



Article

Glutathione Transferases Are Involved in the Genotype-Specific Salt-Stress Response of Tomato Plants

Edit Horváth ^{1,*}, Kitti Kulman ^{1,2}, Bernát Tompa ^{1,3}, Ádám Barnabás Hajnal ^{1,3} , Alina Pelsőczy ^{1,3}, Krisztina Bela ¹ , Ágnes Gallé ¹ and Jolán Csiszár ¹

¹ Department of Plant Biology, Faculty of Sciences and Informatics, University of Szeged, H-6726 Szeged, Hungary; kittikulman30@gmail.com (K.K.); tompa_bernat@yahoo.com (B.T.); hadam996@gmail.com (Á.B.H.); pelsoczi.97@gmail.com (A.P.); bela.krisztina@szte.hu (K.B.); gallea@bio.u-szeged.hu (Á.G.); csiszar@bio.u-szeged.hu (J.C.)

² Agricultural Institute, Centre for Agricultural Research, Eötvös Lóránd Research Network, H-2462 Martonvásár, Hungary

³ Doctoral School in Biology, Faculty of Science and Informatics, University of Szeged, H-6726 Szeged, Hungary

* Correspondence: horvath.edit.03@szte.hu

Abstract: Glutathione transferases (GSTs) are one of the most versatile multigenic enzyme super-families. In our experiments, the involvement of the genotype-specific induction of *GST* genes and glutathione- or redox-related genes in pathways regulating salt-stress tolerance was examined in tomato cultivars (*Solanum lycopersicum* MoneyMaker, Mobil, and Elán F1). The growth of the Mobil plants was adversely affected during salt stress (100 mM of NaCl), which might be the result of lowered glutathione and ascorbate levels, a more positive glutathione redox potential (E_{GSH}), and reduced glutathione reductase (GR) and GST activities. In contrast, the MoneyMaker and Elán F1 cultivars were able to restore their growth and exhibited higher GR and inducible GST activities, as well as elevated, non-enzymatic antioxidant levels, indicating their enhanced salt tolerance. Furthermore, the expression patterns of *GR*, selected *GST*, and transcription factor genes differed significantly among the three cultivars, highlighting the distinct regulatory mechanisms of the tomato genotypes during salt stress. The correlations between E_{GSH} and gene expression data revealed several robust, cultivar-specific associations, underscoring the complexity of the stress response mechanism in tomatoes. Our results support the cultivar-specific roles of distinct *GST* genes during the salt-stress response, which, along with *WRKY3*, *WRKY72*, *DREB1*, and *DREB2*, are important players in shaping the redox status and the development of a more efficient stress tolerance in tomatoes.

Keywords: glutathione transferases; redox homeostasis; salt stress; *Solanum lycopersicum* L.; transcription regulation



Citation: Horváth, E.; Kulman, K.; Tompa, B.; Hajnal, Á.B.; Pelsőczy, A.; Bela, K.; Gallé, Á.; Csiszár, J. Glutathione Transferases Are Involved in the Genotype-Specific Salt-Stress Response of Tomato Plants. *Antioxidants* **2023**, *12*, 1682. <https://doi.org/10.3390/antiox12091682>

Academic Editor: Cecil Stushnoff

Received: 8 June 2023

Revised: 22 August 2023

Accepted: 24 August 2023

Published: 28 August 2023



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1. Introduction

Glutathione transferases (GSTs) are one of the most versatile enzymes, which have a broad range of substrates and participate in various chemical reactions, as well as physiological or developmental pathways [1–3]. In plants, they were initially documented as the members of the detoxification routes involved in maize defense against herbicides [4]. GSTs catalyze a wide range of reactions, of which the most well-known is the conjugation of glutathione (γ -Glu-Cys-Gly, GSH) to electrophilic compounds. Several GSTs also display glutathione peroxidase (GPOX) activity; thus, they are involved in the reduction of organic hydroperoxides. However, GSTs can participate in a variety of other reactions, interacting with numerous alternative substrates [2,3,5–7]. Several studies have shown that they are involved in the response to various stresses [8–13], including salt stress [14–21].

Soil salinization is an increasing threat to agriculture, hampering growth and development of plants and reducing crop yield [22]. The rate of shoot growth decreases immediately if the salt concentration reaches the threshold level. This is mostly the result

of the osmotic effect of the salt around the roots [23]. Ion toxicity occurs when the slow accumulation of sodium in the shoot inhibits the metabolic processes, which may lead to a reduction in growth [22]. The inhibited, early-stage plant development decreases the yield and puts at risk the quality and quantity of the final products [24].

Over the last few decades, plant responses to salinity have been studied extensively, and it has been demonstrated that, when the salt concentration reaches a critical level, it causes osmotic stress, which is accompanied by ionic toxicity if the plants cannot maintain ion homeostasis. Primary stresses can induce other secondary stresses, especially oxidative stress [23,25,26].

Different biotic and abiotic stress conditions can lead to an increase in several reactive oxygen species (ROS), such as superoxide (O_2^-) or hydrogen peroxide (H_2O_2) [27]. These highly reactive molecules may be harmful for the cells, a phenomenon known as oxidative distress; however, plants can utilize ROS as signaling molecules, especially under mild or moderate stress conditions (eustress) [28].

Various essential antioxidant mechanisms have evolved to regulate ROS levels (especially H_2O_2), such as the ascorbate–glutathione (AsA–GSH) pathway and its related enzymes [29]. The total amounts of non-enzymatic antioxidants (e.g., GSH, AsA, and flavonoids) and their redox status (ascorbate/dehydroascorbate: AsA/DHA; and reduced/oxidized forms of glutathione: GSH/GSSG) play crucial roles in cellular processes [30–33]. The preservation of a high GSH level and GSH/GSSG ratio and, thus, a highly negative glutathione redox potential (E_{GSH}), is important for the efficient functioning of cells and organs [34–36]. The maintenance of redox homeostasis and the highly negative E_{GSH} is achieved through the continuous reduction of GSSG by glutathione reductase (GR) enzymes and/or the increased de novo synthesis of GSH [37,38].

Several reactions may contribute to GSH oxidation during oxidative stress, leading to modifications in the status of this potentially important cellular redox signal. Of the molecules able to oxidize GSH to produce GSSG, the most prominent molecules are the ROS. An outstanding GSSG-generating process is the reduction of DHA to AsA, which is catalyzed by the dehydroascorbate reductase (DHAR) enzyme [39]. However, several studies suggest that other enzymes, like glutaredoxins (GRXs) and GSTs, may also contribute to GSH oxidation to GSSG [39]. Previously, using *Arabidopsis* T-DNA insertional *gst* mutants, we found that several specific AtGSTs participate in the control of the E_{GSH} by elevating the GSH content of cells [20,40]. Moreover, a cultivar-specific correlation was found between the redox status of tomato roots and the expression of certain GST genes in different stress conditions [21].

Based on their protein and gene sequences, plant GSTs can be grouped into 10 classes. Among them, the DHAR, tau (GSTU), and lambda (GSTL) classes are plant-specific [2]. The phi class (GSTF) was also considered to be plant-specific; however, homologue sequences have been discovered in some bacteria and fungi [41]. Generally, the GSTU and GSTF isoenzymes, which show GST and GPOX activities, are the most abundant [3,42,43]. In tomato (*Solanum lycopersicum*) plants, 90 *SIGST* genes were identified and, based on the protein sequences, 57 GSTU, 7 GSTL, 6 GSTF, 6 DHAR, 4 GSTT, 3 EF1B γ , 2 zeta (GSTZ), 2 glutathionyl-hydroquinone reductase (GHR), 2 microsomal, and 1 tetrachlorohydroquinone dehalogenase (TCHQD) isoenzymes could be found [11]. Earlier studies have identified tau class GSTs (Bax-inhibitor GST or BI-GST and LeGSTU1-5) in tomato plants that were able to mitigate oxidative-stress-induced cell death in yeast cells [44,45]. Ding et al. described the importance of one of them (SIGSTU24) in tomatoes, which showed elevated GST activity in the attenuation of low-temperature-induced oxidative stress [12]. *Arabidopsis* plants overexpressing the *LeGSTU2* tomato gene (current name: *SIGSTU18*) exhibited higher salt- and osmotic-stress tolerance, due to elevated proline content and increased antioxidant capacity [17]. Sun et al. assessed the transcriptomic profiles of Moneymaker plants and a salt-tolerant wild tomato (*Solanum pimpinellifolium* ‘PI365967’) and found that several GST genes were expressed at a higher level in the salt-tolerant genotype; however, numerous *SIGST* genes upregulated by salt stress were identified only

in Moneymaker plants [46]. A specific set of *GST* genes is induced during a response to salt or osmotic stresses, and elevated *GST* and/or *GPOX* activities form part of successful osmotic-, dehydration-, or salt-stress responses [11,15,21]. Furthermore, increased *GST* and *GPOX* activities, an accumulation of *GSH*, and an elevated *GSH/GSSG* ratio were reported to characterize resilience to stress in tomato plants [21,47].

The aim of this study was to compare the salt-stress responses of three tomato (*S. lycopersicum*) genotypes commonly cultivated in Hungary (cv. Mobil and cv. Elán F1) and extensively used in research (cv. Moneymaker). Experiments were performed to analyze genotype-specific, stress-inducible *SIGSTs*, and glutathione- or redox-related genes, which are involved in the regulation of stress tolerance. Our results indicate that, in these investigated cultivars, various routes can be observed in the achievement of salt-stress tolerance, in which the regulation of the redox status has particular importance.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Three tomato cultivars (*Solanum lycopersicum* L. Moneymaker, Mobil, and Elán F1) were used in this study. The seeds were germinated for three days in the dark and were transferred in a perlite-packed tube into plastic containers filled with modified Hoagland solution (Supplementary Figure S1) [15]. The plants were grown in a controlled environment with a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, a light/dark period of 12 h each, day and night temperatures of 24/22 °C, and a relative humidity of 55–60% for four weeks. The plants were treated for one week by adding 100 mM of NaCl to the nutrient solution. The NaCl concentration was selected based on our earlier results [15]. The shoot and root lengths and fresh weights were determined using a ruler and digital scale, respectively. The samples for physiological, biochemical, or molecular biological analysis were collected after one week of treatment from the second, fully expanded, young leaves and roots.

2.2. H_2O_2 and Malondialdehyde Content Measurements

The H_2O_2 content was determined using xylenol orange (XO) reagent, according to Mátaí and Hideg [48], with some modifications, as described in Gallé et al. [20]. In short, 100 mg of leaf or root tissue was homogenized on ice with 250 μL of 5% trichloroacetic acid, then centrifuged at $10,000 \times g$ for 15 min at 4 °C. A 100 μL clear supernatant was added to 1 mL of the XO chromophore solution, which consisted of 0.125 mM of XO, 100 mM of sorbitol, 0.25 mM of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and 25 mM of sulfuric acid. The ferric–XO assay is based on the fact that H_2O_2 oxidizes Fe^{2+} to Fe^{3+} at an acidic pH, which forms a colored product with XO, and the reaction is enhanced with sorbitol. The absorbance was measured using a Uvikon 930 spectrophotometer (Uvikon 930 spectrophotometer, Kontron AG, Eching, Germany) at a 570 nm wavelength after 30 min incubation in the dark at room temperature. The quantity of H_2O_2 was determined by utilizing a standard curve, prepared with H_2O_2 concentrations ranging from 0.01 to 0.1 mM.

Malondialdehyde (MDA) formation was detected using the modified method of Ederli et al. [49], as was published in Gallé et al. [21]. A quantity of 100 mg of leaf or root tissue was homogenized in 1 mL of 0.1% TCA, in the presence of 100 μL of 4% butylhydroxytoluene to prevent further lipid peroxidation, then centrifuged at $10,000 \times g$ for 20 min at 4 °C. A 250 μL supernatant was assayed by adding 1 mL of 20% TCA containing 0.5% thiobarbituric acid. After incubation at 96 °C for 30 min, the absorbance was measured at 532 nm and corrected for nonspecific absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.3. Ascorbic Acid and Glutathione Content Determination

Non-enzymatic antioxidant contents (ascorbic acid and glutathione) were determined as described in Bela et al. [50]. A total of 300 mg of leaf or root tissue was homogenized in 1.2 mL of 5% TCA. After centrifugation ($10,000 \times g$, 20 min, 4 °C), the supernatant was used for further measurements. In the used colorimetric assay, Fe^{3+} was reduced by AsA to

Fe^{2+} in an acidic solution, then, Fe^{2+} formed a complex with α - α' -bipyridyl [51]. Since only reduced AsA can be detected by this method, to determine the total ascorbic acid content, 100 μL of supernatant was pre-treated with 10 mM of dithiothreitol (Sigma-Aldrich, Darmstadt, Germany). The ascorbic acid concentration was determined spectrophotometrically at 525 nm. Dehydroascorbate (DHA) content was calculated as the difference between the concentration of total and reduced ascorbate.

The total and oxidized glutathione contents were determined using GR enzymatic assay, measured at 412 nm [21]. Reduced glutathione was masked by pre-treating the samples with 4-vinylpyridine. The content of reduced GSH was calculated by subtracting the concentration of GSSG from the total glutathione concentration. The glutathione (GSH/GSSG couple) reduction potential (E_{GSH}) was determined with the Nernst equation, according to Schafer and Buettner [52].

2.4. Enzyme Activity Determination

The protein contents [53] and enzymatic activities [15,38] were measured spectrophotometrically. A phosphate buffer (0.1 mM, pH 7.0) containing 1% phenylmethylsulfonyl fluoride was used to homogenize 200 mg of fresh leaf or root tissue in the presence of 1 mM of polyvinyl-polypyrrolidone on ice, which was then centrifuged at $10,000\times g$ for 20 min at 4 °C. The supernatant was used for the enzyme activity assays. The activity of GR (EC 1.8.1.7) was assessed by monitoring the increase in absorbance at 412 nm, as 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich, Darmstadt, Germany) was reduced by GSH, which was produced from GSSG [54]. The specific activity was determined as the amount of reduced DTNB in $\mu\text{mol min}^{-1}$ protein mg^{-1} ($\epsilon_{420} = 13.6 \text{ mmol L}^{-1} \text{ cm}^{-1}$).

The artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB; Sigma-Aldrich, Darmstadt, Germany), was used to determine the GST (EC 2.5.1.18) activity [55]. A total 1 U was the amount of the enzyme producing 1 nmol of conjugated product in 1 min ($\epsilon_{340} = 9.6 \text{ mmol L}^{-1} \text{ cm}^{-1}$).

The GPOX (EC 1.11.1.9) activity was measured with cumene hydroperoxide (CHP; Sigma-Aldrich, Darmstadt, Germany) as a substrate [56]. The decrease in NADPH was followed by measuring the absorbance at 340 nm. The nonspecific NADPH decrease was corrected by using additional measurements without CHP. A total of 1 U was equal to the nmol of converted NADPH in 1 min ($\epsilon_{340} = 6.22 \text{ mmol L}^{-1} \text{ cm}^{-1}$).

The DHAR (EC 1.8.5.1) activity was assayed as was published in [15], using the method of Edwards and Dixon [5]. The increase in AsA was followed by measuring the absorbance at 265 nm. The enzyme activity was calculated as the increase in AsA content in nmol min^{-1} protein $^{-1}$ ($\epsilon_{265} = 14.0 \text{ mmol L}^{-1} \text{ cm}^{-1}$).

2.5. RNA Purification and Gene Expression Analyses

High-throughput quantitative real-time PCR (HT-qPCR, Avidin Ltd., Szeged, Hungary) and quantitative real-time PCR (qPCR, qTOWER Real-Time qPCR System, Analytik Jena, Jena, Germany) were applied to detect the expression of *S. lycopersicum* genes in the leaves and roots of the three investigated cultivars after one week of 100 mM NaCl treatment. The genes investigated in this study were selected based on our previous results [15,21] and on the number of W-box elements (TTGAC) found in the 5' cis regulatory regions of the GST genes (Table S1). RNA was extracted from 50–100 mg of tissue using a Quick-RNA Miniprep Kit (Zymo Research, Freiburg, Germany), according to the manufacturer's instructions. To eliminate genomic DNA, DNase treatment was applied, and a further purification step was added to the procedure (RNA Clean & Concentrator-25 Kit, Zymo Research, Freiburg, Germany). Reverse transcription was executed by using 1 μg of RNA and random hexamers with RevertAid reverse transcriptase. The primers used for the qPCR are given in Table S2. The tomato *actin2* and *elongation factor* genes were used as internal controls for data normalization. The data from the HT-qPCR and qPCR were calculated using the $2^{-\Delta\Delta\text{Ct}}$ formula [57]. To demonstrate the variations in gene expression levels due to salt stress, the relative transcript levels were normalized to the control samples

for each gene, and then the $2^{-\Delta\Delta C_t}$ values were log2 transformed. The results were then visualized on a heat map. In the untreated plants, the expression levels were normalized to that of the Mobil cultivar for each gene.

2.6. Promoter Analysis

In silico screening was performed via NewPLACE [58] in the cis regulator sequences of the 5' regulatory regions (1500 bp upstream from the start codon) in the selected *GST* genes, sourced from the Sol Genomics Network (SGN) database (<http://solgenomics.net>, accessed on 1 May 2022). Furthermore, a systematic search in published research papers for the transcription factors (TFs) involved in abiotic-stress responses and salt- or drought-tolerance of tomato was performed. According to the promoter analysis and data found in the literature, eight salt- and osmotic-stress-responsive TFs were selected for further gene expression studies (Supplementary Table S3).

2.7. Determination of Pearson's Correlation Coefficients

Pearson's correlation coefficients were computed using Microsoft Excel 2010 to assess the association between the direct values of redox potential (E_{GSH}) and the expression of specific genes (ΔC_t values; Supplementary Table S4) [57].

2.8. Statistical Analyses

All experiments were repeated at least twice, and the samples originated from three biological replicates. The data presented in this study are the means \pm SD from at least three measurements, unless stated otherwise. The statistical analysis was performed using Microsoft Excel 2010 and Student's *t*-tests (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3. Results

3.1. Compensation of Growth Inhibition and Accumulation of H_2O_2 Due to Salinity Are Different between the Tomato Cultivars

The salt sensitivity of the tomato cultivars was evaluated by measuring the growth of the plants and by determining the H_2O_2 and lipid peroxide contents. Under control conditions, the measured growth parameters were similar in all three of the cultivars. After one week of 100 mM NaCl treatments, the shoot and root weights of the Mobil plants decreased more by the applied salt stress than the Moneymaker and Elán F1 plants, indicating that Mobil cultivar is the most sensitive to salt stress (Figure 1). The epinastic symptoms and the chlorotic older leaves of the salt-treated Mobil plants also support this result (Supplementary Figure S1).

Under control conditions, the H_2O_2 and the lipid peroxidation marker MDA contents in the leaves and roots were similar in the investigated cultivars (Figure 2). The salt treatment increased the H_2O_2 contents in all of the plants (Figure 2A,B) but, interestingly, they contained generally less MDA than the controls. Significant decreases were identified in the Elán F1 leaves and in the roots of all three of the cultivars (Figure 2C,D). Decreased MDA levels can be the result of the activation of antioxidant mechanisms. For example, several AtGSTU isoenzymes efficiently scavenge different reactive carbonyl species [59]; thus, these results suggest a cultivar-specific activation of the ROS-processing mechanisms, due to salt stress.

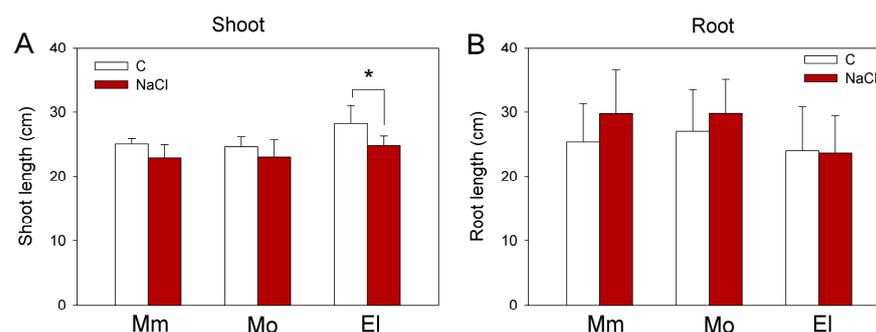


Figure 1. Cont.

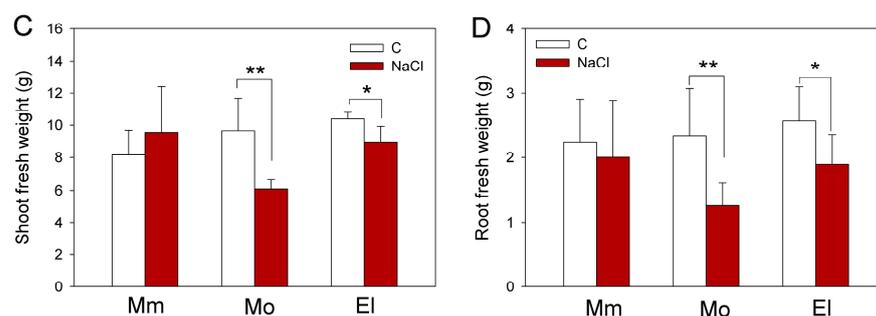


Figure 1. Growth of tomato plants under salt stress. Length and fresh weight of shoots (A,C) and roots (B,D) of five-week-old tomato plants measured under control conditions and after one week of 100 mM NaCl treatment. Mm—cv. Moneymaker, Mo—cv. Mobil, El—cv. Elán F1. Means \pm SD. Significant differences according to the Student's *t*-test (* $p \leq 0.05$, ** $p \leq 0.01$) are indicated.

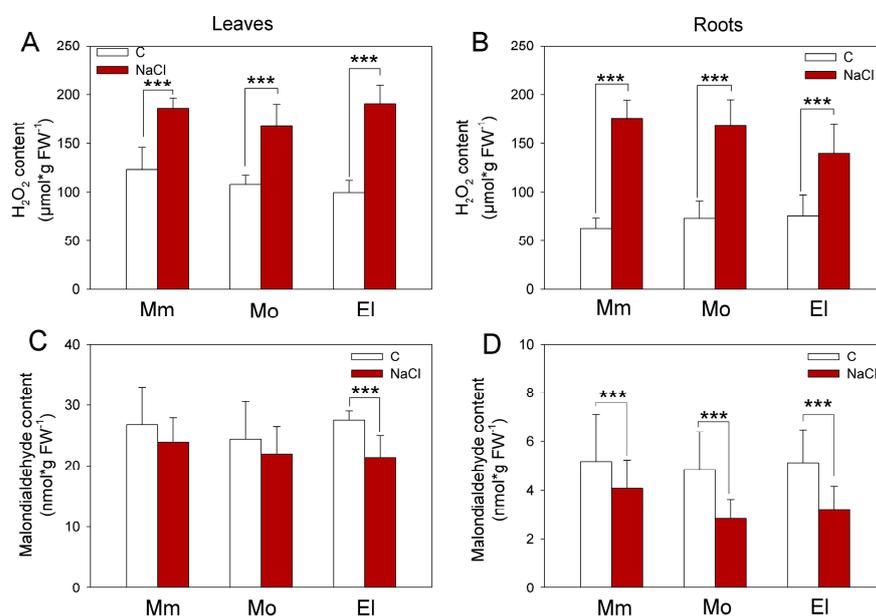


Figure 2. Hydrogen peroxide (H₂O₂) levels (A,B) and malondialdehyde content (C,D) of five-week-old tomato plants under control conditions and after one week of 100 mM NaCl treatment. Mm—cv. Moneymaker, Mo—cv. Mobil, El—cv. Elán F1. Means \pm SD. Significant differences according to the Student's *t*-test (***) $p \leq 0.001$ are indicated.

3.2. The High and/or Stress-Inducible, Non-Enzymatic Antioxidant Levels Promote an Effective Salt-Stress Response

To evaluate the non-enzymatic capacities of the tested tomato cultivars, reduced and oxidized ascorbate and glutathione contents were determined in the control and salt-treated plants. The AsA and GSH levels in the Mobil leaves were significantly lower than those in the Moneymaker and Elán F1 leaves under control conditions and after one-week-long salt stress (Figure 3A,C). Treatment with 100 mM NaCl elevated the AsA content in the roots of all three of the cultivars. The DHA content increased in the Elán F1 roots, thus, the AsA/DHA ratio was lowered; however, it was increased in the Moneymaker and Mobil plants after applying salt stress (Figure 3B). The GSH level was enhanced only in the Moneymaker roots after salt stress. The GSH/GSSG ratio increased in the roots of all of the tomato cultivars compared to the control plants and in the leaves of the Elán F1 plants. The calculated E_{GSH} showed a more negative redox status after salt stress (Table 1), although no significant differences were recorded between the treatments. The Mobil plants had the least reduced E_{GSH} values both in the leaves and roots of the investigated cultivars (Figure 3C,D and Table 1).

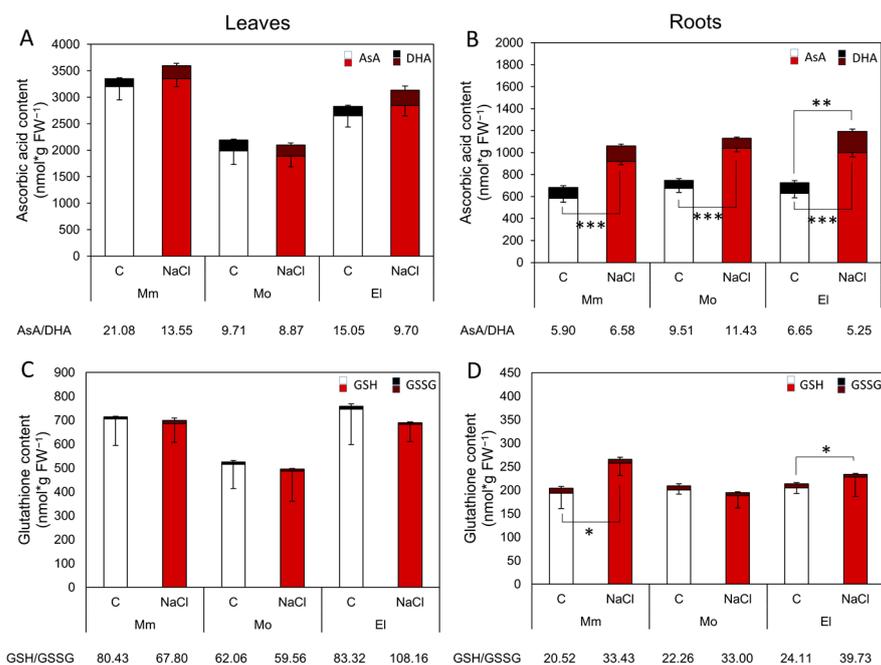


Figure 3. Ascorbic acid (reduced—AsA, oxidized—DHA; (A,B)) and glutathione (reduced—GSH, oxidized—GSSG; (C,D)) contents and their ratios in five-week-old tomato cultivars under control conditions and after one week of 100 mM NaCl treatment. Note the different vertical scales on the leaves (A,C) and roots (B,D). Mm—cv. Moneymaker, Mo—cv. Mobil, El—cv. Elán F1. Means ± SD. Significant differences according to the Student’s *t*-test (* *p* ≤ 0.05, ** *p* ≤ 0.01, *** *p* ≤ 0.001) are indicated.

Table 1. Glutathione redox potential (E_{GSH}) of hydroponically grown five-week-old *Solanum lycopersicum* Moneymaker (Mm), Mobil (Mo), and Elán F1 (El) plants after one week of 100 mM NaCl treatment. No significant differences were detected according to the Student’s *t*-test.

	E_{GSH} (mV)			
	Leaves		Roots	
	Control	NaCl	Control	NaCl
Mm	−321.4 ± 4.5	−319.9 ± 3.0	−286.7 ± 10.7	−292.4 ± 8.4
Mo	−312.7 ± 4.7	−312.9 ± 5.7	−285.5 ± 11.1	−290.1 ± 7.7
El	−323.9 ± 5.2	−326.0 ± 8.8	−287.4 ± 6.7	−292.3 ± 9.0

3.3. Higher Glutathione Reductase, Peroxidase, and Inducible Glutathione Transferase Activities Contribute to the Successful Salt-Stress Response of Tomatoes

In order to characterize the antioxidant enzymes during salt stress, activities of GST, GR, DHAR, and GPOX were determined in the tested tomato cultivars. Under control conditions, the investigations revealed significantly higher GR activities in the Mobil roots, as well as slightly higher GST activities in the leaves compared to the Moneymaker and Elán F1 plants; however, its leaves showed the lowest DHAR activities (Figure 4). After seven days of salt stress, the DHAR activity was inhibited in the roots of the Moneymaker plants. In contrast, elevated DHAR activities were measured in the roots of both the Mobil (by 198.3%) and Elán F1 plants (by 329.8%) (Figure 4B). The one-week-long 100 mM NaCl treatment decreased the GR activities in the leaves of the Mobil plants and in the roots of all of the cultivars, but this decrease was most marked in the Mobil plants (Figure 4C,D). The GST activities were elevated in Moneymaker and Elán F1 leaves (by 160.1% and 91.7%, respectively) and roots (by 44.7% and 81.6%, respectively) after one week of 100 mM NaCl treatment. On the other hand, in the leaves of the Mobil plants, the GST activities decreased, while in the roots, only a slight increase was observed, due to the applied salt stress, compared to the control conditions (Figure 4E,F). The activity of GPOX remained

unchanged in the leaves of all of the genotypes in response to stress but increased in the roots. The least change was observed in the Mobil cultivar (Figure 4G,H).

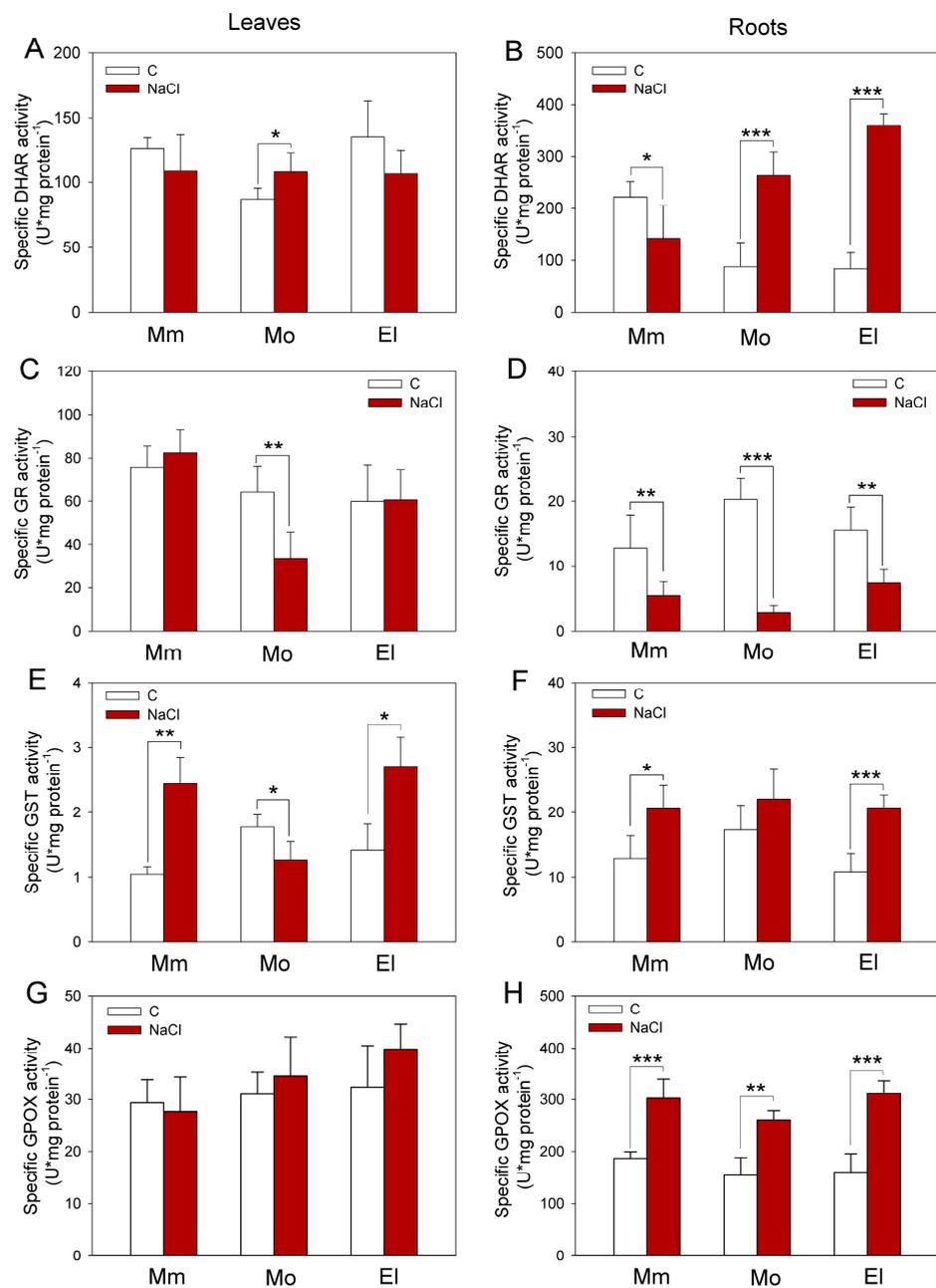


Figure 4. Specific dehydroascorbate reductase (DHAR), glutathione reductase (GR), transferase (GST), and peroxidase (GPOX) activities of tomato cultivars in five-week-old tomato leaves (A,C,E,G) and roots (B,D,F,H), respectively, under control conditions and after one week of 100 mM NaCl treatment. Mm—Moneymaker, Mo—Mobil, El—Elán F1. Means \pm SD. Significant differences according to the Student's *t*-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) are indicated.

3.4. Expression Changes in the GR and Selected GST Genes in Salt-Treated Tomato Cultivars

The transcript levels of the 2 GR and 27 examined GST genes were detected by HT-qPCR from the leaves and roots of five-week-old tomato plants, whether or not they were subjected to the 100 mM NaCl treatment. The expression levels were generally the lowest in the Mobil plants, thus, the data for each gene from the Moneymaker and Elán F1 plants were normalized to those of Mobil. The transcript amount of the investigated genes under control conditions are shown in Supplementary Figure S2.

To represent the effect of the 100 mM NaCl treatment on the transcription of the selected genes, the data were normalized to the untreated leaves and roots of each cultivar and for each gene. The results are displayed as a heat map (Figure 5). In the leaves, four genes (*DHAR3*, *GSTF1*, *GSTU15*, and -32) were downregulated in all of the cultivars after one week of salt stress. Beyond this, six genes showed downregulation in the Moneymaker leaves, two in the Mobil leaves, and five in the Elán F1 leaves, compared to the untreated leaves. At the same time, the expression of *GSTU30* was upregulated in the Moneymaker and Mobil plants, *GSTU54* was upregulated in the Moneymaker plants, and *GR1*, *GR2*, and *GSTU14* were upregulated in the Elán F1 plants (Figure 5A).

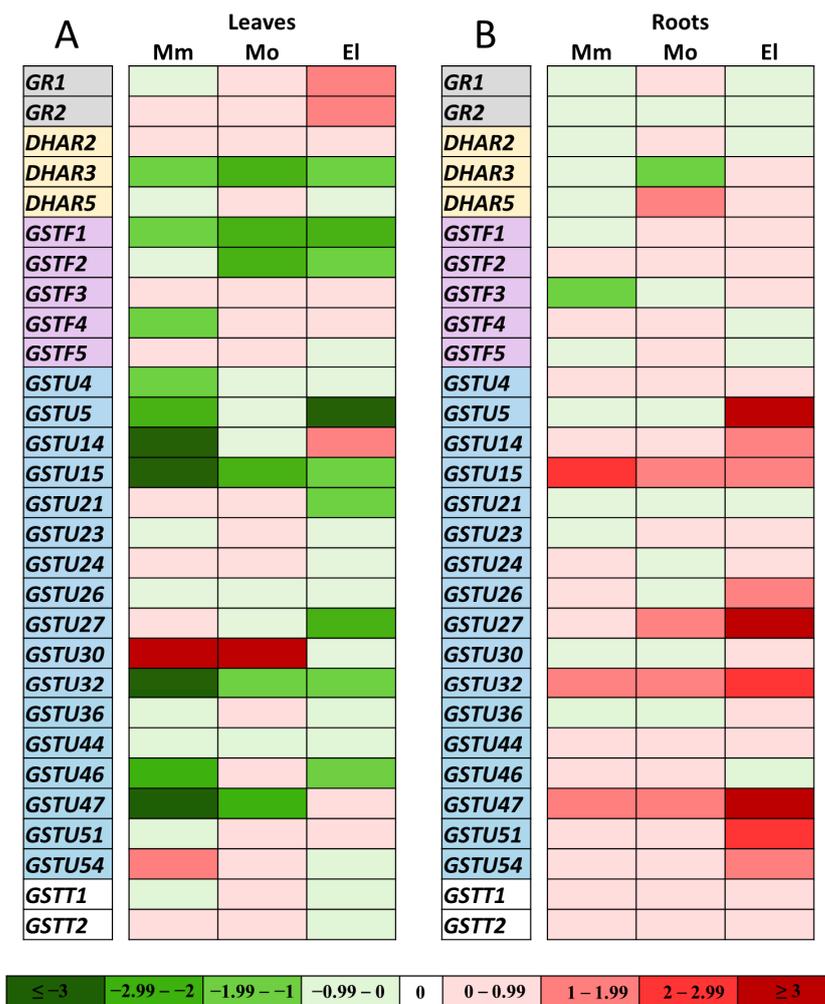


Figure 5. Heat map of the expression levels of 2 *Solanum lycopersicum* glutathione reductases (*GR1* and *GR2*) and 27 selected genes belonging to 4 GST classes (*DHAR*, *GSTF*, *GSTU*, and *GSTT*) determined in salt-treated, five-week-old Moneymaker (Mm), Mobil (Mo), and Elán F1 (El) tomato cultivars. The relative transcript amounts of the genes in the leaves (A) and roots (B) were determined by HT-qPCR after one week of 100 mM NaCl treatment. The expression of the genes was normalized first by the average of *actin2* and *elongation factor 1 α* genes, and second by the average transcript amount of each gene in untreated cultivars. The \log_2 transformation of $2^{-\Delta\Delta C_t}$ data are presented. The green color represents repression, while the red color represents activation, as indicated on the color scale bar. The presented data are from two biological replicates.

In the roots, *GSTU15*, -32, and -47 were upregulated in all three of the cultivars after the salt treatments. In addition, the expression levels of six genes were upregulated in the Elán F1 plants and two in Mobil plants, by comparison with the untreated controls

(Figure 5B). Downregulation was detected only at *GSTF3* in the Moneymaker plants and *DHAR3* in the Mobil plants after the applied salt stress.

3.5. Correlation Analysis of Gene Expressions

To reveal the relationship between the redox potential and the gene expression changes due to the NaCl treatment, Pearson’s correlation coefficients (R values) were determined. We used the direct (negative) E_{GSH} and ΔCt values (Supplementary Table S4) for the calculation, in which a strong positive correlation means that, as the root tissues become more reduced, the expression level of a given gene increases, while in the case of a strong negative correlation, the transcript level of a specific gene increases as the root tissues become more oxidized. The correlation analysis resulted in considerable differences in the R values among the investigated cultivars and organs.

Comparing the R values in the leaves of the cultivars revealed strong positive correlations between the changes in the expression of the selected genes and the E_{GSH} values in almost all cases in the Moneymaker leaves, while in the case of the Elán F1 leaves, strong negative correlations were detected. For example, while *DHAR3*, *GSTU4*, -5, -27, and -46 showed positive correlations with the redox potential in the Moneymaker leaves, a very strong negative correlation was found in the same set of genes in the Elán F1 leaves (Figure 6A). The positive correlations implicate the protective effect of the *GSTs* on the redox potential, while the negative correlations may indicate that the increasing damages enhanced the expression of the *GST* genes.

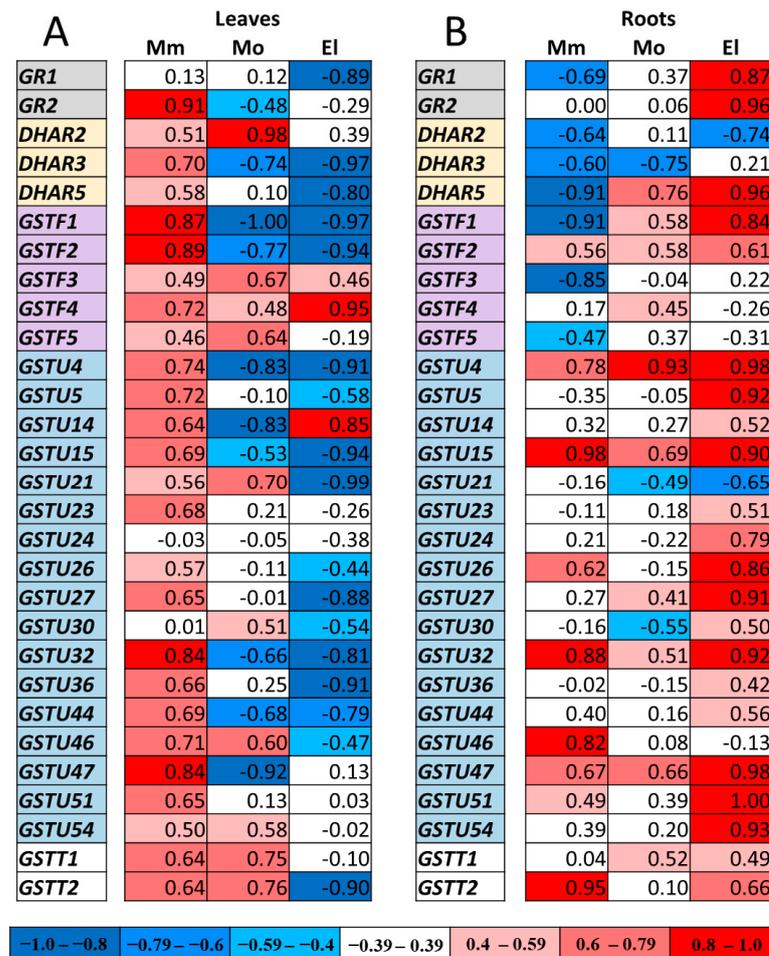


Figure 6. Correlation analysis between the glutathione redox potential (E_{GSH}) and the expression of selected genes (ΔCt values), measured under different conditions in the leaves (A) and roots (B) of Moneymaker (Mm), Mobil (Mo), and Elán F1 (El) tomato plants. Positive correlations are highlighted in red and negative correlations are highlighted in blue.

Regarding the roots, positive correlations were found between the expressions of *GSTF2*, *-U4*, *-U15*, *-U32*, *-U47*, and the E_{GSH} in all of the cultivars (Figure 6B). Interestingly, the transcription of several GSTs related differently to the redox state in the roots rather than in the leaves. As an example, a strong positive correlation was found between the redox potential and the expression of the *GSTF1* gene in the MoneyMaker leaves, while its transcript amount showed a strong negative correlation with the redox state in the roots (Figure 6A,B).

3.6. Involvement of the Cultivar-Specific, Stress-Responsive Transcription Factors in the Salt-Stress Response in Tomato Roots

Based on the results of the gene expression and correlation analysis, *GSTF2*, *GSTU15*, *-U32*, and *-U47* genes were selected for further in silico studies. These sequences were either upregulated (*GSTU15*, *-U32*, and *-U47*) or expressed at control levels (*GSTF2*) after one week of salt treatment in the roots of all of the cultivars. These showed a positive correlation with the further reduced redox potential (Figures 5 and 6), indicating their important involvement in the primary defense mechanisms against salt stress. The in silico analysis of the 5' regulatory region of *GSTU15*, *-U32*, and *-U47* genes revealed the presence of several W-box (TTGAC or TGAC), TGACG-motif (TGACG), and ABRE (ACGTG) elements in their promoter region (Figure 7). Additionally, *GSTU32* had a GCC-box (GCCGCC) and *GSTU47* had a DRE (CCGAC) element. In the promoter region of *GSTF2*, certain redox-responsive cis regulatory elements were also identified (Figure 7).

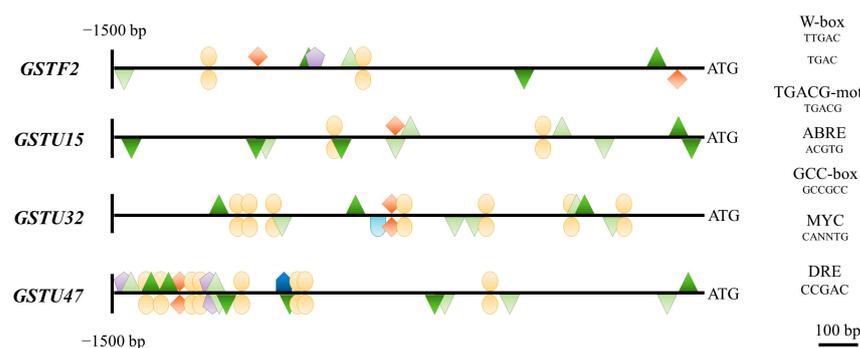


Figure 7. Predicted cis regulatory elements found in the upstream regulatory region of *GSTF2*, *GSTU15*, *GSTU32*, and *GSTU47* using the NewPLACE database (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>, accessed on 25 February 2023).

Accordingly, eight salt- and osmotic-stress-responsive TFs were selected for further gene expression studies, based on the data found in the literature and the results of the in silico promoter analysis (Supplementary Table S3). A comparison of their transcript levels in the leaves and roots of the three cultivars under control conditions showed low levels of differences (Supplementary Figure S3).

However, significant changes occurred in their transcription after one week of salt treatment (Figure 8A,B). In the leaves, several of them were downregulated, compared to the untreated samples (*DREB2* in MoneyMaker; *WRKY7* and *WRKY39* in Mobil and Elán F1; and *WRKY3* and *WRKY74* in the Elán F1 plants). Upregulation was detected only in the case of *WRKY39* and *WRKY74* in the MoneyMaker leaves. In contrast, in the roots, most of the TFs were upregulated after one week of 100 mM NaCl treatment. *WRKY3* and *DREB2* were upregulated in all of the cultivars, *WRKY72* in the MoneyMaker and Mobil cultivars, and *DREB1* both in the MoneyMaker and Elán F1 cultivars, while *WRKY39* and *MYC2* were upregulated in the MoneyMaker and Elán F1 cultivars. Downregulation was only observed in the *WRKY7* gene in the salt-stressed Mobil roots (Figure 8A,B).

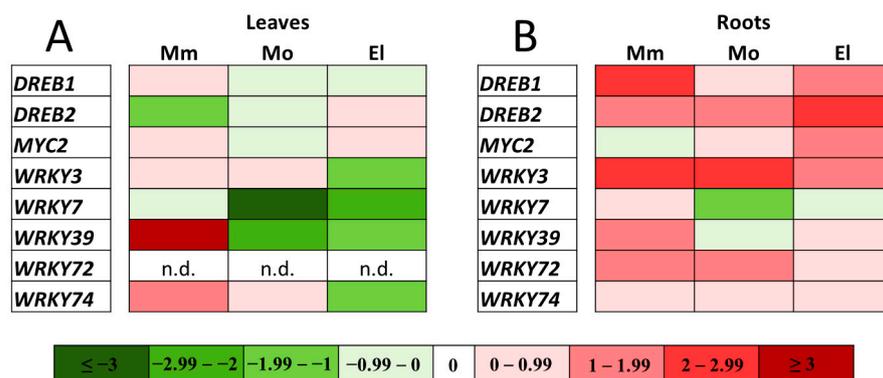


Figure 8. Heat map of the expression levels of eight *Solanum lycopersicum* transcription factor genes. The relative transcript levels of the selected genes were determined in five-week-old tomato (Moneymaker—Mm, Mobil—Mo, and Elán F1—El) leaves (A) and roots (B) by qPCR after one week of 100 mM NaCl treatment. For this, the expression values were normalized, firstly by the average expression of the *actin2* gene, and secondly by the average transcript levels of each gene from the untreated cultivars. The log₂ transformation of the 2^{-ΔΔCt} data are presented as a heat map. The green color represents repression, while the red color represents activation, as indicated on the color scale bar. The presented data have been obtained from two biological replicates. n.d.—not detected.

The correlation analysis performed between the redox potential and gene expression changes in the TFs showed differing results in the cultivars (Figure 9). In the leaves, negative correlations were found between the expression of WRKY39 and the *E_{GSH}* values in all of the cultivars, and that of several other TFs in one or two genotypes. In the roots, a strong positive correlation was found between the more negative GSH redox potential and the upregulation of WRKY3 in all of the genotypes; furthermore, the strong positive correlations found in the case of WRKY72, DREB1, or DREB2 and *E_{GSH}* values in the Moneymaker and Elán F1 plants also suggest their importance in the salt-stress responses (Figure 9).

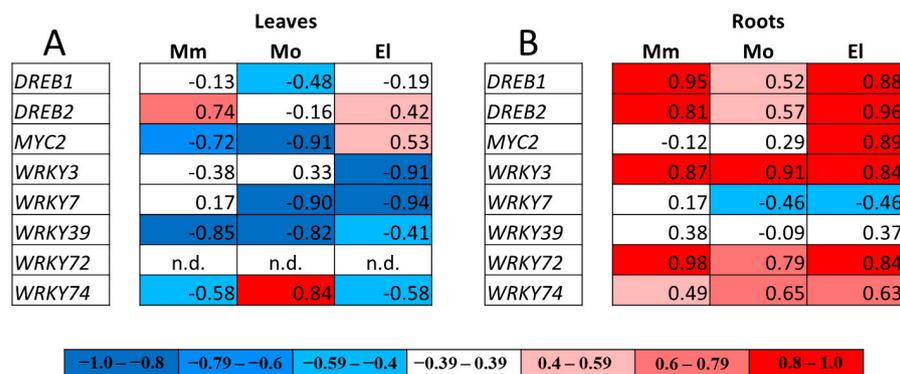


Figure 9. Correlation analysis on the basis of the glutathione redox potential (*E_{GSH}*) and expression of selected genes (ΔC_t values), measured under different conditions in the leaves (A) and roots (B) of Moneymaker (Mm), Mobil (Mo), and Elán F1 (El) tomato plants. Red color highlights the positive correlations and the blue color highlights the negative correlations. n.d.—not detected.

4. Discussion

4.1. Efficient Salt-Stress Responses Include Redox Status Changes and Activation of ROS-Processing Mechanisms to Adjust Plant Growth

It was reported that the growth parameters of the salt-tolerant wild relatives of the tomato (like *Solanum pennellii*, *Solanum galapagense*, *Solanum cheesmaniae*, and *Solanum chilense*) are generally behind the cultivated variants under optimal conditions; however, these can surpass the domesticated tomatoes under high levels of salt stress [60]. An effective salt response was characterized, not only with restored shoot growth, but also with

increased growth and higher root biomass accumulation, under severe salinity stress [61]. In our experiments, a comparison of the salt-stress responses of three tomato cultivars revealed that the Moneymaker cultivar preserved better its growth after one week of salt stress, but significant growth inhibition was detected in the Mobil plants (Figure 1), indicating that the Moneymaker cultivar is the most salt tolerant, while Mobil is the most sensitive cultivar. Although the Moneymaker and Elán F1 cultivars could maintain their growth during the seven days of the 100 mM NaCl treatments, higher H₂O₂ levels were measured in all of the cultivars after salt stress, which was not associated with elevated MDA contents (Figure 2), suggesting that the elevated ROS may serve other, presumably signaling, purposes.

It is well documented that endogenous ROS levels, changes in the redox status, non-enzymatic antioxidants, and enzymes serve as key signaling agents; moreover, the enhancement of GSH and AsA pools promotes adaptation to various types of stresses [31,62–64]. Using the redox-sensitive roGFP redox probe to analyze the in vivo changes in *A. thaliana* roots after NaCl treatment, Jiang et al. have shown that there is a rapid change in the redox profile, but the redox state can be restored within 24 h [65]. Changes in the redox state, even a few mV shift, implies modulation of cellular signaling and alterations in gene expression profiles, including reprogramming the expression of a set of hormone- and defense-related genes [36,65,66]. The GSH redox-state-dependent transcriptional changes in *GST* genes were reported either in plants with low GSH levels [36] or after applying different stress treatments [19–21,66]. However, other than the ROS-processing role of GSTs, their involvement in altering metabolic and growth responses should be elucidated.

4.2. The Upregulation of Several Genes of GSH-Related Enzymes Correlates with the More Reduced GSH Redox Status

The multigenic superfamily of GSTs is one of nature's most versatile enzymes. Besides their well-known reaction, namely, the conjugation of GSH to electrophilic compounds, numerous GSTs act as GSH-dependent peroxidases by reducing organic hydroperoxides [3,67]. The earlier results obtained from salt-treated *Atgstu19*, *Atgstu24*, *Atgstf8*, and *Atgstf9* mutants have shown that *A. thaliana* GST isoenzymes have different roles in determining the level of specific ROS species and participate in the establishment of the root redox status [19,20,40]. In tomato roots, one day of 150 mM NaCl treatment increased the expression of the *GST* genes belonging to the tau, phi, and theta groups, along with GST and GPOX activities [21]. The upregulation of *GST* genes under several stress conditions was related to the elevated total GST enzyme activity [11]. Our results showed that GST activities were elevated in the Moneymaker and Elán F1 cultivars after one week of salt stress, but not in the salt-sensitive Mobil variety (Figure 4). In our earlier study, elevated GST and GPOX activities were detected in the untreated roots of Moneymaker compared to Ailsa Craig cultivars. Moreover, the importance of the redox status and its genotype-specific significance in stress responses were indicated [21]. In this study, differences were found in the pool and redox status of the AsA and GSH of the three tomato cultivars, even under control conditions, which was augmented by salt stress. Lower AsA and GSH levels were measured in the leaves of the Mobil plants, and, compared to the other cultivars, the lowest AsA/DHA and GSH/GSSG ratios and more positive E_{GSH} values were observed in the leaves. While in the Moneymaker cultivar, the accumulation of GSH contributed to the maintenance of redox homeostasis, in the Mobil and Elán F1 plants, the elevated DHAR activities could support the protection of the GSH pool (Figures 3 and 4). DHARs are also involved in balancing redox homeostasis in plants, as they catalyze the GSH-dependent reduction of DHA to AsA [1,68,69]. Moreover, increased AsA levels and the overexpression of DHARs were reported to promote a tolerance to multiple stresses [70–76].

The main basis of the ROS/redox regulation is primarily based on the redox state of Cys residues, and among the main molecules affected are thioredoxin (TRX) and GSH [77]. Regarding GST proteins, DHARs and GSTLs with Cys residues in their active site can be modified by GSH to form mixed disulfides, while GSTZ1, GSTF7, and GSTU19 were

identified as S-glutathionylated (thiolated) proteins in *Arabidopsis* [1,78]. Several GST isoenzymes were annotated as phosphopeptides, while, in others, methionine undergoes sulfur oxidation or cysteines form reversible intramolecular disulfide bonds, which affect the activity of the GSTs [79–81].

Previously, we found that a remarkably high number of *GST* genes were expressed at a higher level in the untreated roots of MoneyMaker plants compared to the less stress-resistant Ailsa Craig cultivar. Furthermore, the transcript levels of several *GST* genes and other genes coding GSH-related enzymes showed a positive correlation with elevated GSH levels and a further reduced E_{GSH} after 24 h of the applied treatments, especially in MoneyMaker plants [21]. In the present experiments, significant but diverse correlations were found between the expression of the investigated *GSTs* and the calculated GSH redox potentials, depending on the organs and genotypes. Positive correlations might indicate that the increased expression of those genes has a protective effect on the tissue redox status. The members of the investigated four *GST* classes are involved in the detoxification of stress metabolites, thus, our results strengthen the idea that several *GSTs* are part of the well-ordered systematic antioxidant response. In the leaves, 22 *GST* genes were expressed at a higher level in the MoneyMaker cultivars compared to the Mobil under control conditions (Supplementary Figure S2A). Although fewer differences were found in the expression of the investigated genes in the roots of the untreated tomato cultivars than in their leaves, our results show that the tolerant genotype possess more efficient protective mechanisms, which might prevent oxidative damages in the MoneyMaker cultivar. In the case of other genotypes, negative correlations were found between the expression of several *SIGSTs* and the redox potential (Figure 6). This suggests that, in such a case, the defense mechanisms might be activated when the oxidative damages were rather high (the tolerance mechanisms positively correlate with oxidative damage).

4.3. Strong and Cultivar-Specific Correlations Were Found in the Changes in Redox Potential and the Expression of TF Genes

In plants, transcriptional responses are regulated either by stress- or ROS-derived changes in the signal transduction mechanisms or by direct or indirect ROS-induced redox regulation [82]. The 5' regulatory regions (1500 bp upstream from the start codon) of the investigated *GST* sequences found on the SGN (<http://solgenomics.net>, accessed on 1 May 2022) homepage were screened for the presence of oxidative stress- and redox-related cis regulatory elements, in particular, using the NewPLACE database. A number of redox-responsive cis elements were found in the regulatory regions of the *GST* genes, such as W-box (TTGAC), TGACG-motif (TGACG), ABRE (ACGTG), and GCC-box (GCCGCC) motifs, potential binding sites for AP2, bZIP, and WRKY-type transcription factors (Supplementary Table S1). Investigating the expression of eight stress-responsive TFs (*DREB1*, *DREB2*, *MYC2*, *WRKY3*, -7, -39, -72, and -74) using qPCR revealed potential regulatory differences between these cultivars. In the leaves, the upregulation of the TF genes due to the applied salt treatment (*WRKY74* and *WRKY39*) was detected only in the MoneyMaker plants. In the roots, upregulated expression levels were found in the case of five, four, and three TFs in the MoneyMaker, Elán F1, and Mobil cultivars, respectively, after salt stress, from which we highlight here the TFs belonging to the WRKY and DREB families.

In tomatoes, 81 *WRKY* genes were annotated, some of which have already been reported to be stress-induced [83]. Among several others, *SIWRKY8*, *SIWRKY39*, *SIWRKY72*, and *SIWRKY74* showed induction during salt, drought, and biotic stresses [83,84]. *SIWRKY39* is a positive regulator in tomatoes' response to biotic and abiotic stresses; moreover, the over-expression of this TF activates the expression of pathogenesis- and environmental-stress-related genes [85]. *WRKY72* has been described to play an important role in conferring drought tolerance in tomato plants [86]. *DREB1* and *DREB2* are dehydration-responsive element-binding TFs, which affect the development of vegetative and reproductive organs, participate in abscisic acid signaling and in the drought-stress response, and, furthermore, mediate salt-stress tolerance in tomato plants [87–89]. The strong positive correlations

between the expression of their genes and the redox potential in tomato roots also indicate their involvement in the maintenance of the redox status of tissues.

A schematic model of the regulatory mechanisms involved in the induced expression of tomato *GSTs* is represented in Figure 10. A relationship was found between the expression of the *WRKY3* gene and several plant hormones, such as salicylic acid, cytokinin, indole-3-butyric acid, and jasmonoyl-isoleucine [90,91]. Based on our findings, *WRKY3*, *WRKY72*, *DREB1*, and *DREB2*, together with other stress-related hormones and redox-state-dependent signaling events, upregulate the expression of *GSTU15*, *GSTU32*, and *GSTU47*, which may be partially responsible for the increased GST activity, leading to more successful stress tolerance.

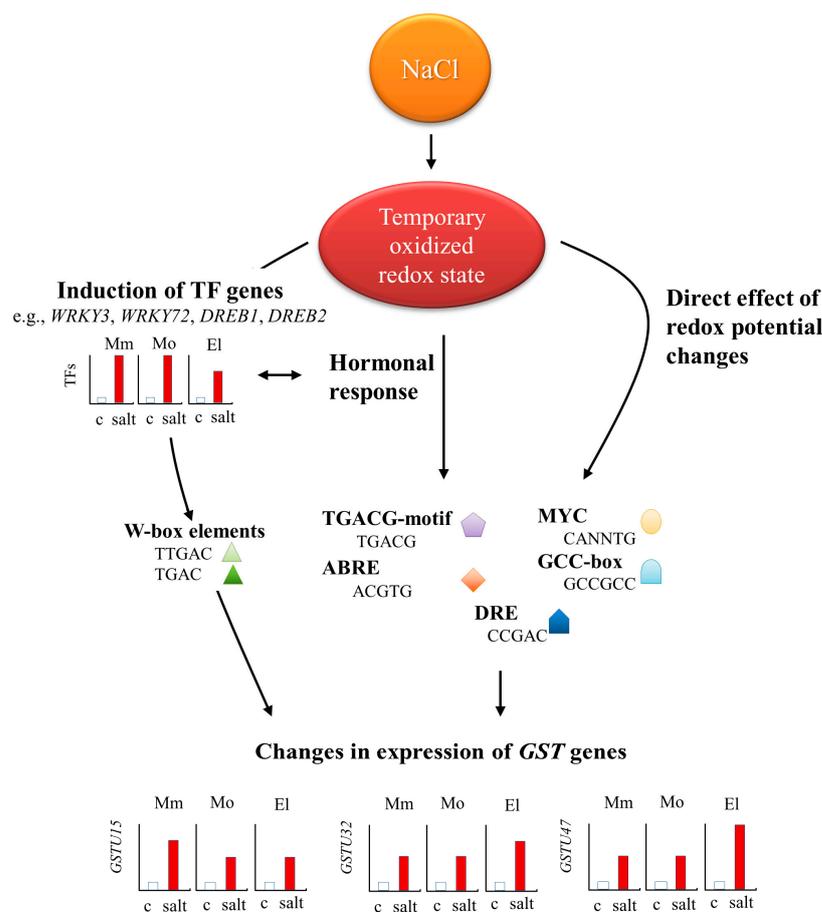


Figure 10. Schematic model summarizing the potential regulators of *GST* gene expression after one week 100 mM NaCl treatments in tomato plants, based on our results and data found in the literature [90,91]. The salt treatment supposedly disturbed the redox homeostasis, which led to a temporary oxidized redox state within the cells. These changes in the redox potential may have had a direct effect on the expression of *GSTs*, but also led to the induction of the TF genes, such as *WRKY3*, *WRKY72*, *DREB1*, and *DREB2*. Presumably, the expression of these genes was also influenced by hormonal changes or, for example, 5' cis regulatory elements could also induce the expression of *GSTU15*, *GSTU32*, and *GSTU47* genes in all investigated cultivars. Abbreviations: Moneymaker—Mm; Mobil—Mo; Elán F1—El; control—C; salt—one week of 100 mM NaCl treatment.

5. Conclusions

Our results indicate that the Moneymaker and Elán F1 cultivars were able to restore their growth during one-week-long salt stress, indicating a more efficient stress response compared to the Mobil cultivar, the growth of which decreased after the stress treatment. These results indicate that lower GSH and AsA levels, more positive glutathione redox potential, and decreased GR and GST activities can negatively influence the growth of

Mobil plants during salt stress. Since the Moneymaker and Elán F1 plants had higher GR and inducible GST activities, along with elevated non-enzymatic antioxidant levels, the combination of these factors is likely to have contributed to the salt tolerance of these plants. Analyses between the redox potential values and the gene expression data revealed several strong and cultivar-specific correlations, highlighting the importance of GSTs in the complex stress responses of tomato plants. Transcription factors *WRKY3*, *WRKY72*, *DREB1*, and *DREB2* can be involved in *GST* gene induction, which may be partly responsible for increased GST activity, leading to more efficient stress tolerance in tomato plants. These results confirm how a specific set of *GSTs* show a genotype-specific spatiotemporal expression pattern in the salt response of tomato cultivars, and these alterations in their expression determine their flexibility under stress conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox12091682/s1>, Figure S1: Image of a five-week-old tomato plant under control conditions and after one week of 100 mM NaCl treatment; Figure S2: Expression of *GRs* and *GSTs* under control conditions; Figure S3: Expression of TFs in control plants; Table S1: Number of redox-related cis regulator sequences in *GSTs*; Table S2: Primer pairs used in this study; Table S3: Selection of the TFs; Table S4: Δ Ct values used in correlation analysis. References [92,93] are cited in the supplementary materials.

Author Contributions: Conceptualization, E.H. and J.C.; methodology, E.H., J.C. and Á.G.; validation, E.H. and J.C.; formal analysis, K.B.; investigation, E.H., K.K., Á.B.H., B.T. and A.P.; writing—original draft preparation, E.H. and J.C.; writing—review and editing, E.H., J.C., Á.G., Á.B.H., B.T. and K.B.; visualization, E.H.; supervision, E.H.; project administration, E.H.; funding acquisition, J.C. and E.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Hungarian National Research Development and Innovation Office, grant numbers NKFIH K 138589 and PD 131909.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article and supplementary materials or are available upon request from the corresponding author.

Acknowledgments: We are grateful to Erzsébet Porkoláb for her exemplary technical assistance. We also wish to say a special thanks to Csaba Hornyák for providing the Mobil and Elán F1 tomato seeds used in the experiments. The authors thank the reviewers for their thorough review and helpful advice on interpretation of the results.

Conflicts of Interest: The authors declare no conflict of interest.

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