to explore which other GST isoenzymes can substitute the eliminated enzymes.

- Dehydroascorbate reductases also belong to the diverse family of GSTs. Tey catalyze the reduction of DHA to ascorbate with the concomitant oxidation of reduced glutathione (GSH) to glutathione disulfide (GSSG) (Dixon et al. 2002). Consistent with their function, they have crucial role in determination of the redox state of ASC and GSH. *Arabidopsis thaliana* contains three DHAR genes, we estimated the role of the cytoplasmic DHAR1 in salt- and osmotic stress responses using *Atdhar1* mutants. According to our results, the insertion mutation of DHAR1 decreased drastically the DHAR activity and resulted in lower levels of ASC and GSH. The stress treatments resulted in less changes in the levels of ASC and DHA in *dhar1* mutants than in Col-0, but increased more the amount of GSH and in this way the total glutathione, especially in the presence of NaCl. Some antioxidant enzymes (APX and GR) exhibited a higher induction due to mannitol treatment in the mutants than in wild type plants. Despite of these compensation processes, the germination frequencies of the mutant plants were lowered significantly by the treatments, especially due to NaCl.

- The half-cell potential values calculated from the concentration of GSH and GSSG showed that the *Atgst* mutants had altered redox homeostasis both under control and stress conditions. Our results suggests that beside the elevated ASC and GSH levels the modified GST and GOPX activities may also play significant roles in its maintenance.

- Using c-roGFP2-containing plants the calculated redox potential values are more negative (Schwazländer et al. 2008, Foyer and Noctor 2011). Evaluation of the redox potential by confocal laser scanning microscope resulted in more oxidised redox state in *Atgstu19* lines than in wild type plants both under control circumstances (with 25.916 mV difference) and after applying 150 mM NaCl stress (the difference was 36.719 mV). Determination of E'_{roGFP2} in *dhar1* mutants revealed not significant effects of the short time (2 hours) 150 mM NaCl or 300 mM mannitol treatments on the redox potential of shoots or roots compared to the Col-0 plants, which can be explained with the increased GSH amounts (data are not shown).

- We concluded that the roGFP2 redox probe can be used for *in vivo* the redox status monitoring of plants with different genotype.

- The different defense mechanisms may complement each other, at least in some degree. There is a relative strong correlation not only between the ASC and GSH levels, but the induction of enzymatic systems also helps the maintenance of the redox balance. For example in the *dhar1* mutant the activity and the expression level of GR, while in *gr1* mutant those of DHAR were increased. Alteration in redox balance is a common consequence of abiotic stresses, leading to additional oxidative damage of the affected cells. Through its interactions with ROS, ascorbate, glutathione, protein thiol-disulfide groups, functions as a central hub in maintaining redox balance with the capacity to modulate other signaling pathways and regulates stress signaling and defence.

The results of these experiments was presented on several conferences (see later), but the main publications in scientific journals are still under preparation.

5. Role of the *Arabidopsis thaliana* glutathione peroxidases under stress conditions

The plant GPX family consists of multiple isoenzymes with distinct subcellular locations which exhibit different tissue-specific expression patterns and environmental stress responses (Bela et al. 2015). These proteins protect against reactive oxygen species, catalyze reduction of hydrogen peroxide (H_2O_2), organic hydroperoxides and lipid peroxides using glutathione or other reducing components, such as thioredoxin. The plant glutathione peroxidases are mostly similar to animal phospholipid hydroperoxide glutathione peroxidases (PHGPX). These PHGPXs play a very important role in protecting against oxidative damage of membranes. At present, the function of these enzymes in plants is not completely understood. The plant GPXs are classified as the fifth group of peroxiredoxins, because it was revealed that they use the thioredoxin- rather than the glutathione-system during the reduction of H_2O_2 and lipidperoxides (Iqbal et al. 2006). The occurrence of thiol-dependent activities of plant GPX isoenzymes suggests that - besides detoxification of H_2O_2 and organic hydroperoxides - they may be involved in regulation of the cellular redox homeostasis by maintaining the thiol⁄disulfide or NADPH⁄$NADP^+$ balance. In addition to the possible antioxidant functions, plant GPXs also participate in redox signaling. Miao et al. (2006) reported that *Arabidopsis* GPX3 (AtGPX3) can interact with ABI1 and ABI2 (abscisic acid insensitive) phosphatases, leading to stomatal closure via activation of cation channels. AtGPX3 can also interact in yeast two-hybrid system with the transcriptional regulator CEO1 protein, which can control several genes involved in plant stress responses (Miao et al. 2006). It has been demonstrated that the redox state of the *Arabidopsis* AtGPX3 is regulated by H_2O_2 and indicated that this GPX is a redox transducer in abscisic acid (ABA) and drought stress signaling. GPXs were suggested to function even as ROS- or redox sensors (Milla et al. 2003), but this effect has been not yet confirmed. However, putative signaling functions were assigned to AtGPX8, as it was supposed that this isoenzyme may take part in the redox modification of nuclear proteins (Gaber et al. 2012).

The *Arabidopsis thaliana* contains 8 GPX isoenzymes, however their role in plant development and stress responses and their exact mechanisms are not well-known. Our aim was to characterize the Arabidopsis GPX enzymes. The substrate specificity and other enzymatic properties were investigated using recombinant proteins, produced and purified from *E. coli*. Other experiments were performed on T-DNA insertion mutants (SALK_128885C; SALK_082445C; SALK_071176C; SAIL_623_F09; SALK_076628C; WiscDsLox321H10; SALK_072007C; SALK_127691C) in different growing and treatment conditions. We determined the *in vitro* malondialdehyde and H_2O_2 contents, the non-enzymatic antioxidant ascorbate and glutathione contents and activity of antioxidant enzymes: glutathione reductase, glutathione transferase, glutathione peroxidase, thioredoxin peroxidase (TPOX), ascorbate peroxidase (APX), guaiacol peroxidase (POX), catalase (CAT), superoxide dismutase (SOD). The growth and germination rate of the different T-DNA insertion mutants during osmotic stress were also detected.

Our results revealed that the affinity of GPX isoenzymes to the lipid peroxides and H_2O_2 differ and it depends on the specific thioredoxin cosubstrates, as well. The drought or osmotic stress experiments conducted on *Atgpx1-8* mutants demonstrated, that the AtGPX3 and 5 have

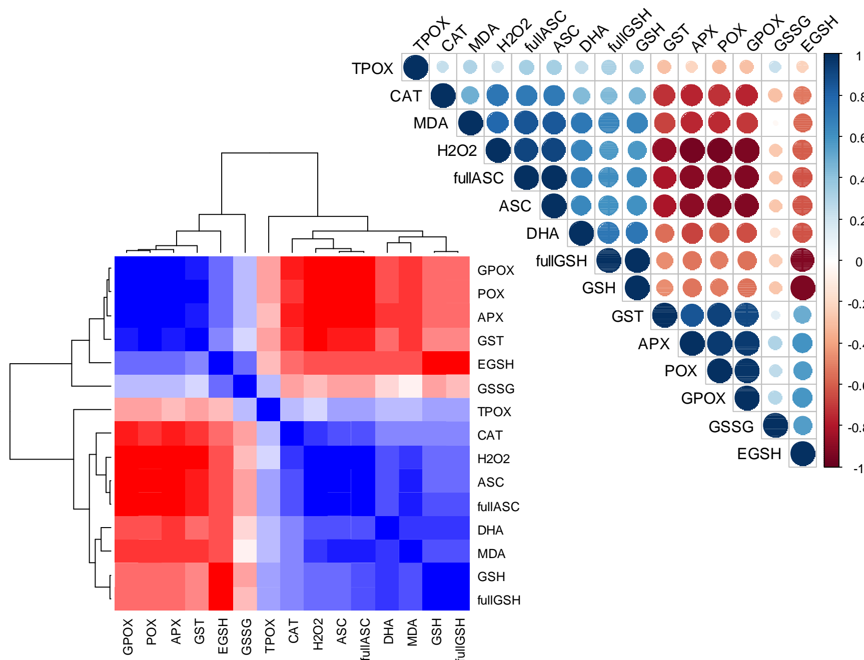
important roles in the development, while the AtGPX2, 4, 6 and 8 especially in the stress responses (Bela et al. unpublished results). In the mutant plants not only the activity of enzymatic antioxidants (GPOX, TPOX, GST) changed, but the level and reduction state of non-enzymatic antioxidants, such as GSH and ASC, too.

Characterization of AtGPX proteins verified their roles in salt- and osmotic stress tolerance. The isoosmotic mannitol, polyethylene glycol (PEG) and NaCl concentrations caused different changes in the antioxidant mechanisms, this may correspond to the specificity of the treatments. Other experiments were conducted to investigate the growth of mutants under salt- and osmotic stress conditions.

Changes in expression pattern of *AtGPXs* after polyethylene glycol (PEG)-triggered osmotic stress - measured by quantitative Real-Time PCR (RT-qPCR)- revealed enhancement particularly in the transcript amount of *AtGPX2, -3* and *-5* genes. More detailed investigations were performed after applying 50-200 mOsm PEG on hydroponically grown 6-week-old wild type and *Atgpx2, Atgpx3* mutant plants. 200 mOsm PEG had severe effects on *Atgpx2* plants (they accumulated the highest amount of H_2O_2 and MDA), but the decrease in their redox potential was the less, because of significant increasing of the reduced and total ASC and GSH levels. Moreover, expression of other *AtGPX* genes (especially *AtGPX4* and *AtGPX8*) was induced, indicating some redundancy of GPXs. Our results showed that the lack of AtGPX2 or -3 affected the redox status, which might influence the long-term growth of plants.

To estimate the redox state of *Atgpxs* under control and stress circumstances, the cytoplasmic roGFP2 was introduced into all *Atgpx* mutants by crossings and also by *Agrobacterium*-mediated transformation. Evaluation of the E'_{roGFP2} in the *Atgpx2-8* mutants revealed that redox potential became oxidized in the highest extent in the shoots of *Atgpx4*, *-6* and *-8* seedlings (by ca. 46 - 62 mV), while in their roots were less changes (ca. 20 - 30 mV, data are not shown). Our experiments performed on *Atgpx* seedlings verified that there are relative small changes in the redox potential of whole plants under different osmotic and salt stress conditions. Meanwhile the activation of antioxidant mechanism could be observed. Among the enzymatic antioxidants the elevation of ascorbate peroxidase (APX), guaiacol peroxidise (POD), GR, GST, DHAR activities was detected e.g. after applying NaCl, polyethylene glycol (PEG), mannitol and SA treatments, indicating their role in stress responses and in maintenance of cell homeostasis. Correlation analysis were performed on data of physiological parameters measured separately in the shoots and roots of the hydroponically grown control and stress treated plants. Figure 3 shows results of a correlation analysis performed on summarized physiological parameters from NaCl and PEG treated sample data. Moreover, detailed analysis were performed on the gene expression levels of all AtGPXs genes and several transcription factors involved in stress responses. The publication of these data is in progress.

NaCl-treated Col-0 and *Atgpx1-8* plants



PEG-treated Col-0 and *AtGPX1-8* plants

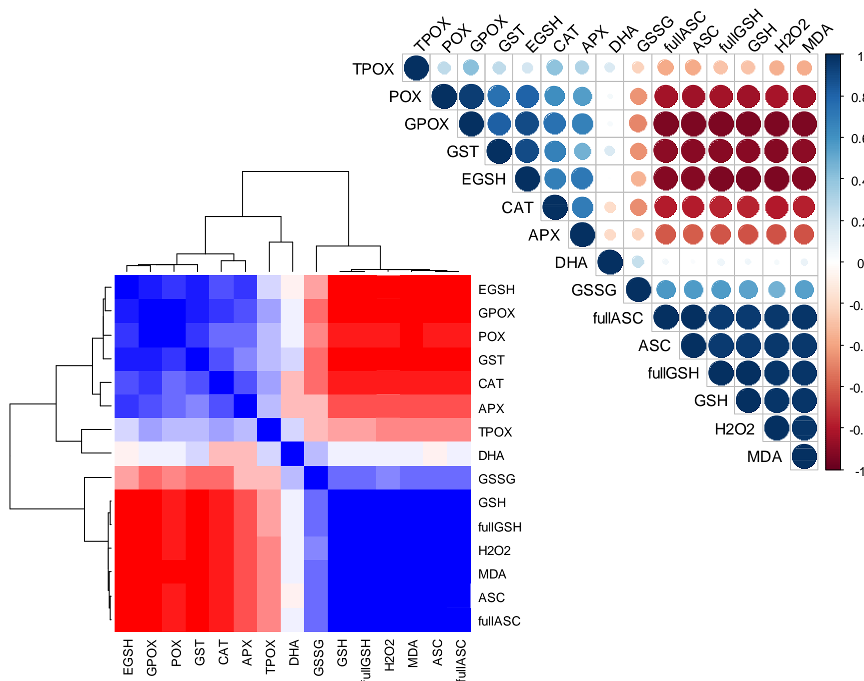


Fig. 3. Comparison of the effect of 100 mM NaCl and 200 mOsm PEG on 5-week-old hydroponically grown Arabidopsis Col-0 and *Atgpx1-8* mutants. The intensity of the blue color symbols the rate of positive correlations, red colors are for negative correlations. Data of individual genotypes were summarized. TPOX: thioredoxin peroxidase activity, POX: guaiacol peroxidase activity, GPOX: glutathione peroxidase activity, GST: glutathione transferase activity, E_{GSH}: GSH reduction potential, CAT: catalase activity, APX: ascorbate peroxidase activity, DHA: dehydroascorbate level, GSSG: odidized glutathione level, Full ASC: total ascorbate level, ASC: reduced ascorbic acid, full GSH: total glutathione level, GSH: reduced glutathione level, MDA: malondialdehyde level.

6. Investigation of the role of PPR40 protein in tomato stress responses

The positive role of a pentatricopeptide domain containing protein, *At*PPR40 in abiotic stress responses has been proven earlier using *Arabidopsis thaliana* insertion mutants and overexpressed lines. The *At*PPR40 protein localised in mitochondria is able to reduce the generation of reactive oxigen species by stabilising the mitochondrial electrontransport (III. complex) during stress treatments and has a protective role against oxidative damages. The salt tolerance of overexpressed lines was observed in *Arabidopsis thaliana* (Zsigmond et. al. 2008, 2012). Determination of the amounts of reduced and oxidised glutathione was successfully applied for Calculation of the half cell redox potential of plants using the formula of Schafer and Buettner (2001) on our earlier data (Zsigmond et al. 2008) resulted in -224.005 – -224.07 mV half cell redox potential in the mitochondria of *Arabidopsis thaliana* Col-0 wild type plants, while it decreased to -260.39 and -278.94 mV in plant's or suspension cultures mitochondria, respectively containing defected mitochondrial AtPPR40-1 protein (pentatricopeptid repeat protein40-1). Our aim was to introduce the *AtPPR40-1* gene into tomato using *Agrobacterium*-mediated transformation to investigate its effect on stress tolerance of plants.

For establishment of tomato regeneration system, five *Solanum lycopersicum* genotypes were introduced in the first year in *in vitro* tissue cultures.Two cultivars (cv. Moneymaker and Rio Fuego) were chosen for the *Agrobacterium tumefaciens*–mediated transformation experiments. Successful hypocotyl transformation and plant regeneration was reached by using the Moneymaker cultivar (Fig. 4, Hurton et al., unpublished).

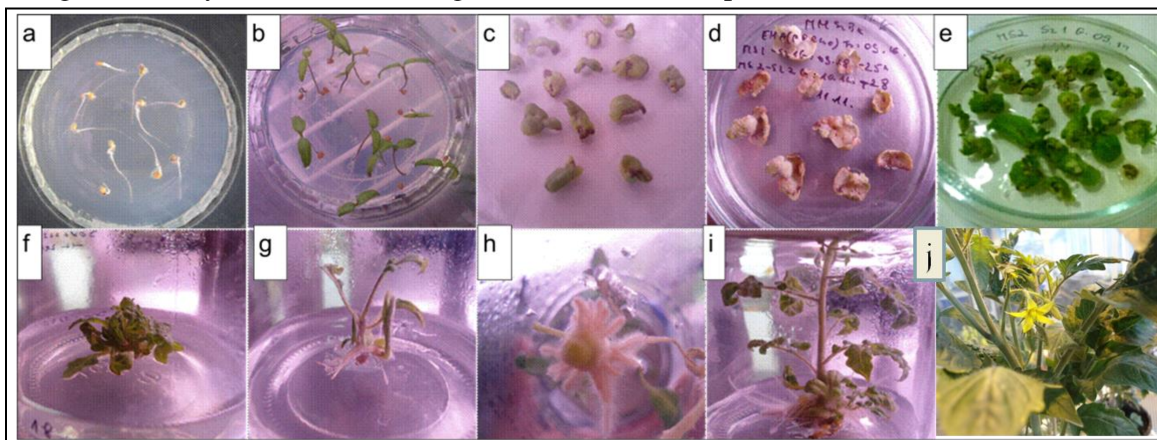


Fig. 4. Steps of tomato plant regeneration system: a: germination of seeds; b: ten days old tomato seedlings with hypocotyls; c: hypocotyls after transformation; d: hypocotyls with calli; e:shoot initiatives; f: shoot planted into glass flask; g,h: rooting stage; i: regenerated plant; j: flowering plant in the greenhouse

To increase the efficiency of genetic transformation we applied plant tissue culture methods and also different *Agrobacterium* lines. The used vector constructions harboring PPR-40 protein coding sequence was the pROK2-PPR40-HA binary vector, the *Agrobacterium* strains were the EHA105(pEHA105) and the GV3101:pMP90 (C58C1, rif, pMP90 (pTiC58ΔT-DNS), Gmr) (Hood et al. 1993, Koncz and Schell, 1986). Even though ca. 30 supposedly transformant plantlets were regenerated, the presence of the *AtPPR40-1* gene was verified

only in 2 tomato plants. The expressed PPR40 protein was identified using Western blot (Fig. 5).

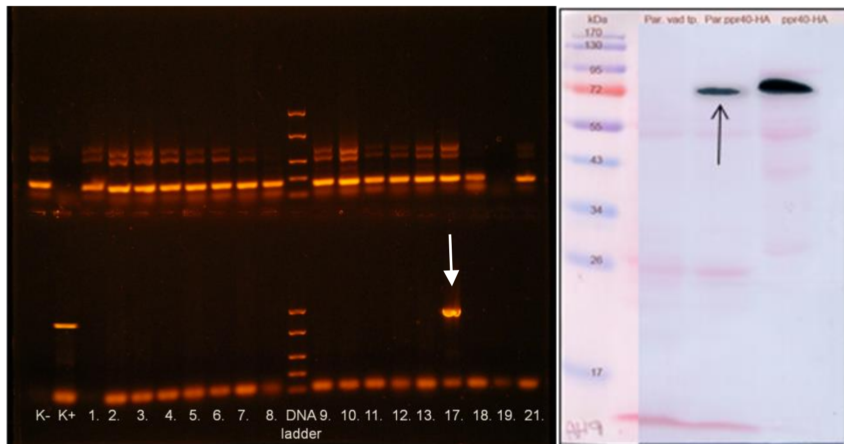


Fig. 5. Identified expressed PPR40 via PCR and Western blot

Several regenerated plants of these transgenic lines were grown in greenhouse to collect seeds. However, analysis of totally ca. 130 plantlets of the line labelled T1 revealed that the transgene is not present in the next generation; the investigation of the filial plants of the other line is still in progress.

The salt- and osmotic stress responses of transgenic tomato lines containing AtPPR40 were estimated in several experiments. Germinating seeds and growing the seedlings on sterile MS media in the presence of 100 mM NaCl for three weeks resulted in no significant differences between their germination or growth compared to the wild type. Leaf discs assays were performed on discs with 8 mm diameter excised either from healthy sterile or greenhouse-grown leaves. Measuring the changes of the photosynthetic pigment contents and some photosythetic parameters (Fv/Fm, Yield) on MS media supplemented with 0-300 mM NaCl or 0-600 mM mannitol after 48 hours revealed no significant differences between the transformant and control lines (unpublished results).

7. Introduction of GRX1-roGFP2 into tomato plants

Important part of the project was the adaption of roGFP technique in tomato to monitor changes of the redox state. Cotyledon transformation was performed on cv. Moneymaker plants using GV3101::pMP90 Agrobacterium strain containing vector constructions with roGFP2-GRX1 sequences with cytoplasmic and mitochondrial localization (c-roGFP2-GRX1 and mit-roGFP2-GRX1, respectively). The presence of transgene was verified either by PCR and fluorescent stereo microscope in 2 lines in the case of cytoplasmic roGFP2 and in 6 lines at mitochondrial roGFP2 (Fig. 6). However, in confocal microscopy applications their fluorescent signals proved to be rather week compared to controls. We want to get homozygous transgenic lines for further appliciations, so several plants from each lines are growing now in the greenhouse to collect seeds, but further transformation experiments were also started.

In planta transformation of tomato plants using floral dip method (Yasmeen et al. 2009) was conducted on cv. Ailsa Craig to introduce c-roGFP2 into tomato plants. The transformation efficiency of this method exhibited high variability depending on the used gene construction (Yasmeen et al. 2009). We have successfully applied this method and had some fruits with transformed seeds. The plantlets were checked for the presence of c-roGFP2 and 4 transgenic lines were identified. Testing of the F2 generation (to get homozygous lines and to use for redox potential determination by confocal laser scanning microscope) is still under process, but according to the preliminary results these plants will be suitable for *in vivo* redox state detection.

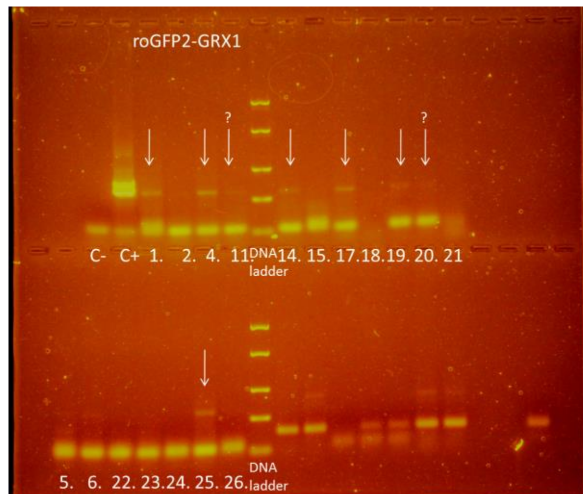


Figure 6. Identification of roGFP2-GRX1 gene in tomato regenerants.

Connecting to the financial costs of the project, the miscellaneous costs (including rental fees of the confocal laser scanning microscope, growth chambers, greenhouse) were significantly less than we planned earlier. We have asked for permission to spent more money for consumables.

There were some changes in the staff of the project compared to our plan:

i) In the original research proposal Edit Horváth was planned to be employed as a junior researcher on the cost of the project for 4 years. In the first year she participated in our researches as a PhD student, and Andrea Megyeriné Pető was employed between 01. 10. 2012. - 31. 08. 2013. (Permitted on 05th of September, 2012).

ii) The technician participated in the project in the first year was Mária Ádámné Meszlényi. However, she has left our University, so Erzsébet Porkoláb took over her tasks. The planned personnel costs were payed for her in the last two years.

iii) Szilvia Brunner PhD student participated in the project as a researcher (0,5 FTE/year), employed not on the project's budget. She left our university in August of 2014, but Krisztina Bela (PhD student) conducted her research work connecting to this project and was took into instead of Szilvia Brunner.

Dissemination of the results

Ten scientific papers were published connected to the project, and another one publication is submitted with results obtained in the project. Our results were presented also in international conferences in the form of 9 lectures and 9 posters. Several BSc, MSc and PhD students participated in the project during their research works. However, most of our main results concerning the application of the roGFP technology and involvement of the particular GSH-related enzymes in maintenance of the redox homeostasis are under publication. Beside one submitted paper, manuscripts reporting the role of AtGPXs in the salt- and osmotic stress responses and in the redox regulation, the characterization of the AtGSTU19, AtGSTU24, DHAR1 isoenzymes and their functions under salt and osmotic stresses are under preparation. Because their publication will take more time I would like to ask to postpone the final evaluation of this proposal with a year, if it is possible.

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Csiszár J, Brunner Sz, Horváth E, Bela K, Ködmön P, Riyazzuddin, Hurton Á, Papdi Cs, Szabados L, Tari I: Stabilization of redox homeostasis is the key element of the salicylic acid-induced priming in salt-stressed Arabidopsis plants. (Submitted to Acta Physiologiae Plantarum, manuscript ID: ACPP-D-16-01134)

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Posters:

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Defended dissertations /thesis connected to this project:

BSc dissertations:

Klára Mári (Biomérnöki BSc, 2013) *In vitro* regeneration of tomato plants and its biotechnological application.

Dorottya Zsuzsanna Csenki (Biomérnöki BSc, 2013) Genetic and physiological characterization of Arabidopsis thaliana *Atgstu24* glutathione transferase mutants genotipizálása és fiziológiai jellemzése

Beáta Radnai (Biológia BSc, 2014) Genotypic and fenotypic characterization of *Arabidopsis thaliana* glutathione transferase mutants.

Györgyi Mándity (Biológia BSc, 2014) Transformation and regeneration of tomato (*Solanum lycopersicum* cv. Rio Fuego) plants

Gábor Csomor (Biológia BSc, 2016) Investigation of osmotic and salt stress tolerance of *Arabidopsis thaliana gstu19* és *gstu24* mutants

Ádám Végi (Biológia BSc, 2016) Genetically modified fluorescent protein based redox probes for investigation plant redox status

MSc dissertations:

Krisztina Bela (Biológus MSc, 2013) Investigation of the role of glutathione peroxidases during oxidative stress using Arabidopsis insertional mutants.

Judit Tárkányné Derdák (Környezettudomány MSc, 2014) Application of genetic transformation on tomato (*Solanum lycopersicum* cv. Moneymaker) plants.

Petra Ködmön (Biológus MSc, 2013): Salt and osmotic stress responses of *Arabidopsis thaliana dhar1* mutants

PhD thesis:

Edit Horváth (2015): Effect of salicylic acid pre-treatment on salt stress acclimation particularly on the role of glutathione transferases in tomato and Arabidopsis plants

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