



Stress-inducible *GmGSTU4* shapes transgenic tobacco plants metabolome towards increased salinity tolerance

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Abstract The involvement of glutathione transferases (GSTs) in plant's tolerance to abiotic stresses has been extensively studied; however, the metabolic changes occurring in the plants with altered GSTs expression have not been studied in detail. We have previously demonstrated that *GmGSTU4* overexpression in tobacco plants conferred increased tolerance to herbicides, partly through its peroxidase activity. Here, we investigated *GmGSTU4* transcriptional response to abiotic and chemical stimuli in soybean. Transgenic tobacco plants overexpressing

GmGSTU4 were also evaluated regarding their phenotypic and metabolomics responses under salt stress. *GmGSTU4* expression was highly induced after salt stress and atrazine treatment. Tobacco plants overexpressing *GmGSTU4* were highly tolerant to 150 mM NaCl in vitro. Metabolomics comparison of plants growing under optimal conditions, indicating a shift of the transgenic plants metabolism towards the metabolic profiles observed under stress, increased concentration of precursors of glutathione biosynthesis and hexose concentration reduction. Under salt stress, transgenic plants maintained their cellular homeostasis in contrast to wild-type plants which exhibited deregulated energy metabolism. The metabolic response of the transgenic plants was characterized by higher concentration of protective metabolites such as proline and trehalose and greater induction of the oxidative pentose phosphate pathway. These results confirm *GmGSTU4* contribution to salt stress tolerance, and outline a regulatory role that primes plants towards the up-regulation of protective and detoxification mechanisms under abiotic stress.

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Introduction

Plants' responses to stress conditions constitute of both sensing the environmental adversity and the downstream activation of signaling cascades (Dietz 2008) which result in adaptive responses such as the up-regulation of the reactive oxygen species (ROS) detoxification network, to counteract oxidative stress, commonly generated under these conditions (Gill and Tuteja 2010; Suzuki et al. 2012).

Glutathione transferases (GSTs, EC 2.5.1.18) superfamily of enzymes forms a major part of plants detoxification network. They are commonly cytosolic, dimeric enzymes, and their major catalytic function is the conjugation of the tripeptide glutathione (GSH; γ -Glu-Cys-Gly) to various electrophilic (and frequently cytotoxic) substrates (Cummins et al. 2011; Edwards et al. 2000). Owing to this function, they were originally described as enzymes facilitating the detoxification of herbicides in crop plants through GSH conjugation (Dixon et al. 1998).

GSTs appear to have a significant role in plants' adaptation under abiotic stress as many isoenzymes are found to be differentially expressed under these conditions (Chi et al. 2011; Sappl et al. 2009). Their protective function can be exerted by both their GSH conjugating activity and GSH-dependent peroxidase activity on cytotoxic alkenals and fatty acid hydroperoxides, respectively, generated under stress (Chronopoulou et al. 2011, 2012).

Multiple evidence concerning GSTs involvement in abiotic stress tolerance exists: first their induced expression after different stress stimuli (Csiszár et al. 2014; Lan et al. 2009), the association of different allelic forms of GST isoenzymes with stress tolerance (Kim et al. 2011) and the second comes by the overexpression of several stress responsive isoenzymes resulting in increased stress tolerance of the respective transgenic plants (Diao et al. 2011; George et al. 2010; Roxas et al. 2000).

Despite the numerous evidences for the involvement of GSTs in plants' protection against abiotic stress, there is a limited knowledge on the underlying mechanisms. It is postulated to be mostly exerted through their GSH-dependent peroxidase activity (Cummins et al. 1999), however, pleiotropic effects have been observed in the activity of antioxidant enzymes (Roxas et al. 2000) and other components of the plants' stress tolerance network (Diao et al. 2011). The cellular redox state is recently acknowledged as a central hub in the coordination of plants' developmental and metabolic processes both in the presence or absence of stress conditions, through post-translational modification of proteins and transcription factors which alter their biological functions (Tanou et al. 2009; Yun et al. 2011). GSTs have been shown to modulate redox homeostasis by alterations in GSH content and redox state (Chen et al. 2012). It is suggested that through this function they can strongly affect plant development (Jiang et al. 2010) as well as tolerance to abiotic stresses (Chen et al. 2012). Given the complexity of plant stress responses, tools for high-throughput analysis such as metabolomics enable the dissection of stress responses and the identification of major modules that underlie increased tolerance (Obata and Fernie 2012). Metabolites are the end products of gene expression and protein activities and therefore are the penultimate regulatory components for growth and

development. It has been shown recently that metabolites apart from their typical properties are also involved in major signaling processes (Liu et al. 2010; Pajeroska-Mukhtar et al. 2012) and therefore can have pleiotropic effects beyond their "regular" function. Moreover, metabolomics can uncover silent phenotypes, which can be informative in gene families which have members with redundant functions, such as the GSTs, as demonstrated by the observation of metabolic perturbations under stress in Arabidopsis plants with multiple silenced GSTs (Sappl et al. 2009). As GSTs increasingly appear to be involved in endogenous metabolic processes (Dixon et al. 2010), metabolomics could be the tool to uncover novel metabolic functions.

The isoenzyme *GmGSTU4* (a tau class GST) from soybean has been well characterized in terms of substrate specificity, kinetic mechanism and 3D structure. *GmGSTU4* which catalyzes a broad range of reactions exhibits high glutathione transferase and glutathione peroxidase activities (Axarli et al. 2009a, b, 2010). We have previously overexpressed the *GmGSTU4* isoenzyme in tobacco plants (Benekos et al. 2010) examining their tolerance against herbicides. Herein, we further investigated *GmGSTU4* transcriptional responses to various stress stimuli in wild-type (WT) soybean. Furthermore, we have evaluated under salt stress, the tolerance and the metabolite profile of transgenic tobacco plants overexpressing *GmGSTU4*. The metabolomics analyses revealed significant metabolic alterations in the transgenic tobacco plants, both under non-stress and salt stress conditions, towards stress anticipation and up-regulation of diverse protective mechanisms and homeostasis maintenance. These findings provide insights into the metabolic alterations underlying GSTs protective effects against salt stress and aid further biotechnological approaches in enhancing salt tolerance in crops by targeting the underlying metabolic pathways.

Materials and methods

Expression analysis of *GmGSTU4* gene in soybean

Soybean seedlings were grown in perlite in a growth chamber under controlled conditions (25 °C, 16 h light/8 h dark cycle) and were subsequently subjected to various stresses as a means to assess the expression of *GmGSTU4* gene. Fourteen-day-old soybean plants were individually transferred into test tubes and exposed to the following xenobiotics: 10 mM atrazine, 10 mM 1-chloro-2,4-dinitrobenzene (CDNB), 10 mM H₂O₂. Osmotic stress was achieved by the addition of 100 mM NaCl. For temperature stress treatments, plants were incubated at 40 and 4 °C.

Plants of the same age that were not exposed to stress were included as experimental controls. Following 4 h of incubation, leaf samples from individually treated and control plants were collected. Samples were immediately frozen in liquid nitrogen and maintained at -80°C .

Total RNA isolation was performed on the collected leaf samples of soybean plants with the SV Total RNA Isolation Kit (Promega). To eliminate contamination of genomic DNA, RNA samples were treated with DNase I at 37°C for 45 min. First-strand cDNA was reverse transcribed using SuperScript II and random hexanucleotides. *GmGSTU4* transcripts were amplified using gene-specific primers. PCR reactions were performed on a Bio-Rad Mini Opticon cycler using Kapa SYBR Fast qPCR kit (Kapa Biosystems). Amplification cycles included a first denaturation cycle of 10 min at 95°C , then 40 cycles composed of 15 s at 95°C and 1 min at 60°C . The expression *G. max* ubiquitin gene (*GmUb*) was used as a control for relative gene expression quantification. Relative expression was calculated with the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Transgenic tobacco plants overexpressing *GmGSTU4*

Wild-type and T_1 transgenic tobacco var. “Basmas” overexpressing *GmGSTU4* plants were grown in vitro as described (Benekos et al. 2010). After preliminary experiments evaluating salt stress tolerance phenotypes of the transgenic lines reported in our previous study (Benekos et al. 2010) transgenic line L3 was used in all the subsequent experiments.

The expression of the *GmGSTU4* gene under the control of the constitutive CaMV 35S promoter was examined in the transgenic tobacco plants. Shoot and root RNA isolation, cDNA synthesis and primers used were carried out as described in Benekos et al. (2010). Quantitative expression analysis of *GmGSTU4* transgene was performed using Kapa SYBR Fast qPCR Kit (KapaBiosystems) in a Corbett Rotor Gene 6000 Real-Time PCR thermocycler. The expression of the tobacco β -actin gene was used as a control for relative gene expression quantification. Amplification of both genes was performed for 30 cycles of 5 s at 95°C , 20 s at 61°C , and 3 s at 72°C . Relative expression was determined as described above.

Salt stress treatments

Three-week-old in vitro grown WT and transgenic tobacco plants were subjected to salinity stress after transplantation to MS medium supplemented with 150 and 300 mM NaCl. Stress tolerance was assessed after 30 days of growth measuring growth parameters such as shoot length and total root fresh weight.

Stress treatments in plants used for the metabolite analysis were slightly modified. Two-week-old plants were transplanted in MS medium containing 100 mM NaCl. Leaves used for the metabolite analyses were harvested 20 days after imposition of stress.

Metabolite extraction, derivatization and GC–MS analysis

Plant leaves were harvested 20 days after imposition of 100 mM NaCl in vitro and immediately frozen in liquid nitrogen and lyophilized. Approximately, 10 mg dry weight of tissue was ground in liquid nitrogen. Ground samples were extracted with 380 μL methanol and 20 μL ribitol in methanol (0.2 mg mL^{-1}). Samples were incubated at 70°C for 15 min with continuous shaking. The addition of 200 μL chloroform was followed by a further incubation at 37°C for 5 min under continuous shaking. After addition of 400 μL ddH₂O, samples were vortexed and then centrifuged at 18,000g for 5 min at room temperature. The aqueous phase containing the polar metabolite fraction was transferred into new eppendorf tubes and dried by nitrogen gas. For derivatization, dried samples were re-suspended in 25 μL methoxyamine-HCl (MOX) (20 mg mL^{-1} in pyridine), and incubated at 30°C for 90 min with continuous gentle agitation. This was followed by addition of 50 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and incubated at 37°C for 30 min with continuous gentle agitation. Finally, 10 μL of n-alkane mix was added for determination of retention indexes (RIs). Gas chromatography coupled to mass spectrometry (GC–MS) measurements were performed in a HP6890 GC coupled to a HP 5973 MS.

Data analysis

For all treatments tested, five biological replications were performed. The chromatograms were evaluated automatically using the AMDIS software and metabolites were identified using the Golm metabolome database (Kopka et al. 2005; Schauer et al. 2005). Results were expressed as a response that corresponds to the ratio between the areas of the target metabolite divided by the area of the reference metabolite (ribitol, m/z 319) and reported relative to the dry weight.

Principal component analysis (PCA) was performed on the relative metabolite concentration data using the Unscrambler 9.5 software (CAMO Software Inc., NJ, USA). Statistically significant differences between genotypes and treatments for each metabolite were validated by the Student's *t* test ($p \leq 0.05$).

Results

GmGSTU4 expression analysis under stress conditions in soybean

Given that the function of GSTs is linked to the detoxification of xenobiotics and the response to abiotic stresses (Chronopoulou et al. 2012, 2014), expression analyses for *GmGSTU4* gene were carried out under normal as well as under stress conditions. Transcript levels were measured by means of real-time RT-qPCR analysis and normalized by the transcript levels of the reference *GmUbiquitin* gene. *GmGSTU4* gene was found to be constitutively expressed at low level in soybean suggesting that the respective protein has a specific functional role under different conditions. To evaluate the expression abundance of *GmGSTU4* in response to different chemical stimulants (atrazine, CDNB, H₂O₂) and stress conditions (osmotic stress, cold and heat shock), young soybean plants were exposed to various stress factors. Osmotic stress, caused by the addition of NaCl, induced an approximately 153-fold increase in transcript accumulation of *GmGSTU4* (Fig. 1). Stress induced by the herbicide atrazine caused 17-fold increase in transcript accumulation. However, the abundance of transcripts was unaffected in response to different chemical stimulants (CDNB, H₂O₂) and heat shock.

Tolerance of tobacco plant overexpressing *GmGSTU4* to salt stress

In a previous study, Benekos et al. (2010) had reported the development of three independent transgenic lines

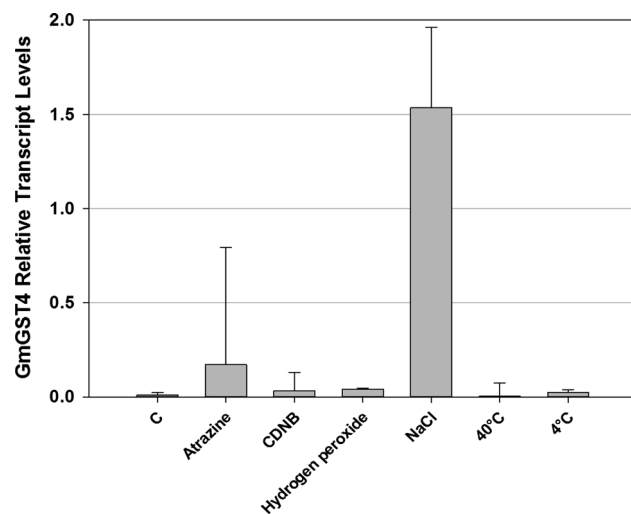


Fig. 1 Accumulation of *GmGSTU4* gene transcripts under various stress conditions. Relative mRNA level was calculated with respect to the respective expression level in the control plants (=1) after normalization with the levels of the ubiquitin (*GmUb*) transcripts. Bars mean \pm SD ($n = 3$)

overexpressing *GmGSTU4*. Line L3 (hereafter mentioned as GST-OX) exhibited the greater increase in GST and GPOX activity and tolerance to herbicide treatments. While this line had no statistically significant difference with other lines tested on salt tolerance (Supp. Fig. 1a), it carried a single transgene insert, and thus it was selected and used in all the subsequent experiments presented here.

Transgene expression, determined quantitatively by real-time qPCR, was found to be 193-fold greater than actin in the shoots of the transgenic plants. Expression in the roots was in average twofold lower than in the shoots (yet, it was still 87-fold higher compared to actin), without being statistically significantly different compared to the shoot. We evaluated the tolerance of transgenic plants to salinity stress in vitro by transplanting three-week-old seedlings to MS medium supplemented with 150 and 300 mM NaCl, respectively. GST-OX plants, when grown in 150 mM NaCl, exhibited increased tolerance compared to WT plants (Fig. 2a), with growth traits measured, such as shoot length and total fresh weight, being almost equivalent with those of plants grown under normal conditions. Particularly, shoot length, total fresh weight and root fresh weight were 48.9, 22.9 and 52.9 %, respectively, greater in transgenic plants compared to WT plants (Fig. 2b). Doubling of the NaCl concentration to 300 mM resulted in severe growth reduction of both WT and GST-OX plants, with the latter exhibiting, although greatly inhibited, 60.5 % increased shoot length, compared to WT plants, while no significant differences were observed for total fresh weight and root fresh weight.

Metabolomics analyses

The increased tolerance of GST-OX plants to salinity stress in vitro led us to further investigate the response of WT and GST-OX plants through the study of their metabolite changes during stress. Plants were exposed to 100 mM NaCl. This concentration has caused significant differences in tolerance between control and GST-OX plants (Supp. Fig. 1a), while at the same time allows us to detect metabolite changes due to stress response and acclimatization, and not due to indirect effects of extreme stress conditions such as toxicity due to plasmolysis caused by high osmolarity (caused by the salt concentration) (Munns 2002) and cell death (Quan et al. 2008). Furthermore, plants were grown for a long period under stress (20 days), to monitor stable changes in metabolite concentration, in contrast to shock and rapid changes that occur under short period of stress, which allows us to study the acclimation and the achievement of cellular homeostasis under stress conditions (Sanchez et al. 2008; Shavrukov 2013; Skiryicz et al. 2011).

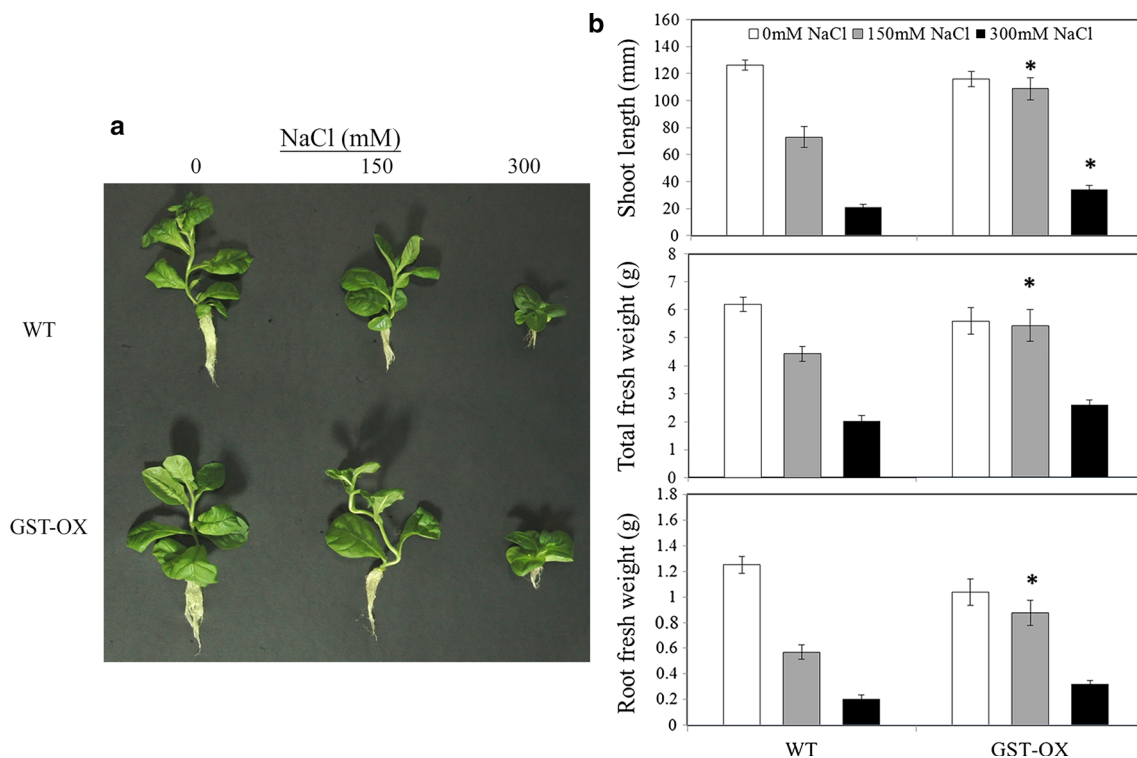


Fig. 2 Tolerance of wild-type (WT) and *GmGSTU4* overexpressing tobacco plants (GST-OX) to salt stress: **a** phenotype and **b** growth traits of WT and transgenic tobacco plants grown for 30 days in MS

Analysis of leaf extracts carried out by GC/MS identified a total of 76 polar metabolites (Supp. Table 1). On this basis, we examined differences in the metabolome between WT and GST-OX plants under control conditions and after salinity stress imposition.

Effect of *GmGSTU4* overexpression on metabolome under non-stress conditions

GmGSTU4 overexpression had a significant effect on plant metabolism under stress-free conditions, as indicated by the 11 out of 76 metabolites (14.5 %) identified having significantly different concentration (Table 1). Metabolites significantly increased in GST-OX plants were acetyl-L-serine, a precursor of the cysteine and glutathione biosynthesis, and glycine (8.1- and 2.2-fold increase, respectively), the TCA cycle intermediate fumaric acid (6.3-fold), the osmoprotectant hydroquinone (5.3-fold) and the precursor of glycerolipid biosynthesis glycerol-3-phosphate (2.9-fold). The levels of hexoses, glucose and fructose were decreased significantly 2.9- and 2.8-fold, respectively, as well as the precursor of ascorbate biosynthesis mannose (8.3-fold). Two metabolites (palatinose, saccharic acid) were specifically identified in the transgenic plants under control conditions. When all the metabolite

medium supplemented with 150 or 300 mM NaCl. Data are the mean \pm SE ($n = 4$). Asterisks significant differences at $p \leq 0.05$ between WT and transgenic tobacco plants at the same treatment

data were subjected to Principal Component Analysis (PCA), the effect of *GmGSTU4* overexpression was revealed, indicating a shift of transgenic plants metabolic profile towards that observed under stress conditions. Thus, the overexpression of *GmGSTU4* has functioned as a signal in the plant that it is under stress and responded accordingly by increasing certain metabolites to protect itself (Fig. 3a).

Effect of salinity stress in vitro in the metabolome of WT tobacco plants

The imposition of salinity stress resulted in a major alteration of WT tobacco plants metabolism, affecting the concentration of 25 % of the metabolites identified, which were distributed in all the different functional categories suggesting strong effect of salt stress in the central metabolism (Table 1). Metabolite data were analyzed with PCA. The two highest ranking principal components accounted for 82 % of the total variance in the metabolite datasets for salinity stress. PCA revealed a clear separation of WT plants metabolome grown in 100 mM NaCl from those grown under non-stress conditions (Fig. 3a).

Table 1 Metabolites that were significantly ($p < 0.05$) altered in the different genotypes and conditions

WT NaCl/WT control		GST-OX NaCl/GST-OX control	
Metabolites	Fold change	Metabolites	Fold change
Mannitol	133.2	Mannitol	65.3
Hydroquinone	8.7	Allantoin	8.4
Glycerol 3-phosphate	6.4	Proline	7.5
Acetyl-L-serine	6	Sedoheptulose	6.9
Allantoin	5.7	Hydroquinone	6.1
Trans-4-hydroxy-L-proline	3.8	Nicotinamide	5.2
Pyrrole-2-carboxylic acid	3.5	Glycerol	5.1
Trans-3-hydroxy-L-proline	2.8	Pyrrole-2-carboxylic acid	4.9
Serine	2.8	Trans-3-hydroxy-L-proline	4.4
		Malonic acid	3
Putrescine	-2.2	Trehalose	2.8
Valine	-2.2	Glycerol 1-phosphate	2.7
Aspartic acid	-2.8	Serine	2.1
Citric acid	-2.8	Trans-4-hydroxy-L-proline	str. sp
Glucose	-6.7		
Fructose	-9.1	Putrescine	-1.8
Maleic acid	-10.4	Valine	-2.5
Sedoheptulose	-10.7	Fructose	-4.8
β -D-1-Thiogalactopyranoside	-15.9		
6-Phosphogluconic acid	ctr.sp		
GST-OX control/WT control		GST-OX NaCl/WT NaCl	
Metabolites	Fold change	Metabolites	Fold change
Acetyl-L-serine	8.1	Sedoheptulose	17.2
Fumaric acid	6.3	Fumaric acid	12.1
Hydroquinone	5.3	Maleic acid	6.2
Glycerol 1-phosphate	2.9	β -D-1-Thiogalactopyranoside	4.8
Glycine	2.2	Hydroquinone	3.7
Threonine	1.8	Glycerol	3.3
		Glucose	2.7
Lactic acid	-2.2	Trans-4-hydroxy-L-proline	2.2
Fructose	-2.8	Glycine	2.2
Glucose	-2.9	Proline	1.8
β -D-1-thiogalactopyranoside	-4.4	Nicotinic acid	1.5
Mannose	-8.2	6-Phosphogluconic acid	GST-ox sp.
		Mannitol	-2.8

Differences between treatments were resolved by PC1, with the salt-treated plants being towards the negative side of PC1 and non-stressed plants on the positive side. Investigation of the loadings for PC1 revealed positive values for sucrose and fructose and negative for serine, meaning that differences can be attributed to the reduction in the concentration of these hexoses and the accumulation of serine in salt-stressed WT plants (Fig. 3b). Comparison of changes in individual metabolites concentration under salt stress using the t test ($p \leq 0.05$) confirmed the results of

the PCA. Specifically of the 19 metabolites whose concentration was significantly altered, nine increased and ten decreased (Table 1). Among the metabolites with increased concentration were the sugar alcohol mannitol (133.2-fold), the amino acid serine and its derivative acetyl-L-serine (2.8- and 6-fold, respectively) and the nitrogen metabolism intermediate allantoin (5.7-fold). On the other hand, a significant decrease was observed in the concentration of hexose sugars glucose and fructose and the heptose sedoheptulose (6.7-, 9.1- and 10.7-fold,

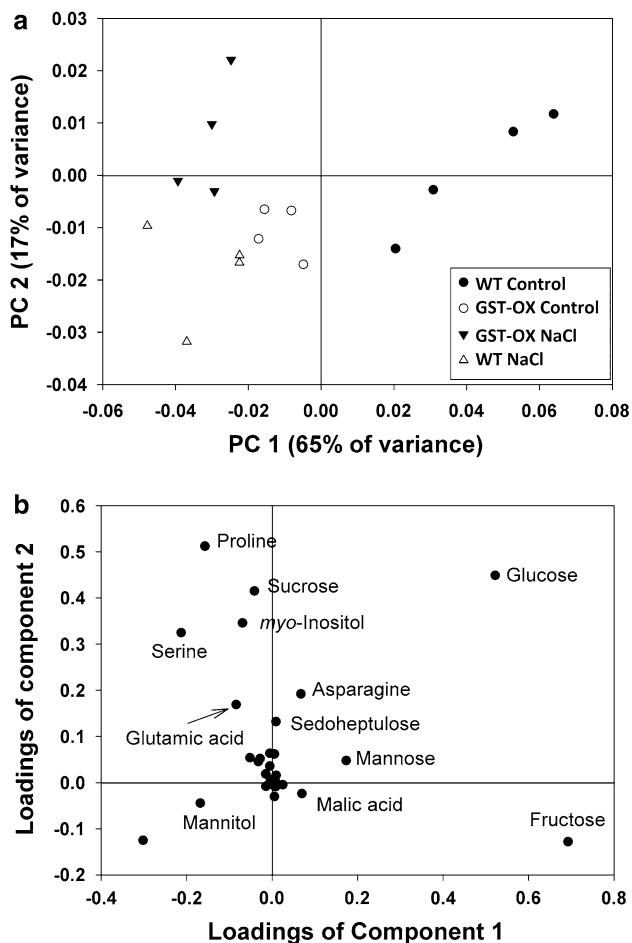


Fig. 3 Principal component analysis (a) of leaf metabolites identified in WT and *GmGSTU4* overexpressing tobacco plants grown for 20 days in MS medium and MS supplemented with 100 mM NaCl. Principal components (PC) 1 and 2 account for 82 % of the variance in the data. **b** Metabolite loadings plot for PC1 and PC2. Loading values for specific metabolites are indicated

respectively), the TCA cycle intermediate citric acid (2.8-fold) and the precursor of various amino acids aspartic acid (2.8-fold).

The metabolic response of GST-OX tobacco plants under salt stress and in comparison to the WT plants

GmGSTU4 overexpression had a profound effect on tobacco plants metabolome compared to WT plants, under salinity stress. A similar number of metabolites were significantly altered in the GST-OX plants in comparison with the WT (17 compared to 19). However, in transgenic plants, there was a significant reduction in the number of metabolites that were decreased under stress (3 compared

to 10, respectively), indicating a protective effect of *GmGSTU4* overexpression to the plants homeostatic mechanisms under salt stress (Table 1).

Comparison of changes in individual metabolites revealed that of the 14 metabolites increased in transgenic plants, six are unique (glycerol, nicotinamide, proline, sedoheptulose, trehalose, malonic acid), indicating distinct metabolic responses in the latter (Supp. Fig. 2a). Almost all of these metabolites have been previously shown to contribute in increased tolerance to salt stress, further justifying the phenotypes observed in GST-OX plants. On the other hand, all three metabolites (putrescine, valine and fructose) that decreased are common with the WT plants (Supp. Fig. 2b). These results point to a distinct response of GST-OX plants to salt stress, and an efficient up-regulation of the protective mechanisms.

Further quantitative comparison with PCA showed that GST-OX plants metabolic profile was clearly separated from that of WT plants under salt stress, with the former being on the positive side of PC2, while the latter on the negative (Fig. 3a). Metabolites that contributed to this separation were mainly glucose and proline whose concentration was significantly greater in GST-OX plants (2.7- and 1.8-fold, respectively). Other metabolites that were significantly increased compared to WT plants were the oxidative pentose phosphate pathway intermediates phosphogluconic acid (GST-OX specific) and sedoheptulose (17.1-fold increase), the osmoprotectants glycerol and hydroquinone (3.2- and 3.7-fold, respectively) and the hydrophilic amino acids glycine and hydroxyproline (both 2.2-fold). Notably, the compatible solute mannitol was 2.8-fold decreased compared to WT plants.

Discussion

The physiological roles of the plant GST superfamily of enzymes still remain to be fully understood, despite the functional characterization of a large number of GST genes from different plant species. Even though their function in herbicide detoxification through GSH conjugation is well characterized both enzymatically (Cummins et al. 2011; Skipsey et al. 2005), and in oxidative stress alleviation through GSH-dependent peroxidase activity (Kilili et al. 2004) their function in a cellular context and its homeostasis both under stress as well as non-stress conditions are not yet well defined (Dixon and Edwards 2010).

Salt stress resulted in a marked increase of the native *GmGSTU4* gene expression in WT soybean plants, indicative of its involvement in salt stress adaptation. *GmGSTU4* induction exhibited a marked specificity in response to different chemical and stress treatments,

suggesting that its regulation is controlled by specific mechanisms (Wagner et al. 2002).

GmGSTU4 overexpressing transgenic tobacco plants displayed a remarkable increased tolerance to salt stress as their growth was not affected at 150 mM NaCl compared to non-stressed plants. These observations support the results from previous studies which have demonstrated increased growth capacity of plants overexpressing GST isoenzymes under salt stress (Diao et al. 2011; Roxas et al. 2000). *GmGSTU4* exhibits increased GSH peroxidase activity, its overexpression in tobacco resulted in increased tolerance to photobleaching herbicides that are not detoxified by GSH conjugation (Benekos et al. 2010), and potentially provides transgenic plants with an efficient antioxidant mechanism to counteract oxidative stress generated under abiotic stresses like the increased salt conditions they were subjected to.

We have further dissected the increased stress tolerance of GST-OX plants using metabolomics. Metabolomics is a recent addition along the other -omics technologies, offering substantial coverage of a large number of metabolites belonging to different structural and functional groups (Hagel and Facchini 2008). As metabolites are the end products of gene expression, metabolomics are invaluable for the dissection of the observed phenotype (Schauer and Fernie 2006). Along with their functional properties as structural molecules or their involvement in biochemical reactions, metabolites have been recently acknowledged as signaling molecules with pleiotropic effects on major developmental and stress acclimation processes, both in the cellular and whole plant context (Wahl et al. 2013; Xiao et al. 2012).

Comparing the metabolome of WT and *GmGSTU4* overexpressing plants under optimal growth conditions with PCA indicated a shift of the metabolism of the latter towards the metabolic fingerprints obtained under stress conditions. Many metabolites were differentially accumulated under control conditions, among them precursors of major cellular redox regulators such as *O*-acetyl-serine and glycine, precursors of glutathione (Noctor et al. 2012) and mannose, precursor of ascorbic acid (Wheeler et al. 1998). Possibly the increased abundance of *GmGSTU4*, which possesses a relatively high GSH-dependent peroxidase activity, perturbs the redox balance even under control conditions, which is translated to altered flux through GSH biosynthesis pathway (Mhamdi et al. 2010; Roxas et al. 2000). The decreased hexose content resembles the metabolic response under oxidative stress (Baxter et al. 2007) and the increased accumulation of osmoprotectants (hydroquinone) further points that transgenic plants metabolic homeostasis was altered towards a stress anticipation state, probably due to the differential response observed between WT and GST-OX plants under salt stress. Thus, apart from the per se protective effect of

GmGSTU4 against oxidative stress that shielded transgenic plants against salt stress, *GmGSTU4* overexpression under non-stress conditions resulted in a primed response that potentially aids for a rapid response against salt stress. This is evident by the metabolic response of WT plants in which salt stress resulted in the accumulation of *O*-acetyl-serine and hydroquinone, metabolites that were already in increased concentration in the transgenic plants under optimal conditions.

Interestingly, glycerol 3-phosphate levels, which have recently been implicated in the induction of systemic acquired resistance (Chanda et al. 2011), were threefold higher in the transgenic plants, and may be the link to the positive contribution of GSTs to disease resistance (Chasot et al. 2008; Dean et al. 2005; Wisser et al. 2011).

Salt stress resulted in the deregulation of major metabolic processes in WT plants, namely nitrogen assimilation, glycolysis and the TCA cycle that are crucial for energy homeostasis and resumption of plant growth under stress (Widodo et al. 2009). On the other hand, none of these perturbations were observed in the GST-OX plants. Moreover, the latter had uniquely upregulated metabolites with unambiguous relationship with stress tolerance such as proline, trehalose, and glycerol. Proline alone has been shown to have multiple functions under stress such as osmoprotective, scavenging of ROS, and maintaining the redox balance (Szabados and Savouré 2010). Interestingly, transgenic tobacco plants overexpressing a GST gene from the halophyte *Limonium bicolor*, also exhibited increased accumulation of proline under salt stress (Diao et al. 2011). Trehalose contribution to adaptation and tolerance to abiotic stresses, through protective and signaling functions, is also established (Paul et al. 2008). In addition, several metabolites with protective function such as hydroquinone and hydroxyproline (Warren et al. 2012) reached higher levels in GST-OX plants as well as metabolites involved in energy homeostasis such as glucose and nicotinic acid compared to the WT plants. Both metabolites are further implicated in signaling functions that regulate plant development (Warren et al. 2012). The accumulation of sedoheptulose and phosphogluconic acid implies increased flux through the oxidative pentose pathway that generates NADPH (Kruger and Von Schaeuwen 2003), a reductant source for major antioxidant pathways in plants. Mannitol was the only osmoprotectant with a decreased concentration in transgenic plants. However, there is evidence that its accumulation is mostly related to susceptibility to stress (Chen et al. 2007), therefore this finding adds up to the fact that transgenic plants were less affected by salt stress.

The compilation of these observations points to the conclusion that the increased stress tolerance of GST-OX was an outcome of the up-regulation of diverse metabolic

components. Thus, the manipulation of plant stress tolerance by overexpression of antioxidant enzymes does not simply involve the effective scavenging of ROS generated under stress but rather complex mechanisms that have pleiotropic effects. In addition to this complexity, we have to consider that GSTs are also implicated actively in endogenous metabolic processes such as binding and transport of anthocyanin, flavonoids and porphyrins (Cummins et al. 2013; Dixon et al. 2010), phytoalexin biosynthesis (Su et al. 2011), as well as binding and glutathione conjugation of reactive electrophile species (Mueller et al. 2008). Almost all those above-mentioned metabolites appear to have protective and/or signaling functions under stress conditions (Böttcher and Pollmann 2009; Phung et al. 2011). As the crystallographic structural characterization of *GmGSTU4* showed a ligand site (L-site) (Ayarli et al. 2009a), we cannot exclude that this enzyme exerts an above-listed function, which can in part result in the metabolic alterations observed under optimal growth conditions and salt stress.

In conclusion, the functional characterization of *GmGSTU4* by its overexpression in tobacco plants confirmed its protective role to salinity stress which was strongly suggested by its strong salt stress expression induction in soybean. Further metabolomics analysis of the transgenic tobacco plants revealed pleiotropic effects of *GmGSTU4* overexpression with multiple pathways affected even under optimal growth conditions, demonstrating that increased tolerance to salt was in part a result of metabolic priming. Moreover, given the signaling functions of many metabolites that were in higher abundance in transgenic plants under salt stress, the protective function

of *GmGSTU4* was potentially further amplified by these pleiotropic metabolic responses (Fig. 4). Biotechnological manipulation and fine-tuning these underlying metabolic alterations may further increase the potential of GSTs in conferring abiotic stress tolerance.

The unbiased identification of GST isozymes by forward genetics screens for both abiotic (Kim et al. 2011) and biotic (Wisser et al. 2011) stress tolerance points their major universal functions under stress and justifies further detailed studies on their physiological roles for crop improvement.

Author contribution statement Christos Kissoudis: performed the experiments, analyzed the data, wrote the paper. Chrissanthi Kalloniati: performed the experiments, analyzed the data. Emmanouil Fletmetakis: performed the experiments, analyzed the data. Nikolaos E. Labrou: designed the study, supervised the study, wrote the paper. Panagiotis Madesis: performed the experiments, analyzed the data, wrote the paper. Athanasios Tsaftaris: supervised the study, wrote the paper. Irini Nianiou-Obeidat: designed the study, supervised the study, wrote the paper.

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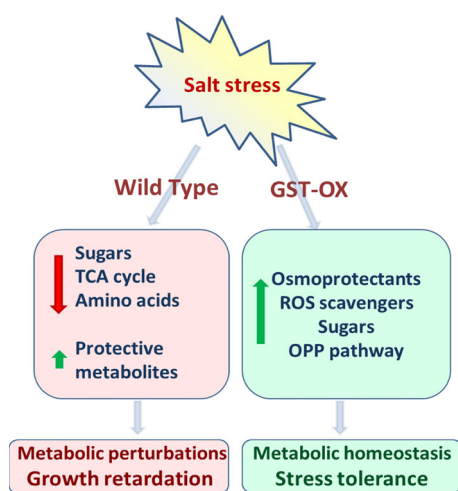


Fig. 4 Schematic representation of differences in metabolic regulation between WT and GST-OX tobacco plants underlying the enhanced salt tolerance of GST-OX plants. *OPP* oxidative pentose phosphate pathway

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