# Closing report of NKFIH K 125265 proposal

# Glutathione transferases as redox transducers?

## I. Scientific background

Reactive oxygen species (ROS), ROS-processing enzymes, antioxidants, and their reduction - oxidation (redox) states all contribute to the redox homeostasis of plant cells. Increased ROS production temporarily shifts the redox status to more oxidized values that will alter the operational controls of many redox-sensitive proteins, thus they play an important role in regulation of growth and stress responses (Tada et al., 2008; Potters et al., 2010; Foyer and Noctor, 2015; Schmidt and Schippers, 2015; Ding and Ding, 2020; Wu et al., 2020). The crucial factor is the oxidation status of the Cys residue of glutathione (γ-Glu-Cys-Gly; reduced form: GSH, oxidized form: glutathione disulfide, GSSG) redox couple that is the main redox buffer in plants. Since GSH/GSSG may reduce/oxidise or de/glutathionylate protein thiols, it has prominent role in the cellular redox control. But besides GSH, all members of the ascorbate-glutathione (ASC-GSH) cycle have specific functions in the metabolism and thus in the growth and development (Foyer and Noctor, 2011, 2015; Kocsy et al., 2013). The glutathione-related enzymes are known as crucial antioxidants having pivotal roles in stress responses and they have been intensively investigated for several decades, but their significance in redox processes received special attention in recent years. They are usually considered to be accompanies of the main non-enzymatic antioxidative compounds, due to their involvement in biosynthesis (such as glutathione synthetases, GSH1, GSH2), reduction of the ascorbate - glutathione cycle (monodehydroascorbate reductase - MDHAR, dehydroascorbate reductase - DHAR, glutathione reductase - GR), or in ROS conversion (glutathione peroxidases - GPXs, glutathione transferases - GSTs) (Csiszár et al., 2016). It has been proposed that members of GPXs and GSTs proteins can be even redox transducers (Miao et al., 2006; Passaia et al., 2014; Laborde, 2010; Meyer et al., 2020; **Gallé et al., 2021**).

GSTs play a crucial role in detoxification of exogenous and endogenous harmful compounds due to their GSH conjugating (S-transferase) and glutathione peroxidase (GPOX) activities, but they also participate in the recycling of antioxidants (e.g., flavonoids). GST proteins have been classified based on sequence similarity, genomic organization, kinetic and physiochemical properties, and immunological cross-reactivity (Dixon et al., 2002; Lallement et al., 2014; Sylvestre-Gonon et al., 2019). In higher plants fourteen classes of GSTs can be found, among them the tau (U), phi (F), lambda (L), DHAR, hemerythrin (H) and iota (I) are regarded to be

plant specific (Lallement et al., 2014). They have diversified cellular function in primary and secondary metabolisms, in development and in cell signalling even under normal physiological conditions, but generally they are strongly induced in response to abiotic and biotic stresses.

We aimed detailed analysis of the role of selected Arabidopsis and tomato GSTs to explore how they influence the ROS levels and redox state of plants and how the changes of redox environment affect their expression. In *Arabidopsis*, AtGSTU19 is thought to be one of the most important in stress responses among the ca. 60 GST proteins (Wagner et al., 2002; Sappl et al., 2004, 2009; Horváth et al., 2015; Xu et al., 2016). Overexpression of *AtGSTU19* gene favoured abiotic stress tolerance by strengthening ROS scavenging activity or maintaining ROS homeostasis by increasing antioxidant enzyme activities (Xu et al., 2016). AtGSTF8 and AtGSTF9 are also involved in detoxification or defence against different stresses (Blanco et al., 2005; Sappl et al., 2009). *AtGSTF8* was identified as an early SA response gene, but it is a marker for early stress and defence responses (Uquillas et al., 2004; Blanco et al., 2005; Sappl et al., 2009).

Tomato (*Solanum lycopersicum)* contains 57 GSTU, 7 GSTL, 6-6 GSTF and DHAR, 4 GSTT, 3 γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ), 2-2 zeta (GSTZ) and glutathionyl- hydroquinone reductase (GHR) and one tetrachlorohydroquinone dehalogenase (TCHQD) isoenzymes (Csiszár et al., 2014; Islam et al., 2017). The *in silico* analysis of the 5' regulatory regions of 30 tomato *GST* genes revealed that most of them (73%) harboured hormone-related *cis*-regulatory elements, while one or more well-known stress-related regulator sequences, like HSE, TC-rich element, MYB-binding site, W-box motif were identified in all of them. Moreover, several *SlGSTs* express on high level in roots, and some of them exhibited root specific transcription (Islam et al., 2017).

Although the function of the plant hormones and several transcription factors in formations of the root meristem has been investigated earlier, the regulatory roles of ROS and redox status emerged only in the recent years (Schnaubelt et al., 2015). It was reported that the high level and proper distribution of ROS in the root tips are also important to the normal growth and development (Dunand et al., 2007; Tsukagoshi et al., 2010; Tognetti et al., 2017). Low level of GSH significantly increased the redox potentials and caused arrest of the cell cycle in roots but not shoots (Schnaubelt et al., 2015). Root is a key organ, and it is crucial for plant performance and crop productivity either under normal and stress conditions when they are forced to adopt by structural and functional modifications (Vives-Peris et al., 2020).

**In this project we have focussed on the role of GSTs in transcriptional reprogramming of genes related to redox status and growth of roots under stress conditions. The main results can be summarized as follows:**

## II. Results

### 1. The Arabidopsis GSTs are involved in the maintenance of root redox homeostasis affecting salt stress sensitivity and meristem size

Detailed analysis of ROS levels and redox potential in longitudinal zones of 7-day-old roots of *Arabidopsis thaliana* L. Col-0 wild type and *Atsgf8* and *Atgstu19* insertional mutants revealed that ***Atgstu19*** **had the most oxidized redox status in all root zones** throughout the experiments (**Horváth et al., 2019**). Significantly higher superoxide radical ($O_2^{\bullet-}$) levels were detected in both *Atgst* mutants than in the Col-0 control. Salt stress caused by 3 hours of 75 or 150 mM NaCl treatment resulted in the highest $O_2^{\bullet-}$ increase in the *Atgstf8* root, while the amount of $H_2O_2$ was elevated most in the case of *Atgstu19*. Parallelly, the vitality was decreased in *Atgstu19* roots more than in wild type under salt stress. The glutathione redox potential ($E_{GSH}$) of the meristematic, transition, and elongation zones was determined by ratiometric measurements of the redox-sensitive cytosolic green fluorescent protein (roGFP2) under control and salt stress conditions. T**he redox status, metabolic activity, and ROS levels of roots showed region-specific changes in response to salt treatment**. Salt stress affected the redox status of the proximal meristem (PM) in all investigated lines dependent on their original redox potentials. **The transition zone (TZ) of *Atgstu19* had the highest redox potential and the lowest vitality but its ROS levels hardly changed in response to salt. The salt treatments increased the $E_{GSH}$ in the elongation zone (EZ) of Col-0, but the already elevated values of the mutants were not altered.** Moreover, the total length of short epidermal cells and their number in the proximal meristem of mutants was lower compared to the wild type, thus **the size of the PM was smaller in the 7-day-old mutants' roots than in the wild type**. It was concluded that AtGSTF8 and AtGSTU19 enzymes are differentially involved in the maintenance of the redox homeostasis of root meristem zones (**Horváth et al., 2019**).

In Arabidopsis, GSTUs include 28 members which can be the result of gene duplication events (Edwards et al., 2010). We have compared the role of AtGSTU19 and AtGSTU24 isoenzymes showing high amino acid identity (Dixon et al., 2009) and playing positive roles in salt stress responses using T-DNA insertion mutants. The two-week-old *Atgstu19* mutants had lower GST

activity and vitality both under control conditions and after two days of 150 mM NaCl treatment than the wild type, but the level of total ROS was similar to the Col-0 plants (**Horváth et al., 2020)**. Interestingly, 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. The physiological characterization of **the mutant seedlings** indicated that they **were able to cope with the applied salt stress to some extent after 48 h**. Analysis of the *AtGSTU* expression patterns revealed that the mutation in a single *AtGSTU* gene was accompanied by the up- and downregulation of several other *AtGSTU*s. The elevated DHAR and GR activities in the untreated *Atgstu24* seedlings suggested that AtGSTU24 may also contribute to the regulation of redox homeostasis. The decreased ROS levels and maintained redox status of plants indicated a successful induction of the antioxidant mechanisms. We have demonstrated that besides the altered *AtGSTUs* expression pattern, the changes in the redox-active antioxidant mechanism might compensate the effect of a mutation in an *AtGSTU* gene (Fig. 1., **Horváth et al., 2020**).

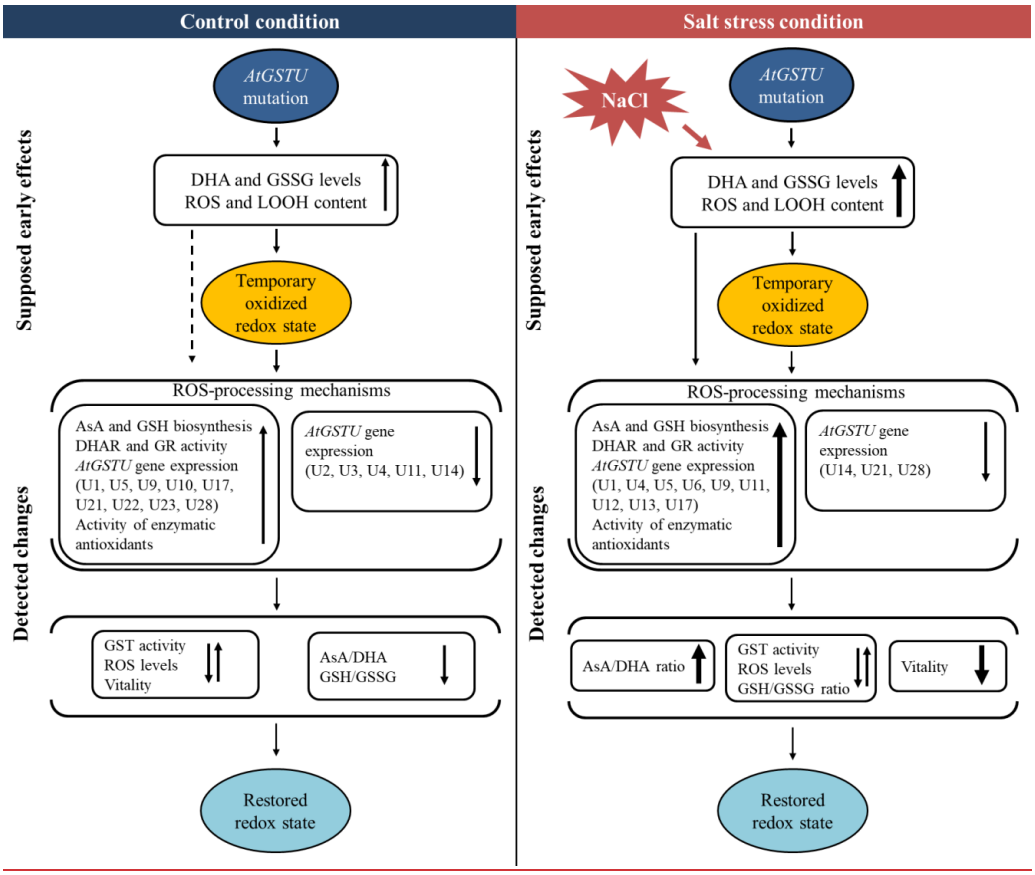


**Fig. 1** Schematic model summarizing the results obtained on *Atgstu* mutants related their involvement in maintenance of redox homeostasis under control conditions and after NaCl treatment (**Horváth et al., 2020**).

## 2. The Arabidopsis glutathione peroxidase-like (GPXL) enzymes are also involved in the investigated processes

Although it was not planned originally in the frame of present project, we performed some experiments on Arabidopsis glutathione peroxidase-like (GPXL) enzymes, which are thiol-based peroxidases catalysing the reduction of $H_2O_2$ or hydroperoxides to water or alcohols. *Arabidopsis thaliana* possess eight GPXL enzymes (Bela et al., 2015). Comprehensive analysis of antioxidant mechanisms in *Atgpxl* mutants under salt- and osmotic stress revealed the significance of the AtGPXL's activities even in roots (**Bela et al., 2018).** The role of AtGPXL5 in development and responses to salt stress was investigated using *AtGPXL5*-overexpressing lines (OX-AtGPXL5) and *Atgpxl5* mutant **(Riyazuddin et al., 2019).** The well-preserved germination rate, seedling growth and chlorophyll content of the OX-AtGPXL5 seedlings in the presence of 100 mM NaCl indicated the increased salt tolerance of *AtGPXL5*-overexpressing plants. In agreement, the *Atgpxl5* knockdown mutants had enhanced salt stress sensitivity in comparison to the wild type. In 6-week-old OX-AtGPXL5 plants, the activity of glutathione peroxidase, thioredoxin peroxidase and most of the main antioxidant enzymes were like in the wild type, but the amount of GSH was increased, thus the redox potential became more negative compared to the wild type. Our results indicated that AtGPXL5 also may have function in the fine-tuning of ROS levels and redox status during salt stress **(Riyazuddin et al., 2019).**

## 3. The GSTs are implicated in salicylic acid-induced transcriptional reprogramming in Arabidopsis

The role of GSTs was investigated further in the transcriptional reprogramming of redox status-related genes in 7-day-old *Arabidopsis thaliana* plants using exogenous SA treatments (**Horváth et al., under publication**). In previous studies it was found that $10^{-4}$ M (100 μM) SA treatment decreased growth of 5-week-old Arabidopsis, leading to death of plants, but $10^{-5}$ M (10 μM) SA pre-treatment promoted the growth and induced hardening, alleviating the effect of subsequently applied salt treatment (Horváth et al., 2015). It was suggested that lower than 50 μM SA is a developmental regulator in *Arabidopsis* roots, while higher concentrations act as stress hormones and induce various changes in auxin biosynthesis and distribution (Pasternak et al., 2019). It is well documented that high endogenous or exogenous SA concentrations induce the accumulation of ROS, but less information is available about redox changes during exogenously applied SA concentrations.

Our aims were to investigate relationships among ROS, redox status, and salicylic acid-induced transcriptional reprogramming. The potential role of AtGSTU19 and AtGSTF8 as redox transducers in these processes was also estimated by introducing *Atgst* mutants into our experiments (Fig. 2). For this investigations Arabidopsis plants were grown on solid half strength MS medium (Murashige and Skoog, 1962) for 7 days and then placed into liquid medium containing 10 μM or 100 μM SA through 24 h. Although there were some changes in the ROS levels and vitality of roots even after 30 min, samples were taken after 1, 3 and 24 h for fluorescent microscopic analysis.

According to our results, elevated $H_2O_2$ levels were detected after 1 h of 100 μM SA treatment in wild type roots, but fluctuations were observed in all root zones over time, suggesting a tight control of $H_2O_2$ levels in SA response. Using the roGFP2 redox probe to study the *in vivo* effects of SA treatments on redox status of GSH revealed that both SA concentrations resulted in a more oxidized redox potential in wild type *Arabidopsis* roots. However, the timing of changes differed. Lower 10 μM SA elevated $E_{GSH}$ after 1 h of treatment in all root zones, but redox potential remained more oxidized in the presence of 100 μM SA compared with 10 μM SA and showed further oxidation in TZ of Col-0 roots after 24 h (Figs. 2, 3).

The lower, 10 μM SA concentration induced only modest changes in the vitality and ROS levels of wild type plants but significantly elevated the $E_{GSH}$ detected by the roGFP2. The 100 μM SA treatment decreased the vitality of all root tips, but it was lower in mutants than in Col-0 plants. The biggest difference in the vitality could be observed in the EZ, where 100 μM SA decreased it by 60%, 80% and 87% in Col-0, *Atgstf8* and *Atgstu19* roots, respectively. The Col-0 roots started to regain their vitality in PM and TZ after 24 h of 100 μM SA treatment.

To investigate the effect of the quick changes in the redox status and $H_2O_2$ level of roots we analysed the expression patterns of some *AtGSTs* and other oxidative stress-inducible genes in wild type and *Atgst* mutants by high throughput quantitative RT-PCR (HT-qPCR) after one hour of 10 μM and 100 μM SA treatment. Roots were collected from approximately 120 seedlings.

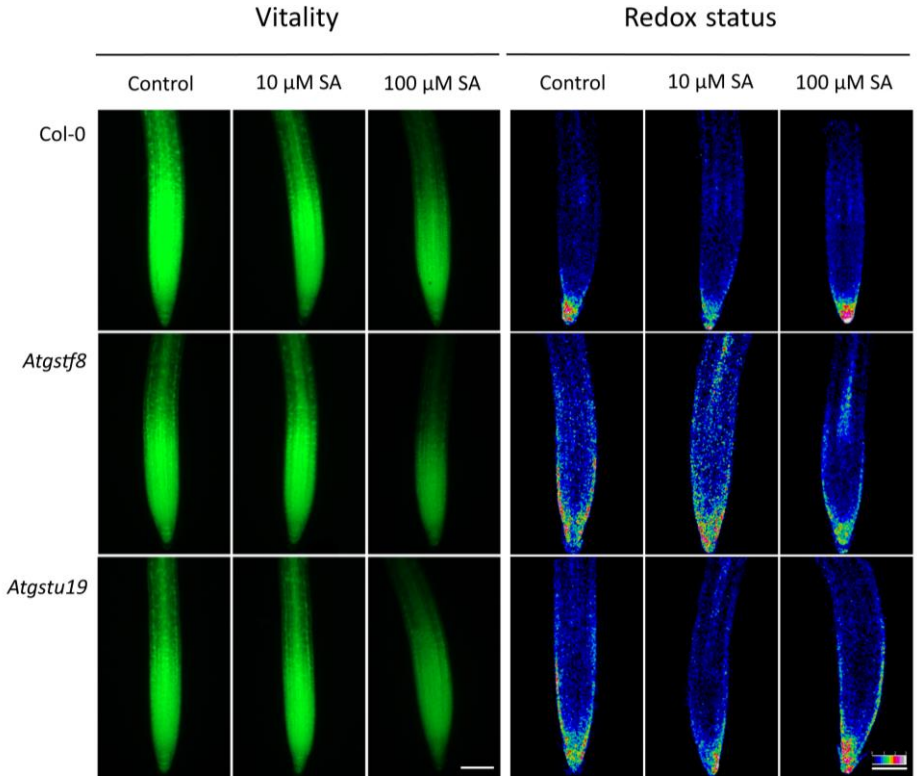


**Fig. 2** Representative images of the fluorescent analysis of the vitality and redox potential in the 7-day-old Arabidopsis Col-0 wild type, *Atgstf8* and *Atgstu19* insertional mutants after one hour of treatment with 10 and 100 µM SA (**Horváth et al., under publication**).

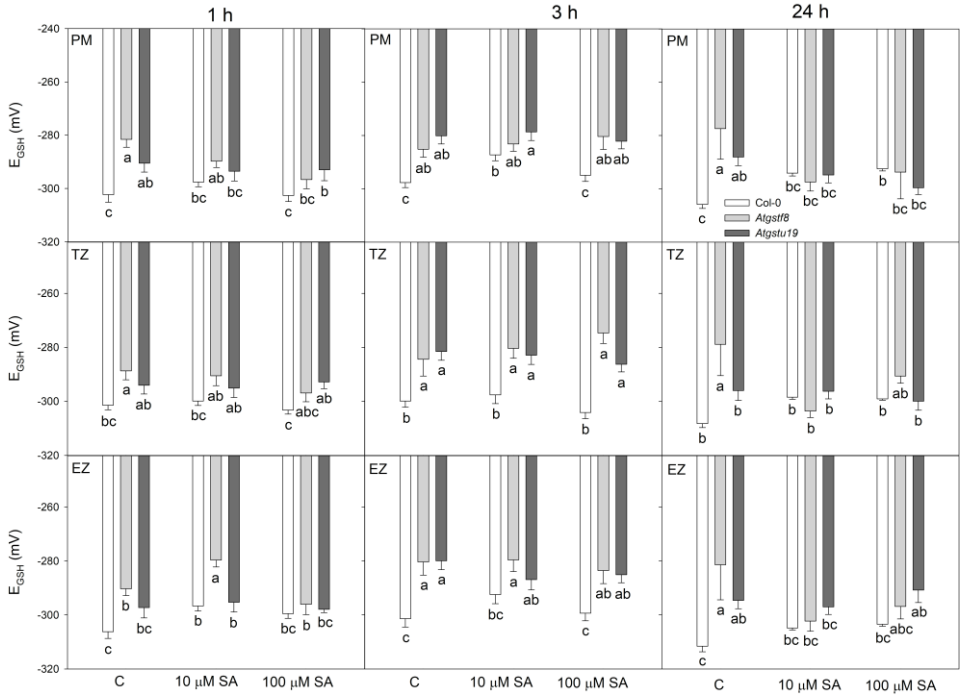


**Fig. 3** Calculated redox potential values of roots expressing cytosolic GRX1-roGFP2 of primary roots of 7-day-old Arabidopsis Col-0 wild type, *Atgstf8* and *Atgstu19* insertional mutants. Data are means ± SE, n = 10. Columns with different letters are significantly different at $p \leq 0.05$, determined by Duncan's test. PM: proximal meristem, TZ: transition zone, EZ: elongation zone (**Horváth et al., under publication**).

Our main findings are:

- Among the investigated 22 genes, the ***WRKY38*** **was induced the most** after 1 h of treatment, both by the 10 and 100 μM SA. Beside this, some elevation of the gene expression was detected in the transcript amounts of ***WRKY46, OXI1, NAC032, HSFA8, GSH1, GSTU7, GSTF8,*** **and** ***UPB1*** **after applying 10 μM SA on the Col-0** wild type. A partially **different set of genes were induced in the presence of 100 μM SA,** and a higher transcript amount was found in case of *WRKY46, GSTU7, GSTU19, GSTU24, GSTU25, GSTF8, GPXL6, NAC032, AKR4C9, WRKY38, OXI1, HSFA8, RRTF1, ZAT10* and *GSH1* genes compared to the control. Lower expression was detected in the expression of *WRKY28* and *MYC2* genes.
- Slightly modified gene expression pattern was measured in control conditions and after applying 1 h of SA treatments in *Atgst* mutants compared to Col-0 roots. Among the investigated genes only *ZAT10* had higher expression in mutant plants than in the wild type. The knock-down **mutation of** ***GSTU19*** **resulted in decreased transcript level** of not only that gene, but that **of** ***GR1, GSTU24, GSTU25*** **and** ***AKR4C9*** also. Short-time SA treatment slightly altered the gene expression pattern of *Atgstu19* compared to the Col-0. For instance, 10 μM SA resulted in lower *GR1* and *GSTU7*, and higher *HSFA8* and *RRTF1* expression.

The schematic model of SA-induced changes in the roots of Col-0 plants after 1 h treatment and the supposed interaction between GSH redox state and $H_2O_2$ level, supported by our results and data found in the literature are summarized in Fig. 4. The enhanced expression of redox-related genes in *Atgst* mutants might be the result of either direct antioxidant role of the concerned proteins or their involvement in redox signalling. Our results indicate that the SA-binding function of AtGSTF8 seems to be more important in SA response than its glutathione transferase or GPOX enzyme activities. Although the direct role of AtGSTU19 in SA perception or signalling was not confirmed by these results, the modified redox processes and gene expression patterns infer its involvement in SA responses may exceed antioxidant functions (**Horváth et al., under publication**). However, estimating its role in SA signalling requires further investigation.

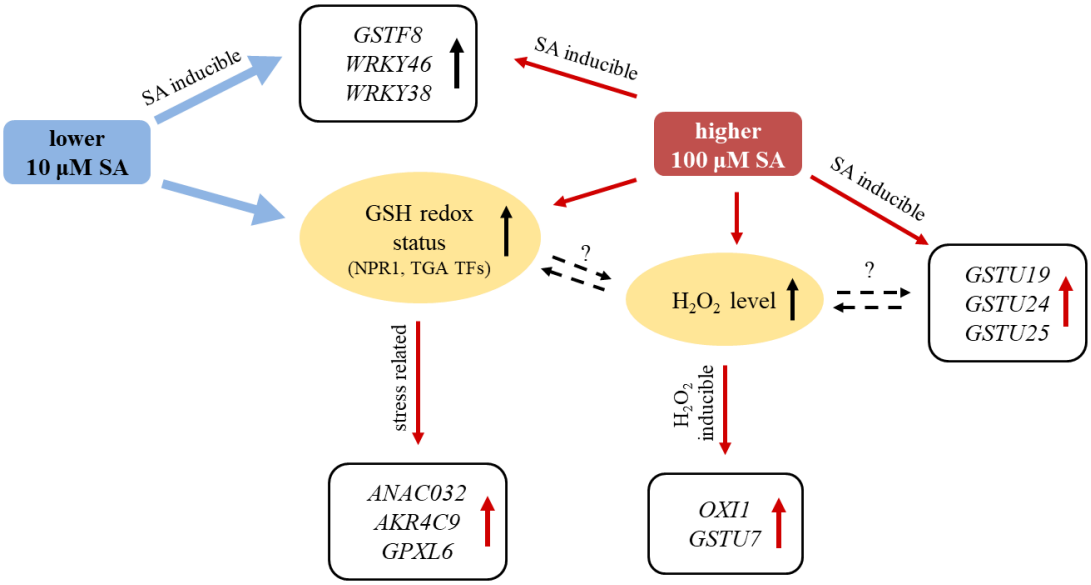


**Fig. 4** Schematic model summarizing the effect of two differing concentrations of salicylic acid (SA) treatment. Gene expression changes after 1 h of SA treatment in Arabidopsis Col-0 wild type plants and their supposed regulation by SA, glutathione (GSH) redox status and $H_2O_2$ levels are depicted. (An upward arrow indicates increase, the thickness of the arrows refers to the extent of the changes (**Horváth et al., under publication**).

### 4. Relationship between tomato *GSTs* and redox state

Since the most abundant AtGST proteins are implicated in the fine-tuning of the redox homeostasis, a tight interaction was supposed between GSTs and the redox signalling even in tomato. Henceforth we have compared the responses of two tomato cultivars (*Solanum lycopersicum* cvs. Moneymaker and Ailsa Craig) by treating 4-week-old plants with 100 and 150 mM NaCl, 200 and 300 mM mannitol, $10^{-7}$ and $10^{-4}$ M SA for 3 and 24 hours **(Gallé et al., 2021)**. The glutathione half-cell reduction potential ($E_{GSH}$) was calculated from the concentration of GSH and GSSG according to Schafer and Buettner (2001). The expression level of *SlGSTs* involved especially in removing the toxic stress metabolites and genes involved in re-reducing the main redox-active antioxidants (46 *SlGSTU*, 5 *SlGSTF*, 4 *SlGSTT*, 3 *DHAR*, two *GR* and one *GSH1)* was detected by HT-qPCR after 24 h of 300 mM mannitol, 150 mM NaCl and $10^{-4}$ M SA treatments.

Generally, 'Moneymaker' had lower ROS and lipidperoxide content, higher GST and GPOX activities than 'Ailsa Craig'. It was suggested that the higher GSH and ASC contents of the 'Moneymaker' might be responsible for the more efficient defence against different abiotic stresses. The more MDA measured in 'Ailsa Craig' roots compared to 'Moneymaker' indicateed that operation and/or activation of the antioxidant mechanisms in ‘Ailsa Craig’ due to stresses

did not prevent the accumulation of ROS and lipid peroxides as efficiently as that in the 'Moneymaker' cultivar. The $E_{GSH}$ showed a more oxidized redox state in 'Ailsa Craig' than in 'Moneymaker' even under control conditions, and it became more positive due to treatments (Table 1, **Gallé et al., 2021**).

**Table 1.** Ratio of ascorbic acid and dehydroascorbate (ASC/DHA), reduced and oxidized glutathione (GSH/GSSG) and glutathione redox potential ($E_{GSH}$) of hydroponically grown 4-week-old *Solanum lycopersicum* 'Ailsa Craig' and 'Moneymaker' after 24 h of stress treatments

| Treatments (24h) | Ailsa Craig | Moneymaker |
|---|---|---|
| **ASC/DHA** | | |
| Control | 0.64 ± 0.03 | 0.82 ± 0.10 |
| 200 mM mannitol | 1.13 ± 0.07 | 0.95 ± 0.09 |
| 300 mM mannitol | 0.48 ± 0.07 | 0.76 ± 0.11 |
| 100 mM NaCl | 0.77 ± 0.08 | 0.97 ± 0.07 |
| 150 mM NaCl | 1.01 ± 0.05 | 0.70 ± 0.03 |
| $10^{-7}$ M SA | 0.53 ± 0.05 | 0.81 ± 0.08 |
| $10^{-4}$ M SA | 0.62 ± 0.10 | 0.76 ± 0.07 |
| **GSH/GSSG** | | |
| Control | 8.18 ± 1.15 | 12.63 ± 1.33 |
| 200 mM mannitol | 7.36 ± 0.46 | 17.80 ±1.69 |
| 300 mM mannitol | 7.85 ± 0.21 | 16.43 ± 1.12 |
| 100 mM NaCl | 5.75 ± 0.57 | 16.14 ± 1.49 |
| 150 mM NaCl | 9.18 ± 0.63 | 14.65 ± 1.27 |
| $10^{-7}$ M SA | 10.77 ± 2.15 | 11.41 ± 0.77 |
| $10^{-4}$ M SA | 7.52 ± 1.01 | 11.70 ± 0.72 |
| $E_{GSH}$ | | |
| Control | -214.55 ± 1.00 | -226.52 ± 1.49 |
| 200 mM mannitol | -213.45 ± 2.75 | -238.01 ± 0.95 |
| 300 mM mannitol | -214.42 ± 0.24 | -235.17 ± 0.40 |
| 100 mM NaCl | -206.06 ± 2.76 | -235.00 ± 0.48 |
| 150 mM NaCl | -218.51 ± 1.60 | -233.47 ± 0.39 |
| $10^{-7}$ M SA | -221.36 ± 0.73 | -225.56 ± 0.41 |
| $10^{-4}$ M SA | -214.18 ± 0.90 | -225.99 ± 1.69 |

It was found that under control conditions almost half of the investigated genes expressed at higher level, and only seven *SlGSTs* showed lower transcript abundance in 'Moneymaker' than in 'Ailsa Craig'. In some cases, there was more than 100 times bigger differences in the relative transcript amount between the two cultivars (like in case of *GSTF1*, *GSTU41* and *GSTT3*). Analysis of the expression pattern of the totally 58 *SlGSTs* after different stress treatments revealed that all the selected genes responded in roots to one or more stresses. Except with a few examples (such as *GSTU18*), their expression was induced, but the changes were largely

stress- and genotype specific. Interestingly, most of the *SlGSTs* expressing on very high level in un-treated 'Moneymaker', such as *SlGSTU27*, *SlGSTU41*, *SlGSTF1*, were strongly induced in 'Ailsa Craig' by different stress treatments **(Gallé et al., 2021)**.

To check the relation between the redox potential and the expression of the investigated genes, we determined the Pearson's correlation coefficients. Our results indicated that several genes (*GR1, -2, GSTF1, GSTT2, -4, GSTU19, -20, -22, -23, -35, -37, -38, -47*) have a very strong positive correlation with the redox potential in both cultivars. As we used $E_{GSH}$ and ΔCt values for calculation, strong positive correlation means that as the root tissues become more reduced, the expression level of a given gene increases, while in case of strong negative correlation the transcript level of a given gene increases as the root tissues become more oxidized. Numerous cases stronger correlation was found between the expression level and redox potential changes in 'Ailsa Craig', than in 'Moneymaker' (e.g., *SlGSTU27, SlGSTU41*). The stronger redox dependency of *SlGSTs* observed in 'Ailsa Craig' might indicate that alteration of the redox status and/or ROS level was more important component of the *SlGST* expression induction in the 'Ailsa Craig' than in 'Moneymaker' cultivar **(Gallé et al., 2021).**

**The more oxidized environment, the increased GSSG, the low GSH level all might induce transcription of *GST* genes** (Schnaubelt et al., 2015). It is widely accepted that GSTs influence the redox state of GSH and ASC due to their GSH conjugating, GPOX or DHAR activities. However, **our results highlighted the importance of the high level of GSH and the more negative redox potential in induction of *SlGST* expression. This** relationship **indicates that these GSH-utilising enzymes may have redox status-directed feedback in their transcriptional regulation**.

***In silico* analysis** performed on 5' regulatory region (-1500 bp) of *SlGSTU* genes with the **strongest** positive and **negative correlation** to calculated redox potential **revealed that the** promoter of the latter group **contained four or five W boxes**, **which are among the PLETHORA- (PLT) regulated sequence elements.** The PLT1 and PLT2 APETALA-2 transcription factors are required for the proper root growth and differentiation by controlling distal cell division and stem cell maintenance (Aida et al., 2004). It was reported that the redox balance may affect the functions of the key transcription factors, such as PLT (Licausi et al., 2013). Since **the PLT-regulated W-box sequence elements can be found in *SlGST* promoters, we may assume that in this way some GSTs can be part of the gene regulatory network controlled by PLT, thus might be the link between growth processes and antioxidant mechanisms (Gallé et al., 2021).** According to a recent concept, the transcription

factor-mediated gene expression network downstream of ROS ensure the right response to the changing environmental conditions (Mase and Tsukagoshi, 2021). Whether these GSTs are under ROS/redox control through the PLT and/or other transcription factors that was explored in quiescent centre (Wang et al., 2021), needs further investigations.

## 5. *In silico* identification of stress-, hormone- and light-responsive elements in the 5' regulatory regions of additional *GSTs*

There are several evidence that GSTs, through involving in numerous redox, hormone and stress responses, cross-talks between these different signalling pathways. Our previous analysis of the *cis*-acting regulatory sequences in the 5' promoter regions of the selected *SlGSTs* revealed the presence of many motifs connected to light responsiveness beside the regulatory elements involved in stress responsiveness (Csiszár et al., 2014). Based on this earlier results, four tau class GST genes (*SlGSTU6*, *SlGSTU23*, *SlGSTU24*, *SlGSTU26*) were selected according to their catalytic activity and their inductions to various environmental stresses, and the diurnal changes in their transcription and the total extractable GST activity were measured in hydroponically grown 'Ailsa Craig' tomato cultivar. Our findings underlined the circadian fluctuation of the GST activity with a maximum late in the light period, but the expression of selected *SlGST* genes was also highly affected by dark (**Gallé et al., 2018**). It was reported that GSH content shows diurnal changes, and it plays a role in the daytime/light-dependent redox balance in plants (Zechmann, 2017). The light-regulated induction and dark inactivation of GSTs indicate that these enzymes can also participate in the signal transduction of visible and UV light (Fig. 5). The known information connected to the relationship among GSTs and light was summarized in a review paper **(Gallé et al., 2019).**

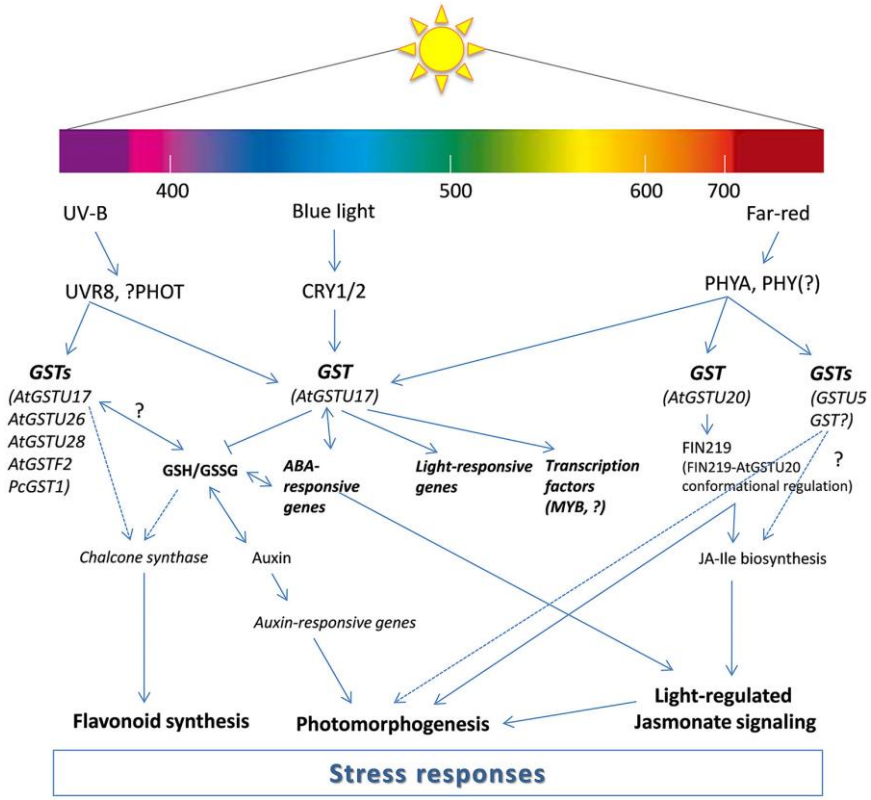


**Fig. 5** Proposed model for the participation of GSTs in light signal transduction illustrating transcriptional and post transcriptional regulation of GSTs by light (UV B, blue and far-red) and possible function of GST proteins in the light induced signalling pathways. AtGSTU17 was reported to fine tune GSH homeostasis and GSH/GSSG ratio and regulate auxin, ABA and light response. AtGSTU20 is having a role in jasmonate (JA) signalling as a conformational regulator of FIN (FR-insensitive 219). Other GSTs (AtGSTU26, ATGSTU28, AtGSTF2 and PcGST1) are also parts of light- (UV B) regulated signalling which possibly affect chalcone synthase transcription (**Gallé et al., 2019**).

The *in silico* analysis of the 5' promoter regions of *AtGSTF8* and *AtGSTU19* genes were also performed (**Horváth et al., 2019)**. Beside numerous *cis*-acting regulatory sequences associated with light responsiveness, several common regulatory elements involved in defence and abiotic/biotic stress responses (e.g., ARE, TC-rich repeats, MBS motif, ABRE elements) were found. It was suggested that each type of ROS has unique chemical properties and targets a specific set of signalling routes therefore activates specific set of genes (Møller et al., 2007; Bindoli and Rigobello, 2013; He et al., 2018). These ROS-activated *cis*-regulatory elements (like AREs) are parts of the ROS signalling pathways (He et al., 2018). However, motifs involved in hormone regulation (auxin, gibberellin, methyl jasmonate, ethylene, SA) were also identified with different number in the investigated *AtGST* regions (-1500 bp). For example, while TGA-box (TGACGTGGC) sequences were present in both genes, the auxin-responsive TGA-element (AACGAC) could be found in the 5' regulatory region of *AtGSTU19*. Moreover, other *cis*-

acting regulatory sequences associated with the control of leaf morphology and seed development were also identified in the 5' upstream regions of one or both genes, indicating the complex interaction between different signalling inputs controlling their expression (**Horváth et al., 2019**).

**6. Experiments on functional analysis of the *SlGSTU24* in root**

We also aimed to investigate the involvement of redox potential changes in the expression of cell type-specific developmental- and stress inducible genes (e.g., *GSTs*). We have optimized and applied the single cell genomic methods at first on the tomato PM cells. To estimate tissue- or cell type specific transcriptions we apply laser capture microdissection (LCM) to select and collect specific cells from tomato roots. This method is used by project participants at the Dept. of Physiology, Anatomy and Neuroscience, University of Szeged, and Laboratory of Functional Genomics, BRC, Szeged on fixed brain section samples. Our important task was to develop the methods used for fixation, embedding, sectioning of roots and identification of specific cells by fluorescent microscope. . Although we had some successful tests and results (Fig. 6), during these experiments several problems occurred. **In our simplified method** worked out lately, the fresh plant material is dyed and sectioned by cryostat (Fig. 7, **Gallé Á, Faragó N et al., unpublished results**). The second, 13 μm thick **cross-section taken from the tip of tomato roots cover the PM**. After identification of the specific cells by fluorescent microscope and collection by LCM (ARCTURUS VERITAS 704, Themo Fisher) single cell quantitative real-time PCR (scQPCR) is performed. This method **is applied to compare expression of *SlGSTU23, SlGSTU24, PLT1* and *WOX5* genes in hydroponically grown control and 150 mM NaCl treated 'Moneymaker' roots** (**Gallé Á, Faragó N et al., unpublished results**). This focussed genomic analysis, when we can detect the expression levels of unique and small number of genes originated from root tips, has high importance that enables outstanding sensitivity will give significant new information by using it to analyse regulatory networks.

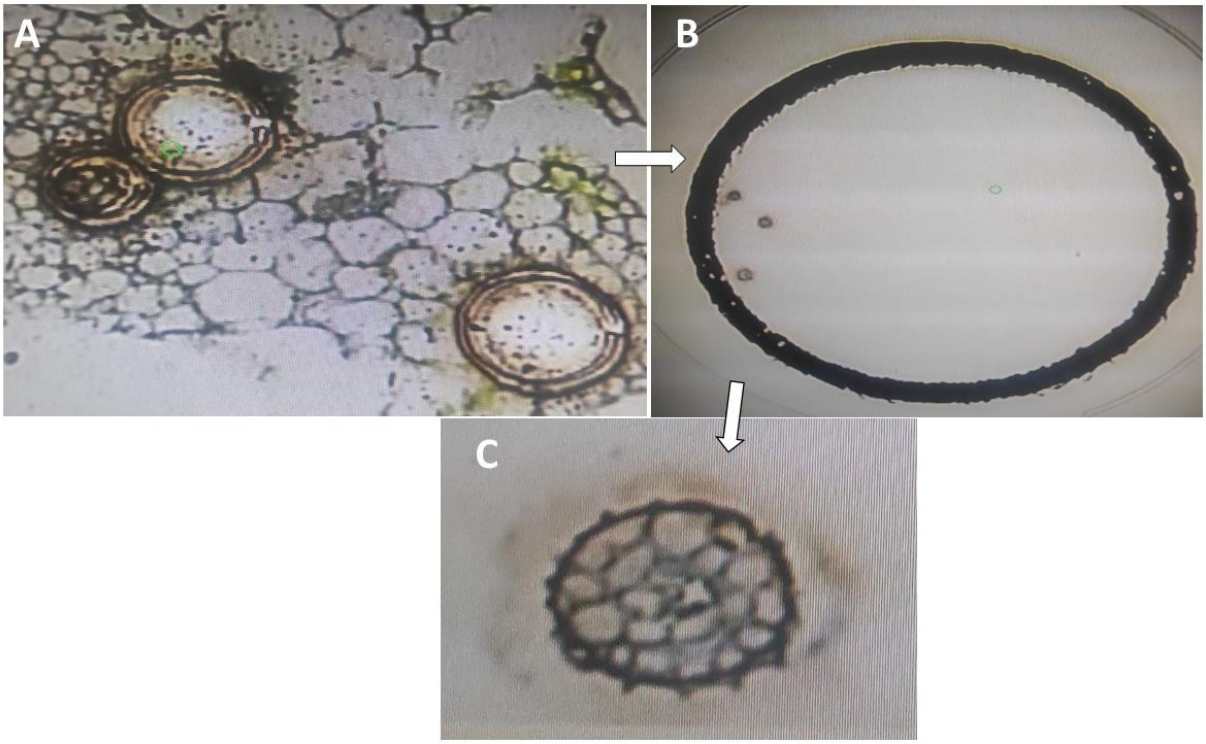


**Fig. 6** Adaptation the laser capture microdissection (LCM) in tomato. A: Tomato stem segments after laser capture. B, C: Laser captured tissue segments in the microtube lid **(unpublished results)**.

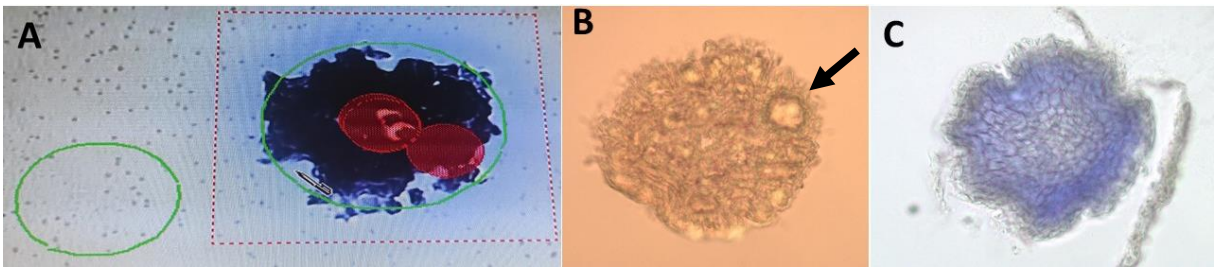


**Fig. 7** Applying the laser capture microdissection (LCM) in tomato root. A: Tomato root segment ready to laser capture. B: Fixed tomato root segment after laser capturing. C: Dyed tomato root segment **(unpublished results).**

Another approach to investigate the individual role of the SlGSTU24 enzyme aimed to apply the CRISPR/Cas9 system to induce loss-of-function mutations. The SlGSTU24 consist of several domains with known function and structure of the coding sequence. During designing the adequate construction, we were looking for the most crucial sites responsible for GSH binding, dimerization, and substrate binding. Considering the requirements of applying the CRISPR/Cas9 system, we designed three different constructs for inducing mutations in *SlGSTU24* gene. Because we did not have success by using the first available vector

constructions, the designed gRNAs were cloned into pKSE401G vector (provided by Cheng Dai and Yan Guambo, from Huazhong Agricultural University, Wuhan, China). This is a modified pKSE401 vector that contains additional 35S::eGFP insert next to the required CRISPR-Cas9 sequences, which enables a more efficient selection of transformant plantlets by GFP signal (Fig. 8).

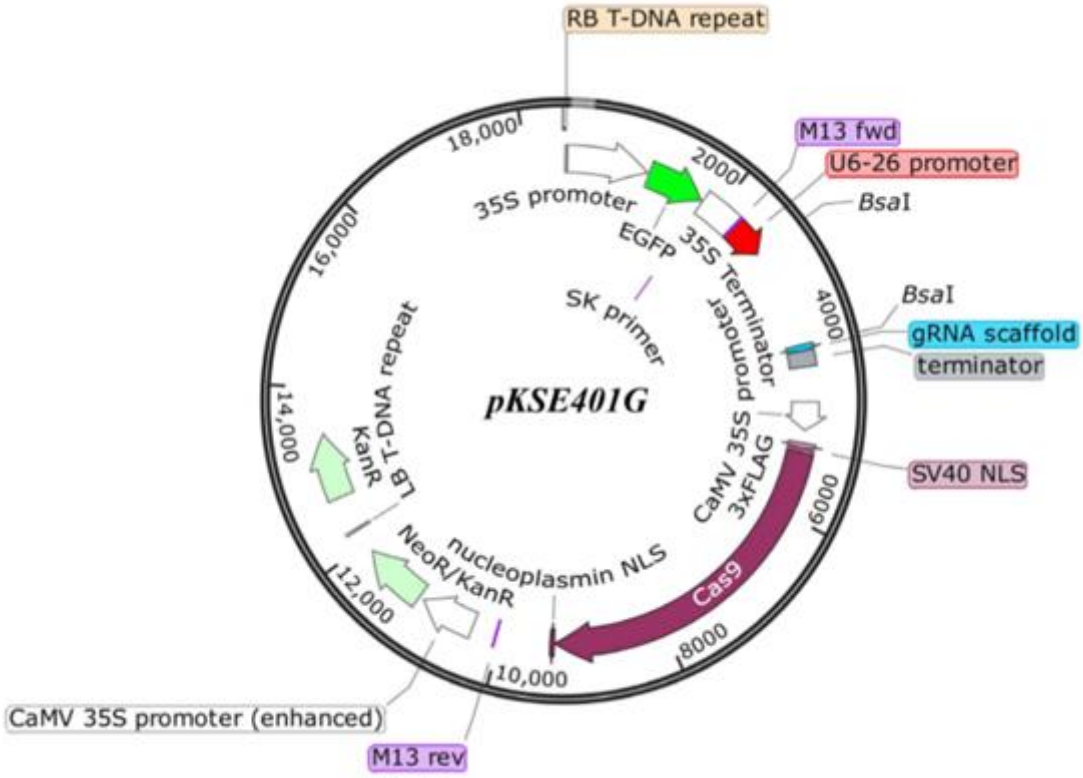


**Fig. 8** The vector construction used for introduction precise mutation in *SlGSTU24* by applying CRISPR/Cas9 system (Tang et al., 2018).

Our three constructs were the following: PAM6, PAM12 and PAM19 (named after the numerous protospacer adjacent motif, PAM sites found in the coding sequence). Constructs named PAM6 and PAM12 targeted different sequences within dimerization domains, while PAM19 was targeting a sequence within the substrate binding domain. We successfully introduced all the three plasmid constructs to *Agrobacterium tumefaciens* LBA4404 strain, performed numerous transformation experiments on *Solanum lycopersicum* cv. Moneymaker, and successfully regenerated new plants from calluses grown from infected cotyledons. However, after selection/regeneration processes, we have faced several difficulties. First, we

could detect GFP signal from ca. 50 plants, but later we couldn't get signal from the regenerated plants, even though we proved the presence of the gene by PCR reaction (for example, in 55,5% of the PAM19 mutants). Moreover, we couldn't evince the effect of the Cas9 enzyme as we monitored the more than 100 regenerants for mutations within the *SlGSTU24* gene through Sanger-sequencing. We may conclude that **although the CRISPR/Cas9 system was successfully introduced by the performed transformations, the wanted modifications were not generated.** Accordingly, the CRISPR/Cas9 transformation protocol in cv. Moneymaker tomato has to go through serious improvement and optimalization or we have to apply an improved CRISPR technology (Biswas et al., 2021; Zhang et al., 2021). However, in the frame of this project **the tomato transformation and regeneration system became a routine method at our department,** which enables us to continue our research work even in an another NKFIH 1 K project (K 138589, Relationship between GSH-related antioxidants and redox processes, their regulation in roots).

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## IV. Dissemination of the results

### Publications:


1. 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. Env Exp Bot 150: 127-140.
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### Manuscripts under publication:

Horváth E, Bela K, Kulman K, Faragó N, Riyazuddin R, Gallé Á, Puskás L, Csiszár J: Role of redox changes in salicylic acid-induced gene expression reprogramming in Arabidopsis roots.
Riyazuddin R, Bela K, Poór P, Szepesi Á, Horváth E, Rigó G, Szabados L, Fehér A, Csiszár J: Crosstalk between the Arabidopsis glutathione peroxidase-like 5 isoenzyme (AtGPXL5) and ethylene

### Book Chapter:

Bela K, Riyazuddin R, Horváth E, Hajnal Á, Gallé Á, Bangash SAK, Csiszár J (2020) A növényi glutation-peroxidáz-szerű enzimek szerepe az oxidatív stresszválaszban. In: Poór P, Mézes M, Blázovics A (szerk.) Oxidatív stressz és antioxidáns védekezés a növényvilágtól a klinikumig. Magyar Szabadgyök-Kutató Társaság kiadványa, ISBN 978-615-6203-00-7. pp. 12-19.

**Lectures:**

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5. Riyazuddin, R; Krisztina, Bela, K; Edit, Horváth, E; Poór, P; Szepesi, Á; Rigó, G; Szabados, L; Fehér, A; Csiszár, J (2021) Crosstalk between the membrane localised Arabidopsis glutathione peroxidase-like isoenzymes-5 (AtGPXL5) and ethylene signaling In: Györgyey, János (szerk.) XIII. Magyar Növénybiológiai Kongresszus : Összefoglaló kötet p. 36.
6. Csiszár, J; Gallé, Á; Horváth, E; Bela, K; Hajnal, Á; Erdei, L; Tari, I; Fehér, A (2021) A stresszválaszok és redox folyamatok fiziológiai és molekuláris vizsgálatai az SZTE Növénybiológiai Tanszéken. In: Györgyey, János (szerk.) XIII. Magyar Növénybiológiai Kongresszus: Összefoglaló kötet. p. 40.
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**Posters:**

1. 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 (szerk.) 11th International Conference on Plant Functioning Under Environmental Stress. Cracow, Polish Academy of Sciences. Abstracts p. 58.
2. 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 (szerk.) 11th International Conference on Plant Functioning Under Environmental Stress. Cracow, Polish Academy of Sciences. Abstracts p. 120.

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**PhD dissertations:**

Bela Krisztina: Növényi glutation peroxidáz enzimek vizsgálata lúdfűben
Riyazuddin Riyazuddin: Overexpression of the Arabidopsis glutathione peroxidase-like 5 gene (AtGPXL5) resulted in altered plant development and redox status

**MSc dissertations:**

Holinka Botond: Investigation of salt stress responses of Arabidopsis *gstf8* and *gstu19* glutathione transferase insertional mutants using fluorescent microscopy)
Hajnal Ádám Barnabás: A redox folyamatok és szabályozásuk paradicsom növények stresszválaszában
Kulman Kitti: Glutation transzferázok szerepe a paradicsom fajták sóstressz válaszában
Hajnal Ádám Barnabás **won 1st place on the 35th OTDK competition and participated on the "OTDK+" conference** (the title of his presentation: Növényi stresszválaszok redox folyamatai és regulációjának vizsgálata két paradicsomfajtában

**BSc dissertations:**

Hajnal Ádám: Optimization of *Agrobacterium*-mediated transformation for introducing the roGFP2 gene into tomato)
Kulman Kitti: Arabidopsis *gstf8* és *gstu19* glutation transzferáz inszerciós mutánsok redox állapotának változása szalicilsav kezelés hatására
Fazekas Cintia: Arabidopsis glutation transzferáz mutánsok (Atgstf8 és Atgstu19) genotipizálása és stresszválaszuk vizsgálata
Horváth Adrienn: Redox szabályozás fotoszintetikus szervezetekben; a glutatioriláció
Horváth Mátyás: A glutation transzferáz enzimek génexpressziós változásai exogén stresszorok hatására paradicsom növényekben
Nagy Dorina Alexandra: Glutation a növényekben

## V. Justification of the modifications compared the original research plan and work plan

**Research activities:**

The research work of the project was performed mostly according to the workplan. Some modifications are the following:

1. We aimed to compare the effect of salicylic acid (SA) hormone and the SA-analogue benzothiadiazole (BTA) activator on redox state of Arabidopsis and tomato seedlings, however the higher concentrations applied at SA treatments could not be prepared accurately in case of BTA. Although we have performed some experiments, the very low water solubility of BTA (in water 7.7 mg/l, in methanol, for example, is 4.2 g/l; https://pubchem.ncbi.nlm.nih.gov/compound/Acibenzolar-S-Methyl#section=Solubility).

Since BTA in higher concentrations precipitated during dilution with water and the used methanol also has serious effect on reactive oxygen species (ROS) levels of plants, most of the experiments were conducted only with SA.

2. Some experiments were performed connected to Arabidopsis glutathione peroxidase (AtGPXL) enzymes. Both plant GSTs and GPXLs may possess glutathione peroxidase activity and are involved in redox processes.
3. Some of our investigations dealt with the light responsiveness of GSTs. It was reported recently that GSH content shows diurnal changes, and it plays a role in the daytime/light-dependent redox balance in plants, moreover the importance of the light responsiveness of GSTs emerged in recent years. Our results and information found in literature highlighted that GSTs, through involving in numerous redox, hormone and stress responses, cross-talks between these different signalling pathways.
4. Some delay occurred at the experiments connecting to establishment StGSTU24 mutants using the CRISPR/Cas9 technique and in applying the single cell methods on roots because of the Covid-19 pandemic.

**Personnel changes:**

In the budget employment of one junior researcher (Krisztina Bela) was planned full-time for 4 years. She had a Postdoctoral NKFI project that provides her salary from. 01. 12. 2019, and because of her other tasks she could participate in this project with less FTE, other colleagues were called in to do the research work (Riyazuddin, Riyazuddin, Hajnal Ádám Barnabás and Kocsis Ágnes Katalin). The requested changes (submitted on 04. 2020. and 06. 2020.) were permitted by the President of the Council of Complex Environmental Sciences.

**Changes in the Budget plan:**

We could not travel to international conferences, thus the sum planned for per diem allowances was not spent. Moreover, in the meantime, the legal contribution rate of salaries was decreased. The remaining personnel sum was shared among the participants according to the fulfilled research work.