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Research article

Glutathione transferase supergene family in tomato: Salt stress-regulated expression of representative genes from distinct GST classes in plants primed with salicylic acid

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ABSTRACT

A family tree of the multifunctional proteins, glutathione transferases (GSTs, EC 2.5.1.18) was created in *Solanum lycopersicum* based on homology to known *Arabidopsis* GSTs. The involvement of selected *SlGSTs* was studied in salt stress response of tomato primed with salicylic acid (SA) or in un-primed plants by real-time qPCR. Selected tau GSTs (*SlGSTU23*, *SlGSTU26*) were up-regulated in the leaves, while GSTs from lambda, theta, dehydroascorbate reductase and zeta classes (*SlGSTL3, SlGSTT2, SlDHAR5, SlGSTZ2)* in the root tissues under salt stress. Priming with SA exhibited a concentration dependency; SA mitigated the salt stress injury and caused characteristic changes in the expression pattern of *SlGSTs* only at 10^{-4} M concentration. *SlGSTF4* displayed a significant up-regulation in the leaves, while the abundance of *SlGSTL3*, *SlGSTT2* and *SlGSTZ2* transcripts were enhanced in the roots of plants primed with high SA concentration. Unexpectedly, under high salinity the *SlDHAR2* expression decreased in primed roots as compared to the salt-stressed plants, however, the up-regulation of *SlDHAR5* isoenzyme contributed to the maintenance of DHAR activity in roots primed with high SA. The members of lambda, theta and zeta class GSTs have a specific role in salt stress acclimation of tomato, while *SlGSTU26* and *SlGSTF4*, the enzymes with high glutathione conjugating activity, characterize a successful priming in both roots and leaves. In contrast to low concentration, high SA concentration induced those GSTs in primed roots, which were up-regulated under salt stress. Our data indicate that induction of GSTs provide a flexible tool in maintaining redox homeostasis during unfavourable conditions.

1. Introduction

Plant glutathione transferases (EC 2.5.1.18) are a diverse group of multifunctional enzymes that generally use reduced form of glutathione (GSH; γ-glu-cys-gly) in distinct catalytic reactions and they have a role in the detoxification of xenobiotics and toxic lipid peroxides, reduction of dehydroascorbate, in primary metabolism and biochemical reactions of secondary products such as flavonoid derivatives (Marrs, 1996). In addition to their enzymatic role, glutathione transferases (GSTs) also function as non-catalytic carrier proteins, which are required for vacuolar uptake of anthocyanins (Kitamura et al., 2004). They are also involved in signalling mechanisms following exposure to UV light (Loyall et al., 2000) and participate in the regulation of plant growth and development (Jiang et al., 2010).

The structure and sequences of GST genes show high variability, but the GST proteins share a structural homology based on the thioredoxin/glutaredoxin-like N-terminal and a larger C-terminal domain that can be considered common structural elements characterizing the GST superfamily. The GSH-binding site (G site) is localized near the N-terminus of the molecule and GSTs promote proton abstraction from the sulfhydryl group of GSH by a serine residue in the active site. The co-substrate binding site (H-site) is positioned adjacent to the G-site and these enzymes exhibit

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; DHAR, dehydroascorbate reductase; GPOX, glutathione peroxidase; GST, glutathione transferase; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance.

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glutathione conjugating activities along with isomerisation and peroxidase reactions. GSTs belonging to dehydroascorbate reductase (DHAR) and lambda classes (GSTLs) contain a cysteine residue in the G site in the place of serine (Dixon and Edwards, 2010a). The Arabidopsis genome contains 55 GST genes which can be divided into eight classes: tau, phi, theta, zeta, lambda, DHAR, tetrachlorohydroquinone dehalogenase (TCHQD) and microsomal GSTs with 28, 13, three, two, three, four and one–one members, respectively (Edwards et al., 2010). Recently, based on structural similarities, the γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ) has been regarded as a member of the plant GST family (Lan et al., 2009; Liu et al., 2013). There have been extensive duplications of the plant-specific phi and tau class GST genes, which are often stress inducible, they have xenobiotic-detoxifying activities and/or are involved in defence-related secondary metabolism (Dixon and Edwards, 2010a; Dixon et al., 2002a). The theta class has a putative role in detoxifying oxidised lipids, the zeta class functions in tyrosine catabolism, while the lambda and DHAR GSTs are possibly glutathione-dependent reductases (Dixon and Edwards, 2010b).

Direct roles of GSTs in reducing oxidative damage were reported in several plant species (Cummins et al., 1999; Edwards et al., 2000; Kilili et al., 2004), and transgenic plants overproducing a GST gene exhibited significant oxidative stress tolerance (Roxas et al., 2000; Zhao and Zhang, 2006; Ji et al., 2010), furthermore their involvement has also been suggested in controlling the programmed cell death (Kampranis et al., 2000). Using the mammalian pro-apoptotic Bax protein to detect tomato genes involved in the protection against Bax-induced cell death, Kampranis et al. (2000) identified a tau class tomato GST. This "Bax-inhibitor GST" (BI-GST, SlGSTU24) possesses GSH conjugating (GST) and weak glutathione peroxidase (GPOX) activity, and it was found that its expression in yeast significantly enhanced resistance to H_2O_2-induced stress, re-established the mitochondrial membrane potential and brought the total GSH levels back to normal. Five GSTU (LeGSTU1-5) proteins which readily formed heterodimers with BI-GST have been identified later in tomato, these proteins were able to protect yeast cells from prooxidant-induced cell death (Kilili et al., 2004).

Other GSTs have an important role in hormone metabolism or action and they can be induced by auxin, ethylene, abscisic acid, jasmonic acid and salicylic acid (Marrs, 1996; Moons, 2005).

Salicylic acid (SA) is a common phenolic compound that can function as a plant growth regulator affecting various processes from growth, development, interaction with other organisms to environmental stress responses (Raskin, 1992). SA is considered to be an important signalling molecule, which is involved in local and systemic acquired resistance (SAR) induced by various pathogens and wide range of abiotic stresses (Hayat et al., 2010). Exogenously applied SA may be used as a priming or hardening compound to enhance the resistance of plants to biotic and abiotic stresses (Hayat et al., 2010; Joseph et al., 2010). In our earlier results we found that priming of tomato plants with SA mitigated the salt stress injury in a concentration dependent manner by increasing the activities of enzymatic and non-enzymatic antioxidant mechanisms and by osmotic adjustment (Szepesi et al., 2005, 2009; Tari et al., 2010).

The complexity of the plants responses to salt stress can be partially explained by the fact that salinity imposes both ionic and osmotic stresses as well as nutritional imbalance in the tissues (Munns, 2005). Salt stress also increases the production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) that are detrimental to cells at higher concentrations because they cause oxidative damage to membrane lipids, proteins and nucleic acids and alter the redox homeostasis (Hasegawa et al., 2000; Foyer and Noctor, 2009). Several mechanisms, like ion extrusion, osmotic adjustment, synthesis of compatible osmolytes and activation of antioxidative mechanisms, contribute to the maintenance of growth and development in saline environments (Zhu, 2001; Mittova et al., 2003; Parida and Das, 2005; Miller et al., 2010). The primary components of the system combating excess ROS in plants are non-enzymatic antioxidants (such as ascorbate, glutathione, carotenoids, tocopherols) and enzymes which are involved in the elimination of ROS (superoxide dismutase, SOD; catalase, CAT; peroxidases), in the re-reduction of the members of the ascorbate–glutathione cycle, or in the maintenance of cellular redox state (e.g. ascorbate peroxidase, APX; glutathione reductase, GR) and other proteins with general or specific roles in stress responses, such as glutathione transferases (Mittler, 2002; Jaleel et al., 2009; Potters et al., 2010). The enhanced level of antioxidant capacity is usually associated with higher tolerance to stress including high salinity (Taleisnik et al., 2009; Joseph and Jini, 2011), but ROS can also function in signalling that mediate responses to different stressors. The importance of reactive oxygen forms was demonstrated to enhance tolerance to other stresses, which is defined as cross-tolerance (Dat et al., 2000; Mittler et al., 2004; Van Breusegem et al., 2008; Jaspers and Kangasjarvi, 2010).

GSTs may have an important role in salt stress acclimation of plants, but the results concerning the expression level of various isoenzymes are contradictory. Analysis of the lambda GST family in rice revealed that *OsGSTL1* was down-regulated, while *OsGSTL2* and *OsGSTL3* had no change in expression in rice during salt stress (Kumar et al., 2013a). Chen et al. (Chen et al., 2012a) found that the knock out mutant of *GSTU17* in Arabidopsis exhibited an enhanced salt tolerance which could be attributed to increased GSH and abscisic acid (ABA) levels in the mutant plants. This suggests that the contribution of various isoenzymes to stress acclimation is a complex phenomenon.

Comparison of the transcriptomic profiles of a salt-tolerant wild tomato (*Solanum pimpinellifolium* 'PI365967') and a salt-sensitive domesticated cultivar (*Solanum lycopersicum* 'Moneymaker') revealed that several GSTs were expressed at a higher level in the salt tolerant genotype (Sun et al., 2010). In addition, several glutathione transferase genes up-regulated by salt stress were identified only in the sensitive cultivar. The authors concluded that these genes may have lost their high expression capacity during domestication, but can be induced in response to salinity and reached similar expression level than in the tolerant wild genotype. However, the role of GSTs belonging to distinct groups has not been identified in salt stress acclimation of tomato yet.

In the present work, a phylogenic tree of putative GST proteins of tomato was constructed and divided into eight classes. Eleven GST-coding sequences were selected representing the main groups, and their involvement in the salt stress response was estimated and confirmed by real-time quantitative PCR (RT-qPCR). It is of interest if there is a special role of the most frequently studied tau GSTs or other isoenzymes from lambda, phi, theta and zeta groups in salt stress acclimation of tomato. The promoter analysis of these genes suggests that their transcription can be associated with responsiveness to a wide range of signals including oxidative stress, SA, ABA and light.

We are also interested in how this expression pattern changes after priming with low and high concentrations of SA. During the past two decades, our understanding about the mechanism of priming has been broadened, and it was found that several epigenetic mechanisms could be involved in the process but the function of the GSTs in SA-induced chemical hardening to abiotic stresses has still not been elucidated in details.

2. Methods

2.1. Plant material and growth conditions

S. lycopersicum 'Rio Fuego' plants were raised from seeds in moist vermiculite in the greenhouse under 300 μmol $m^{-2}s^{-1}$ light intensity (F36W/GRO lamps, Sylvania, Germany) and 12/12 h day/night photoperiod. At the cotyledon stage, tomato plants were transferred to a Hoagland solution (Gémes et al., 2011). After three weeks under control conditions, the plants were treated hydroponically with 10^{-7} M or 10^{-4} M SA and 100 mM NaCl was added to the nutrient solution from the 7th week. Samples were taken from the roots and the second fully expanded young leaves after one week of salt treatment, on the 8th week. The measurements were performed in three biological replicates and the experiments were repeated at least two times.

2.2. Screening of databases, phylogenetic analyses

Tomato glutathione transferase (SlGST) sequences were identified *in silico*. Screening for SlGST coding sequences were performed at the SOL Genomics Network (SGN) database (http://solgenomics.net). In order to group GSTs, an approximately 200 amino acid long region containing the conserved residues and domains was aligned by ClustalW (Thompson et al., 1994) and the phylogram tree was drawn by the Neighbour-Joining method using the MEGA5 and Dendroscope3 softwares (Tamura et al., 2011; Huson et al., 2007). Arabidopsis GST sequences (AtGSTs) were retrieved from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org) database. *Cis*-acting regulatory elements present in the 1.5 kbp of the 5′ regulatory regions from the translational start sites of the selected genes were identified using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.3. RNA purification, expression analyses with real-time RT-PCR

The expression rate of tomato GST genes was determined by RT-qPCR after the purification of RNA from 100 mg plant material according to Chomczynski and Sacchi (1987). DNase digestions were applied and first strand cDNA was synthesized using MMLV reverse transcriptase (Fermentas). Primers were designed for the selected SlGST coding sequences using the Primer3 software, and were synthesised in the Nucleic Acid Synthesis Laboratory, Biological Research Center (Szeged, Hungary). The primers used for the quantitative RT-PCR are listed in Table S2 [Supplementary Information]. Representative PCR products were confirmed by sequencing. The expression rate of GST genes was monitored as published earlier (Gallé et al., 2009). Each reaction was carried out in three replicates using cDNA synthesised from independently extracted RNAs and the experiments were repeated two times. The 18S ribosomal RNA and tomato elongation factor 1 α subunit (EF1-α) genes were used as high and low internal controls, respectively (Leclercq et al., 2002; Lovdal and Lillo, 2009). The EF1-α exhibited constant expression in our experiments, thus it was used for data normalization. To demonstrate the differences between changes in the expression levels of different *GST*s, the relative transcript level in the control samples was arbitrarily considered as one for each gene.

2.4. Determination of enzyme activities

Crude protein extracts were prepared by homogenizing 0.25 g of tissues on ice in 2 mL of extraction buffer (0.1 M phosphate buffer pH 7.0, containing 1 mmol L^{-1} phenylmethylsulfonyl fluoride and 1% polyvinyl-polypirrolidone), the extracts were centrifuged at 10000 *g* for 15 min. The glutathione transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GPOX) enzyme activities were measured spectrophotometrically using the artificial 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroperoxide (CHP) substrates, respectively, as published earlier (Csiszár et al., 2004). The used reagents were purchased from Sigma–Aldrich. DHAR activity was determined by the method of Edwards and Dixon (Edwards and Dixon, 2005). Specific activity (U g^{-1} fresh weight) was used for characterization of the enzyme activities. Data in the figures are usually from one representative experiment with three replicates.

2.5. Statistical analysis

The means ± SD were calculated from the data of at least 3 measurements. Statistical analysis of enzyme activity measurements was carried out with SigmaStat 3.1 software by Duncan's test and the differences were considered significant at $P \leq 0.05$. Data of RT-qPCR was calculated using $2^{(-\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001).

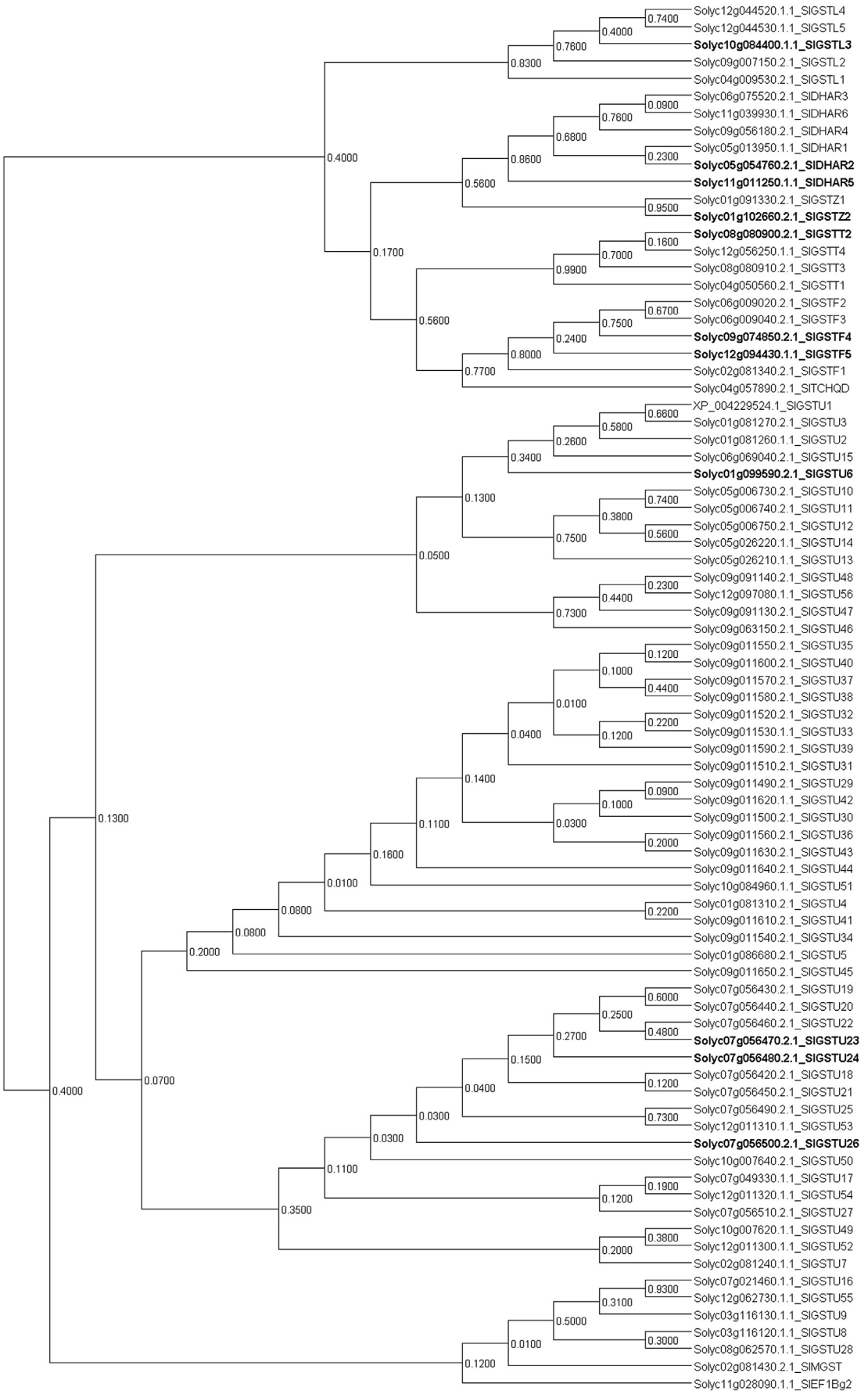
3. Results

3.1. Searching for tomato GST coding sequences and constructing a phylogenetic tree

Tomato glutathione transferase coding sequences were identified using an *in silico* approach. Sol Genomics Network (SGN) database (Bombarely et al., 2011) was used for the identification of tomato GST genes. A homology based family tree was created after selecting ca. 200 amino acid long sequences found at the SGN database using MEGA5 (Tamura et al., 2011) program. Based on homology to known AtGSTs already assigned to GST classes and conserved sequences used for classification of GST proteins (Edwards et al., 2002), we could identify eight classes of soluble tomato GSTs. Screening for the gene sequences of the SGN database led to the identification of 81 full-length or partial tomato GST sequences. Among these putative GST coding gene sequences, the tau class of tomato GSTs (GSTUs) is the most heterologous comprising 56 members, the phi (GSTF) and lambda (GSTL) groups are represented by 5 sequences, the DHAR, theta (GSTT) and zeta (GSTZ) groups contain 6, 4 and 2 genes, respectively. Beside one tetrachlorohydroquinone dehalogenase (SlTCHQD) enzyme there is one γ-subunit class of the eukaryotic translation elongation factor 1B GST (SlEF1Bγ) and one membrane associated GST (SlMGST) (Fig. 1). This tree does not contain three other GST-like sequences (Solyc06g083770.2.1, Solyc09g009820.2.1 and Solyc12g036560.1.1), which bear the thioredoxin-like fold domain involved in GSH binding (Dixon and Edwards, 2010a). To avoid confusion about tomato GST sequences, those found in SGN database were named according to conventions suggested by Edwards et al. (Edwards et al., 2000). The mapping of the *S. lycopersicum* (*Lycopersicon esculentum)* genome has allowed the GSTs to be numbered according to their organization and position within the chromosomes beginning with the sequences found on chromosome one and contiguous numbers were given to the GSTs belonging to the same class. The genome locus of each gene, the fully deduced coding sequences and the former (if any) and new names of tomato GST proteins and their sequences are shown in FASTA format in Table S1 [Supplementary Information].

3.2. Selecting the tomato GST coding sequences

To investigate the transcript amount of specific tomato GSTs during salt stress response and/or long-time hardening effect of SA,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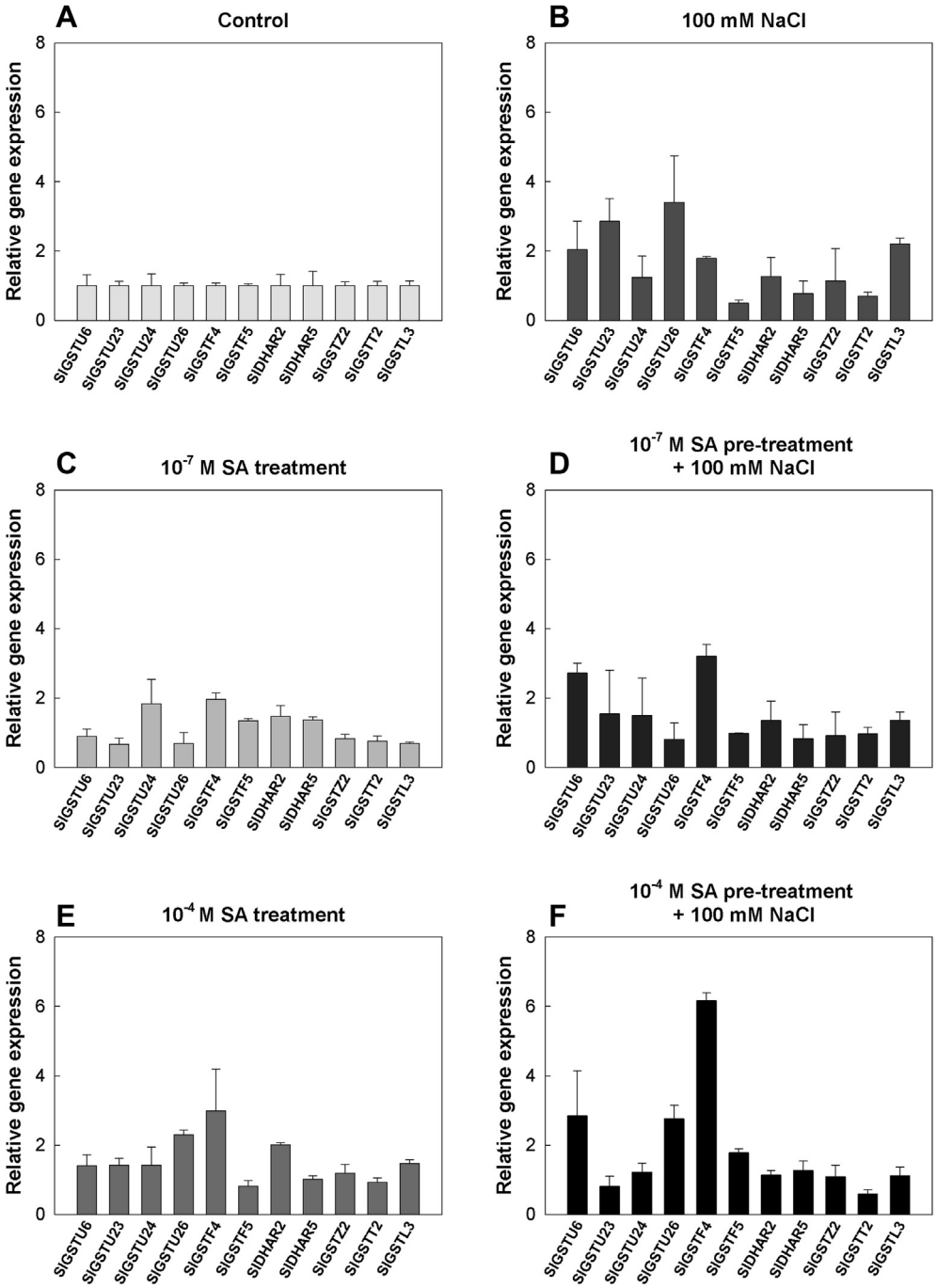

Fig. 2. Effect of 3-week salicylic acid (SA) pre-treatment on the transcript levels of selected tomato GST genes in leaves of 8-week-old tomato plants after applying 100 mM NaCl for one week. Data were normalized using the tomato elongation factor α subunit (EF-1) gene as internal control, the relative transcript level in the control samples was arbitrarily considered as one for each gene. Data consist of means ± SD, $n = 6$.

information found in literature and databases was taken into consideration. Although several tomato GST proteins involved in the oxidative stress responses were earlier characterized (Kilili et al., 2004; Kampranis et al., 2000) or their involvement was reported in salt stress responses as a results of microarrays or proteomic studies (Sun et al., 2010; Zhou et al., 2007; Manaa et al., 2011), much more information is available about the expression, regulation and function of GSTs in Arabidopsis. Dixon, Edwards and their co-workers published several papers and reviews with detailed characterization of AtGSTs (Dixon et al., 2002b; Dixon et al., 2009; Edwards and Dixon, 2009, 2010; Dixon et al., 2010; Dixon et al., 2011; Edwards et al., 2011).

Fig. 1. Grouping of tomato GST coding sequences extracted from SOL Genomics Network (SGN) database (http://solgenomics.net). The protein sequence of SlGSTU1 was taken from NCBI database (http://www.ncbi.nlm.nih.gov) instead of truncated sequence found in SGN. An approximately 200 amino acid long region containing the conserved residues and domains was chosen and the phylogram tree was drawn in MEGA5 program by the Neighbour-Joining method. Numbers in the figure indicate the relative phylogenetic distance. Sequences chosen for further investigation are labelled.

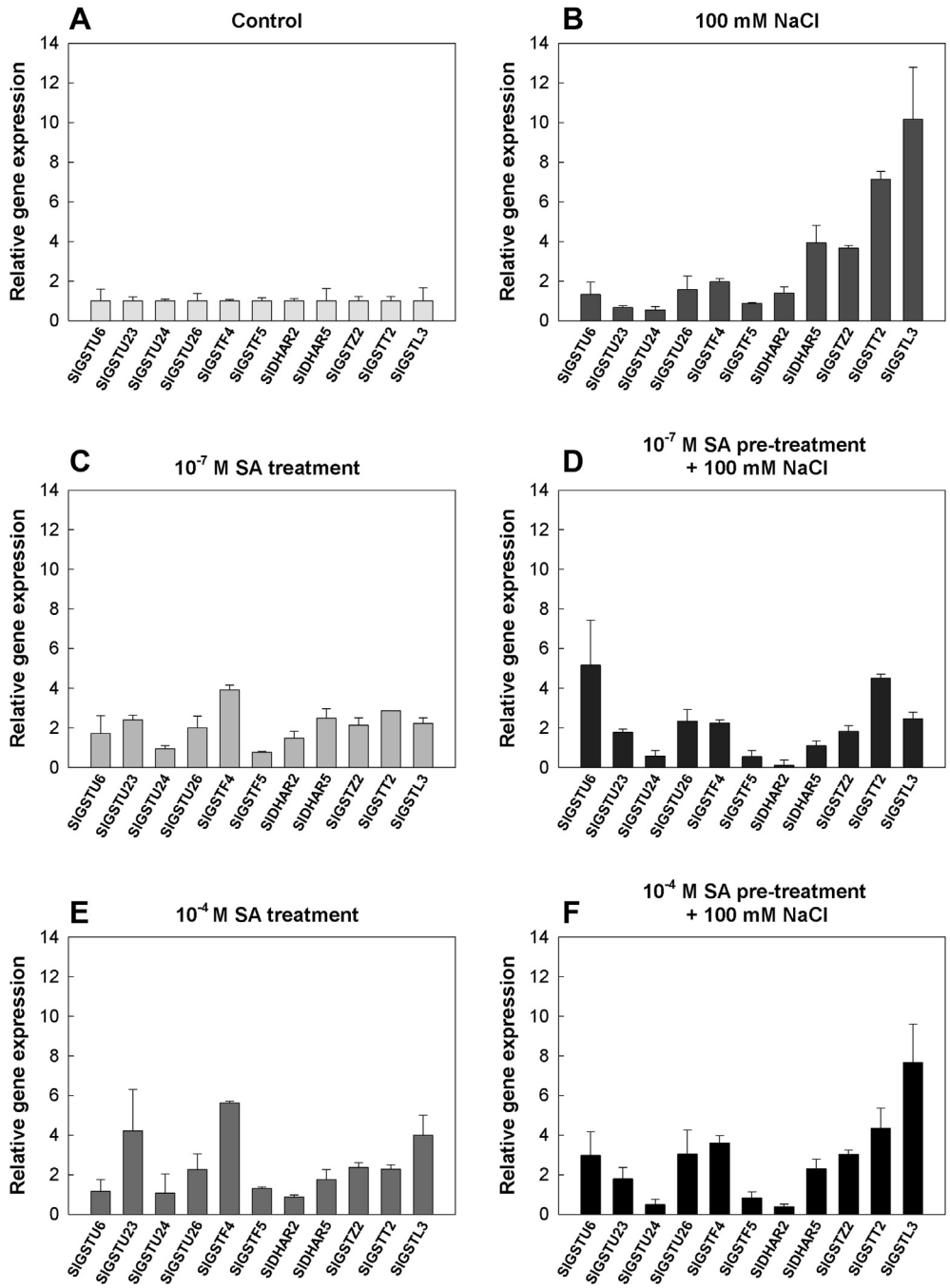

Fig. 3. Effect of 3-week salicylic acid (SA) pre-treatment on the transcript levels of selected tomato GST genes in roots of 8-week-old tomato plants after applying 100 mM NaCl for one week. Data were normalized using the tomato elongation factor α subunit (EF-1) gene as internal control, the relative transcript level in the control samples was arbitrarily considered as one for each gene. Data consist of means ± SD, $n = 6$.

Sappl et al. (2004, 2009) identified more than twenty Arabidopsis GST genes which are induced by short-time treatment of different oxidants and/or salicylic acid. Eleven different tomato GST coding sequences belonging to 6 different GST classes were selected in our experiments (Fig. 1) based on the cited literature suggesting that tomato GST proteins or their orthologs in Arabidopsis may play an important role in oxidative stress- or SA responses [Supplementary Information, Table S2]. To compare the phylogenetic relationship of tomato GST coding sequences and Arabidopsis GSTs, an approximately 200 amino acid long region containing the conserved residues and domains (Wagner et al., 2002) was chosen and a similarity tree was constructed containing the AtGSTs and the selected tomato GST sequences [Supplementary Information, Fig. S1].

The main aspects of selection among *SlGSTs* for gene expression investigations and the primer pairs used are shown in Table S2 [Supplementary Information].

3.3. Changes in transcript levels of selected SlGST genes in tomato plants under salt stress with and without priming

Transcript amounts of selected tomato GSTs were investigated by RT-qPCR in 8-week-old tomato plants after one week of salt stress with or without SA pre-treatments. In order to compare the

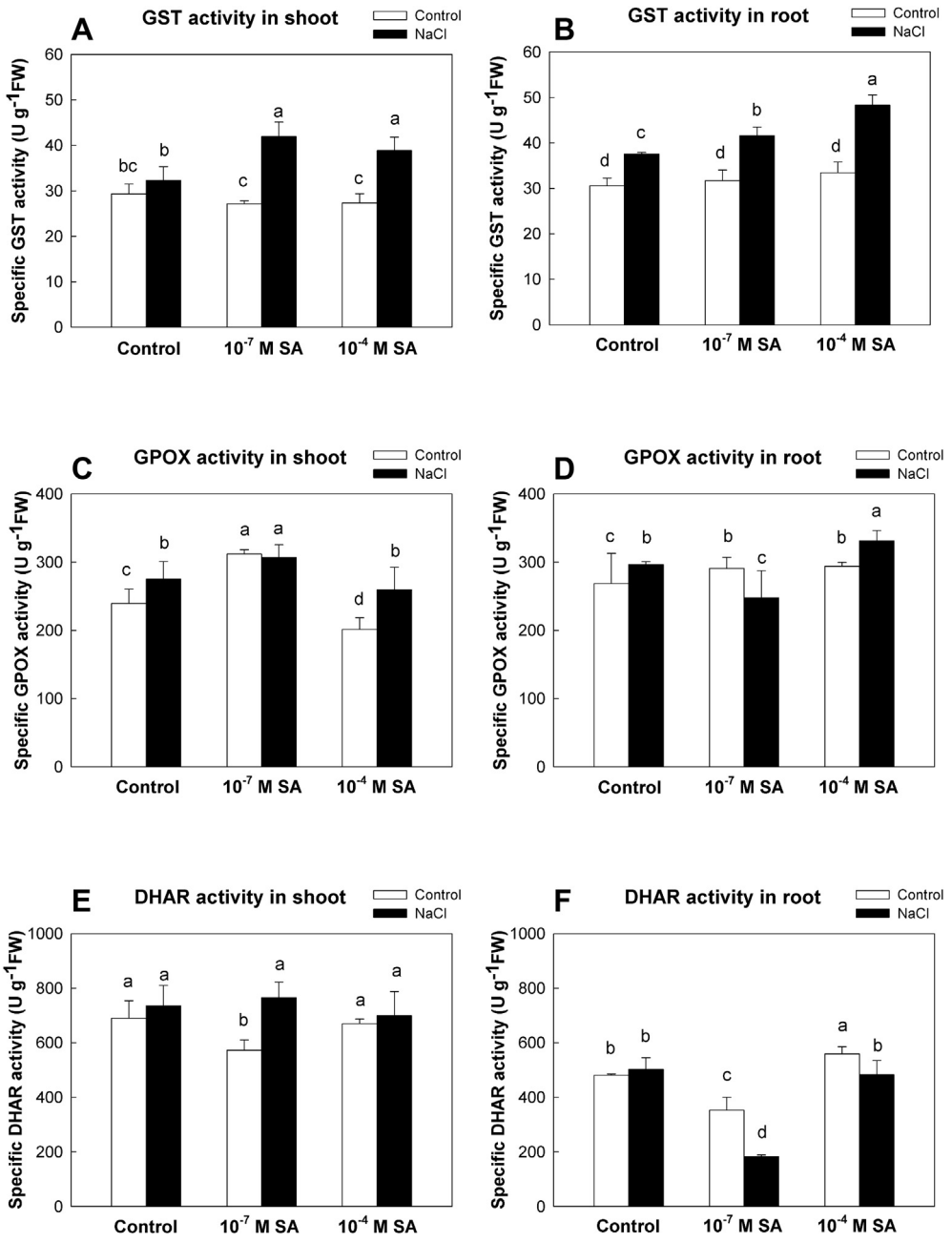

Fig. 4. Effect of 3-week salicylic acid (SA) pre-treatment on glutathione S-transferase (GST), glutathione peroxidase (GPOX) and dehydroascorbate reductase (DHAR) activities in leaves and roots of 8-week-old tomato plants after applying 100 mM NaCl for one week. Data consist of means ± SD obtained from at least 3 measurements. Means denoted by different letters are significantly different at $P \leq 0.05$ as determined by Duncan's test.

changes in transcription of individual GSTs after the treatments, the relative transcript level measured in leaves and roots of control samples was equalled to one for each gene.

100 mM NaCl applied for one week induced the transcript accumulation of several GSTs. The transcript amount of selected tau GSTs (*SlGSTU23*, *SlGSTU26*) and to smaller extent *SlGSTL3* were increased in the leaves, while certain lambda, theta, DHAR and zeta class GSTs, *SlGSTL3, SlGSTT2, SlDHAR5* and *SlGSTZ2* exhibited a very significant up-regulation in the root tissues during salt stress.

SA treatments for four weeks in total affected the expression of selected tomato GST genes in a concentration-dependent manner. In the presence of 10^{-7} M SA, we detected up to twofold increase in transcript amount of GSTs in the leaves (in the case of *SlGSTF4* and the *BI-GST,* which is named as *SlGSTU24*), while in roots the expression level of *SlGSTF4* increased fourfold, and that of six other sequences (*SlGSTU23, SlGSTU26, SlDHAR5, SlGSTZ2, SlGSTT2* and *SlGSTL3*) twofold (Figs. 2 and 3). Treatment with 10^{-4} M SA induced the expression of more *SlGSTs* and their transcript amounts were 2-6-fold higher than those in controls (especially *SlGSTF4*, *SlGSTU23* and *SlGSTL3*).

Priming with SA exhibited a concentration dependency (Gémes et al., 2011; Poór et al., 2011); SA mitigated the salt stress injury and caused characteristic changes in the expression pattern of GST genes only at high concentration. It can be observed that it was a phi type GST, *SlGSTF4*, which exhibited a significant up-regulation compared to salt stressed controls in the leaves of primed plants. Generally, in leaves the representatives of tau (*GSTU6, GSTU26*) and phi class GSTs were induced by both NaCl and SA treatments, while

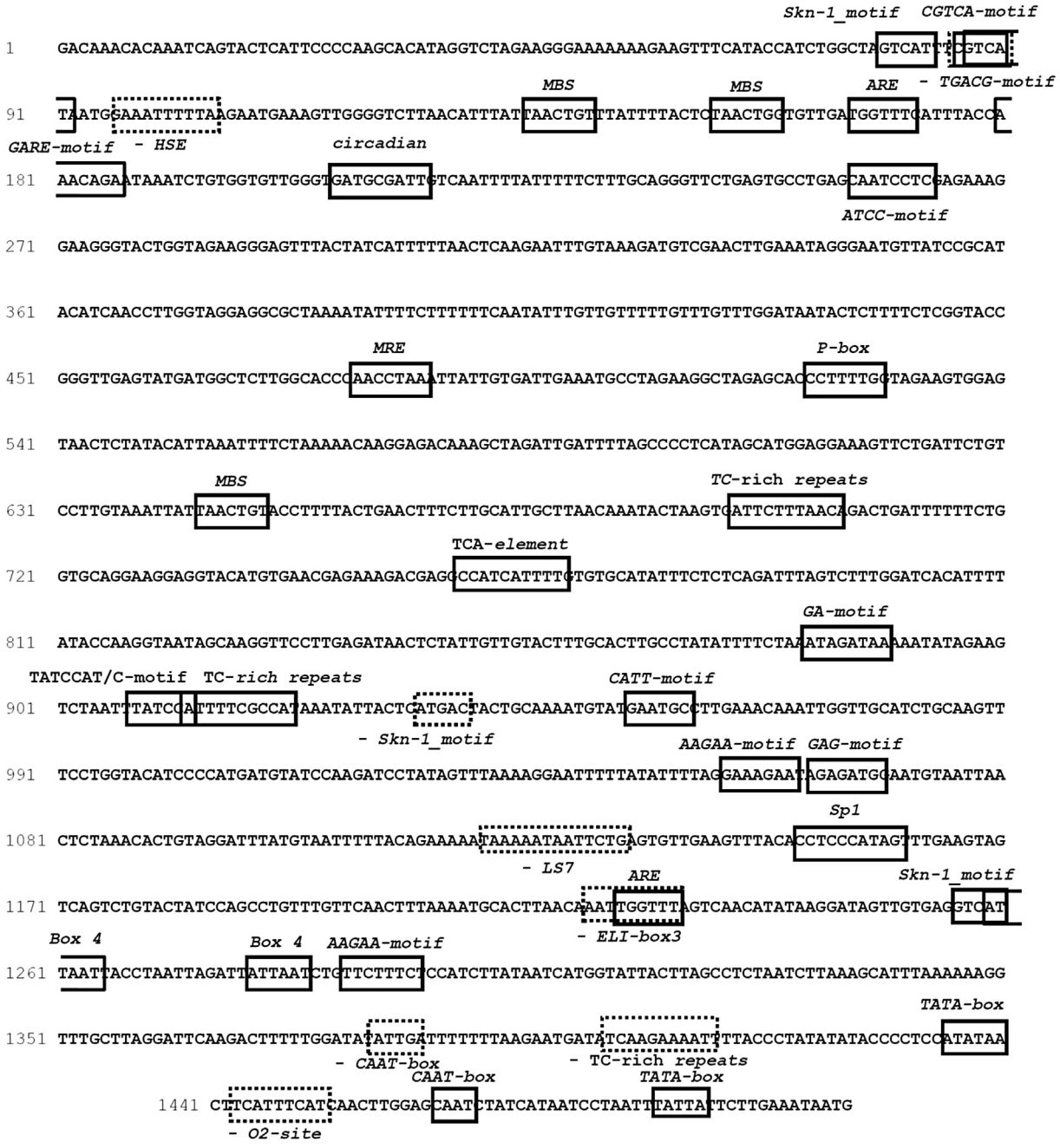

Fig. 5. The main predicted *cis*-regulatory elements found in the upstream regulatory region of *SlGSTF4*. The predicted function of motifs: AAGAA-motif: unknown; ATCC-motif, Box 4, CATT-motif, GA-motif, GAG-motif, LS7, Sp1: parts of conserved DNA modules involved in light responsiveness; ARE: *cis*-acting regulatory element essential for the anaerobic induction; CAAT-box: common *cis*-acting element in promoter and enhancer regions; CGTCA-motif, TGACG-motif: *cis*-acting regulatory elements involved in the MeJA-responsiveness; circadian: *cis*-acting regulatory element involved in circadian control; ELI-box3: elicitor-responsive element; GARE-motif, P-box: gibberellin-responsive elements; HSE: *cis*-acting element involved in heat stress responsiveness; MBS: MYB binding site involved in drought-inducibility; MRE: MYB binding site involved in light responsiveness; O_2-site: *cis*-acting regulatory element involved in the regulation of zein metabolism; Skn-1 motif: *cis*-acting regulatory element required for endosperm expression; TATA-box: core promoter element around −30 of transcription start; TC-rich repeats: *cis*-acting element involved in defense and stress responsiveness; TCA-element: *cis*-acting element involved in salicylic acid responsiveness; TATCCAT/C-motif: *cis*-acting regulatory element involved in sugar repression responsiveness.

the abundance of *SlGSTL3*, *SlGSTT2* and *SlGSTZ2* transcripts remained enhanced in the roots suggesting that the products of these genes may have an important role in the responses to the investigated stress factors and can contribute to the high GST activity. Interestingly, applying 100 mM NaCl to 10^{-4} M SA pre-treated plants resulted in lower transcript levels in case of several *SlGSTs*, among them the *SlGSTU24* (*BI-GST)* and *SlDHAR2* in roots (Figs. 2 and 3). Although the *SlDHAR2* expression decreased in primed roots compared to the salt stressed plants, the up-regulation of *SlDHAR5* isoenzyme may contribute to the maintenance of DHAR activity in the roots primed with 10^{-4} M SA concentration during salt stress.

3.4. GST-related enzyme activities in tomato plants under salt stress with and without priming

The SA- and salt-induced changes in the GST, GPOX and DHAR activities were compared in the leaves and roots of 8-week-old

tomato plants pre-treated with 10^{-7} M and 10^{-4} M SA and after one week of salt stress. The total GST activities measured by CDNB substrate were similar to the controls at the end of the 4-week-long SA treatments both in the fully expanded young leaves and in the roots. However, this enzyme worked at a significantly higher level after one week of salt stress in the SA-pre-treated plants than in controls, indicating that detoxification mechanisms were activated due to primed status in SA-hardened plants (Fig. 4A, B).

The GPOX activity measured with the CHP substrate was enhanced in the roots of SA-pre-treated tomato plants, but this activity remained high only in the 10^{-4} M SA-hardened roots after applying the salt stress, suggesting that these plants have a very effective defense system against damage caused by lipid peroxides (Fig. 4D). The total GPOX activity was enhanced only by 10^{-7} M SA in the leaves and was reduced by 10^{-4} M SA, but the primed plants could maintain or increase the enzyme activity during salt stress.

As it could be expected from the gene expression pattern of *SlDHAR2* and *SlDHAR5* genes, the total DHAR activity decreased in the roots of 10^{-7} M SA pre-treated plants, while it was elevated or remained at the control level in plants treated with 10^{-4} M SA (Fig. 4F). The changes were different in the leaves, where the DHAR activities of SA pre-treated plants were similar to the controls after one week of salt stress (Fig. 4C, E).

3.5. *In silico analysis of cis-acting regulatory elements of selected tomato GST genes*

As the tomato genome sequence became available, it provided an easier access to the *in silico* screening of *cis*-regulator sequences in the 5′ regulator regions of the investigated genes (1500 bp upstream from the start codon) found in the SGN (http://solgenomics.net) by PlantCARE (Lescot et al., 2002). The main regulatory elements found in the promoter region of *SlGSTF4*, as an example, are shown in Fig. 5. Our result show that two TCA-elements which may be involved in SA responsiveness, can only be found in the promoter region of *SlGSTU6, SlGSTT2* and *SlGSTZ2* genes, however, other motifs possibly involved in defense (TC-rich repeats) are present in almost every regulator region (exception is *SlGSTT2*), and usually in more than one copy. Several other *cis*-acting regulatory elements involved in systemically acquired resistance (TGACG), regulation of flavonoid biosynthetic genes (MBSII site) or the other MYB binding site important in drought inducibility (MBS motif) or the ABRE element (responsible for ABA inducibility) were identified in the investigated region of the genes. Interestingly, numerous motifs connected to light responsiveness are present in the 5′ upstream regulator regions of the *SlGST*s indicating the complex interaction between different signalling inputs. The list and description of the main nucleotide motifs discovered in the 5′ regulator regions of the investigated genes are shown in Table S3 [Supplementary Information].

4. Discussion

Salt tolerance is believed to be affected by many different factors, such as transport selectivity and ion extrusion, ion compartmentation, synthesis of compatible solutes and activation of ROS scavenging mechanisms (Zhu et al., 2007; Jiang et al., 2007; Munns and Tester, 2008; Papdi et al., 2008). It was shown that high salinity generates ROS which disturbs the cellular redox system causing a shift towards more oxidized forms. In addition, the activity of antioxidative systems and redox status of antioxidants during salt stress have been correlated with plant salt tolerance (Meneguzzo et al., 1999). In tomato, salinity decreases ascorbic acid (AA) and GSH contents and induces lipid peroxidation (Mittova et al., 2004). Maintenance a high ratio of GSH/GSSG was shown to play an important role in salt and drought tolerance of tomato, maize and wheat (Shalata and Neumann, 2001; Kocsy et al., 2002). GSTs with GSH-dependent reductase activity (DHAR and GSTL) may participate in the maintenance of reductant pools (AA, α-tocopherol, anthocyanins) while other isoenzymes catalyse the detoxification of reactive metabolites (Dixon and Edwards, 2010a). At the same time the GST activity consumes reduced GSH, this is the reason why GST overproduction competes with other antioxidant mechanisms. This also explains the interesting findings of Chen et al. (Chen et al., 2012b) on elevated GSH and ABA levels in *GSTU17* knock out Arabidopsis plants.

Salicylic acid, which is involved in the activation of defense mechanisms against biotic and abiotic stress factors, may mitigate the damaging effects of a wide range of oxidative stressors (Shirasu et al., 1997; Horváth et al., 2007; Ashraf et al., 2010). Exogenous application of SA at a suitable concentration could be a powerful tool for the reduction of stress sensitivity through its hardening effect, which as a long-term response contributes to the maintenance of a dynamic homeostasis of metabolic pathways and efficient survival. In our earlier works we observed that priming with SA was very effective in increasing salt stress tolerance in tomato. SA treatments activated the enzymatic and non-enzymatic antioxidant systems (Szepesi et al., 2009; Gémes et al., 2011), enhanced the CO_2 fixation rate, the accumulation of various carbohydrates (Poór et al., 2011) and compatible osmolytes (Tari et al., 2010) in plants exposed to high salinity. Both endogenous and exogenous SA treatments may result in temporary increase in H_2O_2 levels (Gémes et al., 2011; Janda et al., 2003), which has been proposed to act as a secondary messenger and can even lead to induction of antioxidant responses in plants, thus promoting elimination of excess ROS (Mittler, 2002). Several mechanisms have been proposed for inducing the accumulation of H_2O_2 by SA, such as inhibition of CAT and APX enzymes after direct binding of SA to the enzyme proteins (Hayat et al., 2010). The rise of SA-free radicals inhibits heme-containing antioxidative enzymes e.g. CAT and peroxidases (Durner et al., 1997). Screening for differentially expressed proteins in cucumber seedlings treated with 5×10^{-5} M SA revealed that among the 59 proteins identified to date the largest functional category included proteins involved in antioxidative reactions (23.7%), and they exhibited the largest change in their relative abundance 5 days after the exposure to exogenous SA (Hao et al., 2012).

In addition, SA as well as SA-induced H_2O_2 may act as potential intermediate in signal transduction pathways involved in defense-related gene expression (Durner et al., 1997; Chen et al., 1993; Poór and Tari, 2012). ROS signalling is highly integrated into numerous other signalling networks that regulate plant acclimation, including that of ethylene, ABA and jasmonic acid (Foyer and Noctor, 2009; Miller et al., 2010). ROS can be sensed, transduced, and translated into appropriate cellular responses by redox-sensitive proteins that can undergo reversible oxidation/reduction and may switch 'on' and 'off', depending on the cellular redox state. The ROS-induced redox changes may also have a regulatory role in SA signalling pathways. For example, the reduction of intermolecular bisulfide bonds of the cytosolic oligomer NPR1 (non-expressor of pathogenesis-related genes 1), a transcription regulator of SA signalling results in monomerisation in the presence of SA. The resulting monomers are then able to translocate to the nucleus and activate the expression of defense genes in the NPR1-dependent pathway (Mou et al., 2003). However, recent evidence suggests that H_2O_2-dependent changes in the glutathione pool can activate SA-dependent defense responses independently of NPR1 (Han et al., 2012). Deef (2007) reported that pre-treatment of wheat and barley kernels with SA resulted in elevated levels of glutathione, as well as in salt tolerance of seedlings. Furthermore, it was found that

a gene encoding salicylic acid-binding protein 2 (SABP2, a protein that has been shown to play a vital role in SAR), was up-regulated in a resistant tomato genotype under salt stress, suggesting a possible role of SA in the salt response of *Solanum* genus (Sun et al., 2010).

In our experiments, application of 100 mM NaCl for 7 days on 7-week-old tomato plants resulted in elevated total measurable GST and GPOX activities in the roots, but in the case of shoots the activation was significant only at GPOX activity. The changes were higher in the SA-pre-treated plants (Fig. 4), indicating that activation of GSTs during the priming is an important part of the process and may contribute to enhanced resistance of plants in the long-term responses. Other investigations using the same experimental system revealed that adding SA to hydroponic cultures of tomato initially can cause rather drastic changes in ROS formation and operation of antioxidant mechanisms, although the differences became smaller during the 3-week acclimation process (Gémes et al., 2011). Because the GST enzyme family has divergent functions, they may promote the enhanced stress tolerance of SA-primed plants by several different ways, among others via the more efficient removal of lipid peroxides and other harmful endogenous compounds and the maintenance of reductant pools in the cells.

The GST family of tomato consist of 81 members, among them 78 belonging to the six main classes of soluble plant GSTs. The plant-specific tau class is the largest and most of the work on the field of stress acclimation has been done by the isoenzymes of this family (Kilili et al., 2004; Thom et al., 2002).

In our experiments, we found that the expression levels of several selected tomato *GST*s were elevated after one week of salt stress, but the expression patterns were different in the leaves and roots.

The expressions of *SlGSTU23* and *SlGSTU26* were highly enhanced in the leaf but not in the root tissues during salt stress. The members of this class showed GSH-conjugating activities with various xenobiotics in rice (Yang et al., 2009) and in poplar (Lan et al., 2009), thus they presumably participate in the conjugation of toxic metabolites to GSH in tomato leaves during salt stress. In primed leaves the expression of *GSTU23* decreased during salt stress, but it was substituted by the activation of *SlGSTU6*. In the root tissues, however, an increased expression of *SlGSTL3*, *SlGSTT2*, *SlDHAR5* and *SlGSTZ2*, the representative genes from lambda, theta, dehydroascorbate reductase and zeta classes, was found.

The lambda GSTs in plants, similarly to DHARs, catalyse the glutathione-dependent reduction of the substrates and *in vivo* they can bind flavonols and flavonol derivatives as well as oxidized derivatives of tocopherols that can be an important step in maintaining the redox status of the antioxidant pool of the tissues (Dixon et al., 2011). Our results contradict to the findings with rice lambda GSTs, because their expression was down-regulated or did not change during salt stress (Kumar et al., 2013b). This can reflect the differences in salt stress acclimation of a sensitive rice and of a moderately salt tolerant tomato genotype such as cv. Rio Fuego. It was also found that *AtGSTL3*, the Arabidopsis ortholog of *SlGSTL3*, appears to be a constitutive cytosolic enzyme (Dixon et al., 2011), but this enzyme in tomato was induced both by SA and high salinity.

Classes of zeta and theta GSTs are found both in plants and mammals and function in primary metabolism such as isomerisation of maleylacetoacetate, in tyrosine catabolism (zeta class) and in the detoxification of hydroperoxides formed during oxidative stress (theta class) (Dixon et al., 2002a). Up-regulation of the expression of selected *SlGST*s belonging to these classes after one week of salt stress only in roots suggests that these biochemical reactions have special importance in the root tissues during salt stress.

SA pre-treatment caused a concentration dependent change in GST expression. At low concentration (10^{-7} M), except for *SlGSTF4,* the SA-induced changes in the expressions of *SlGSTU23*, *SlGSTZ2*, *SlGSTT2* and *SlGSTL3* were not very significant in the leaves, but their expressions were enhanced in the root tissues. High SA concentration (10^{-4} M) enhanced the *SlGSTF4* and *SlGSTU26* mRNA abundance both in the leaf and root tissues, and also up-regulated the expression of *SlGSTU23*, *SlGSTZ2, SlGSTT2* and *SlGSTL3* in the root tissues. However, the expressions of most GST genes were lower in the primed plants during high salinity than those of salt stressed controls. The most striking difference is the higher mRNA abundance of two phi *GST*s, *SlGSTF4* and *SlGSTF*5, in the leaves and that of *SlGSTU26* and *SlGSTF4* in the roots. In Arabidopsis, AtGSTF8 is a major phi-type GST, which contains a chloroplast localisation signal. However the majority of *AtGSTF8* transcripts are spliced, the signal peptide is removed and the protein remains in the cytosol (Thatcher et al., 2007). This enzyme catalyses the glutathione conjugation of an oxylipin, ((15Z)-12-oxophyto-10,15-dienoic acid), which is a component of jasmonic acid biosynthesis (Dueckershoff et al., 2008), thus, by reducing the jasmonate pool it can activate the SA-induced signal transduction pathway. According to the SGN database, two homologues of *AtGSTF8* have been found in tomato [Supplementary Information, Table S1], but only *SlGSTF4*, which has closer relationship to *AtGSTF8* in our phylogenetic tree [Supplementary Information, Fig. S1] displayed increased expression both in leaves and roots of plants primed with SA.

Unexpectedly, the expression of *SlGSTU24*, the 'Bax-inhibitor GST', which was very effective against prooxidant-induced cell death, was not up-regulated under stress conditions, instead, a close homologue, *SlGSTU26* was significantly up-regulated in the primed plants exposed to high salinity.

These changes in GST expression pattern seem to be the most characteristic for SA priming. In contrast to low SA, priming with high SA concentration maintained the high transcript abundance of *SlGSTZ2*, *SlGSTT2, SlDHAR5* and *SlGSTL3* as it was found in the salt-stressed control, suggesting that the GSTs from lambda, theta, DHAR and zeta classes play an important role in salt stress acclimation in tomato. The theta class enzymes are particularly efficient as glutathione peroxidases, thus elevated transcript abundance of *SlGSTT2* (a homologue of *AtGSTT1*) may contribute to GSH-dependent elimination of lipid hydroperoxides in peroxisomes (Dixon and Edwards, 2010a).

The DHAR group of GSTs in tomato contains 6 members. While in the leaves the expression of *SlDHAR2* and *SlDHAR5* increased slightly or remained unchanged under salt stress as compared to the untreated control both in primed and un-primed plants, the transcript amounts of *SlDHAR2* declined below the control levels in the roots. Thus, in these plants the measured DHAR enzyme activity can mainly be confined to *SlDHAR5*, which exhibited a high expression level under salt stress. The DHAR activity of stressed plants remained approximately at the control level in the roots of 10^{-4} M SA pre-treated plants in contrast to its decreased activity in 10^{-7} M SA pre-treated tomatoes, which corresponds well with the more effective hardening effect of the 10^{-4} M SA treatments (Gémes et al., 2011). DHARs reduce dehydroascorbate to ascorbate while oxidising GSH to glutathione disulfide and they have a role in maintaining the reduced ascorbate pool. Consequently, the GST enzymes can participate in the maintenance of the redox state, metabolism, function and structure of cells (Dixon and Edwards, 2010a; Dixon et al., 2002b). SlDHAR5 is a close homologue of the AtDHAR3, which was shown to be a chloroplastic enzyme. Since DHAR activity is an important part of the ascorbate–glutathione cycle in the chloroplasts, this correlates well with the improvement of photosynthetic performance by SA during stress conditions (Poór et al., 2011).

Since GST activity consumes GSH as a substrate for the detoxification processes, a fine control of GST expression is necessary for maintaining the pool and redox status of GSH during stress conditions. This may be the reason why GST isoenzymes are frequently down-regulated during abiotic stress acclimation and new isoenzymes appear which have other substrate specificities, kinetic parameters and compartmentation.

In summary, our results suggest that GSTs are important participants in adaptation to changes in environmental signals. The altered expression levels of *SlGSTs* and the increased or repressed GST enzyme activities with diverse functions may be the part of the stress response, fine-tuning of ascorbate and glutathione homeostasis and redox status, thus may also participate in the hardening effect of SA and improving the salt stress tolerance in tomato plants.

5. Contributions

Jolán Csiszár: designed and coordinated the experiments, wrote and edited the manuscript, Edit Horváth: carried out the phylogenetic analysis, participated in laboratory experiments, data analysis and creating Figures, Zsolt Váry: laboratory experiments and data analysis, Ágnes Gallé: RT-qPCR analysis, Krisztina Bela: *in silico* promoter analysis, Szilvia Brunner: statistical analysis, Irma Tari: designed and coordinated the study, wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.02.010.

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