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A Putative Lambda Class Glutathione S-Transferase Enhances Plant Survival under Salinity Stress

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Regular Paper

In a survey of candidate genes located in the salinity tolerance locus of soybean, we identified a putative glutathione S-transferase (GST) gene (*GmGSTL1*) which was up-regulated in response to salt treatment. Phylogenetic analyses revealed that this putative GST belongs to the Lambda class, a plant-specific group with unknown functions. We expressed *GmGSTL1* in heterologous systems, including tobacco BY-2 cells and *Arabidopsis thaliana*, to test its ability to protect the cell/plant against salinity stress. Compared with the control, we observed a marked reduction of reactive oxygen species (ROS) accumulation in transgenic cells under salt treatment, and their survival rate was also improved. Similarly, expression of *GmGST1* in transgenic *A. thaliana* also alleviated stress symptoms under salt treatment. To address further the possible protective mechanisms of GmGSTL1, we identified two candidate flavonoid interactants (quercetin and kaemferol) of the GmGSTL1 protein from soybean leaf extract. Exogenous application of quercetin could reduce salinity-induced ROS accumulation in BY-2 cells and leaf chlorosis in *A. thaliana*.

Keywords: Glutathione S-transferase • Oxidative stress • Salinity • Soybean.

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DAB, diaminobenzidine tetrahydrochloride; EIC, extracted ion chromatogram; ESI, electrospray ionization; FTMS, Fourier transform mass spectrometry; GSH, glutathione; GST, glutathione S-transferase; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MS medium, Murashige and Skoog medium; ROS, reactive oxygen species; WT, wild type.

Introduction

Glutathione S-transferases (GSTs) constitute an ancient and highly diverse group of proteins essential for enzymatic detoxification, cell signaling and other cellular processes in microbes, animals and plants (Hayes et al. 2005, Atkinson and Babbitt 2009, Gill and Tuteja 2010). Despite their structural and functional heterogeneity, the common enzymatic activity is to catalyze the nucleophilic substitution or addition of reduced glutathione (GSH) to the electrophilic center of various non-polar compounds (Hayes et al. 2005). GSTs can be further classified into structurally distinct polyphyletic families (Atkinson and Babbitt 2009). In *Arabidopsis thaliana*, there are 54 GSTs documented (Dixon et al. 2010). The Phi (F) and Tau (U) classes are believed to be plant specific (Edwards et al. 2000). The F class is auxin responsive (Dixon et al. 2002) and participates in the proanthocyanidin transportation pathway (Katsuhara and Kawasaki 1996), while the U class is well known for its xenobiotic-conjugating property (Dixon et al. 2002). The Theta (T) and Zeta (Z) classes share close similarities with the mammalian system and are involved in hydroperoxide reduction and tyrosine catabolism, respectively (Dixon and Edwards 2006, Dixon et al. 2009). The function of Lambda (L) class GSTs, however, remains largely unknown.

One indirect function of GSTs is to remove reactive oxygen species (ROS), a collective term including superoxide radicals, hydroxyl radicals, alkoxy radicals, hydrogen peroxide and singlet oxygen, which are continuously produced in 'energy factories' (Dixon et al. 2010). In plants, the chloroplast PSI and PSII and the mitochondria complex I and III of the electron transport chain are prime sites for the generation of ROS (Moller 2001, Moller et al. 2007, Miller et al. 2010). Abiotic stresses, such as salinity, drought and heavy metals, could lead to the overproduction of ROS, resulting in oxidative damage to membrane proteins, lipids, DNA and other cytosolic components (Gill and Tuteja 2010). Leaf chlorosis, which is the first obvious indicator of senescence-associated programmed cell death, is a well-known consequence of ROS accumulation (Lim et al. 2007). The up-regulation of antioxidant genes and enhanced antioxidative enzyme activities, such as those of GSTs, indicate an evolutionarily conserved response to oxidative stress in various organisms (Hayes et al. 2005). There are reports showing that the overexpression of enzymes with general GST activity improves abiotic stress tolerance in various plant models (Zhao and Zhang 2006, Diao et al. 2010, Z.Y. Wang et al. 2012). However, unlike GSTU that catalyzes the GSH conjugation of exogenous xenobiotics, there is little evidence for GST/GSH conjugation to secondary metabolites (Dixon et al. 2010).

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In this study, we identified a putative Lambda class GST in soybean (*GmGSTL1*). Two candidate flavonoids, namely quercetin and kaemferol from soybean leaf extracts, were found to bind to GmGSTL1 protein. Either the ectopic expression of *GmGSTL1* or the supplementation of exogenous quercetin could functionally improve plant tolerance toward salinity stress. In sum, we present here the molecular and physiological evidence supporting the involvement of Lambda class GSTs in salinity tolerance of plants.

Results

Cloning of *GmGSTL1*

In a survey of candidate genes located in a salinity tolerance locus of soybean (Lee et al. 2004, and unpublished data), we identified a gene (*GmGSTL1*) encoding a GST (Phytozome: Glyma03g33340.5) which may play a role in stress tolerance. We cloned the cDNA containing the full-length coding region of *GmGSTL1* from a salinity-tolerant soybean. Phylogenetic analysis (**Supplementary Fig. S1**) revealed that the GmGST protein belongs to the Lambda class GSTs and shares 79.1, 76.2 and 72.8% sequence identity with the AtGSTL3, AtGSTL1 and AtGSTL2 proteins from *A. thaliana*, respectively. The N-terminus of GmGSTL1 consisted of the TRX domain (Atkinson and Babbitt 2009) which harbors the GSH-binding consensus (G-site) and the well-conserved cysteine in the active site (**Fig. 1A**). The C-terminus consisted of the typical α-helical domain of Lambda class GSTs (**Fig. 1A**), containing the hydrophobic substrate-binding pocket (H-site) for endogenous and xenobiotic alkylating agents such as carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress (Edwards et al. 2000, Dixon et al. 2010). Gene expression analyses showed that *GmGSTL1* is mainly expressed in leaves (**Supplementary Fig. S2**), and its expression in the leaf tissue can be further induced by salt treatment (**Fig. 1B**, **C**).

Ectopic expression of *GmGSTL1* in transgenic BY-2 cells reduced ROS accumulation and enhanced cell survival under salinity stress

To test the protective role of *GmGSTL1* in plant cells, the full-length coding region was ectopically expressed in the tobacco BY-2 cell line for gain-of-function tests. Expression of the transgene was confirmed by reverse transcription real-time PCR (**Supplementary Fig. 3A**, **B**). Under salinity stress, the accumulation of ROS was monitored by the fluorescent probe H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) and cell death was visualized by trypan blue stain. Without NaCl treatment, wild-type (WT) cells exhibited the basal fluorescence signal (**Fig. 2A**) and the survival rate approached 100% (**Fig. 2B**). Addition of 100 mM NaCl induced ROS accumulation. Under 100 mM NaCl treatment, a marked increase in fluorescence intensity was observed in the WT and the empty vector transgenic control (V7) cell lines,

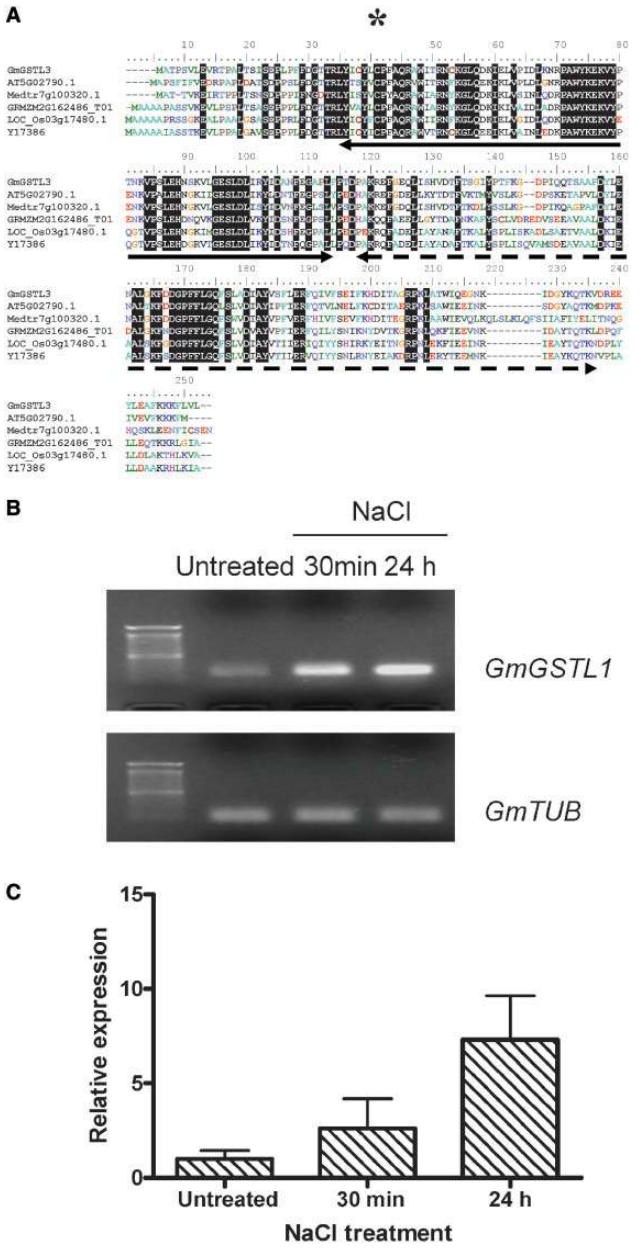

Fig. 1 Cloning of *GmGSTL1*. (A) Sequence alignment of GmGSTL1 and its homologs in *A. thaliana*, *Medicago*, maize, rice and wheat. The N-terminal domain consisted of the TRX domain (solid arrow) which contained the GSH binding motif and the active site cysteine (asterisk). The C-terminal domain consisted of the typical α-helical domain of class Lambda GST (dashed arrow) with a hydrophobic substrate binding pocket which is for substrate binding. The shaded region represents conserved residues. (B) Expression of *GmGSTL1* was induced in soybean leaf under 0.9% NaCl treatment. The expression of *GmGSTL1* was monitored by reverse transcription–PCR and visualized by ethidium bromide staining after 30 PCR cycles for *GmGSTL1* and 25 cycles for the soybean tubulin gene (*GmTUB*). (C) Relative expression was calculated by the ΔΔCT method (Livak and Schmittgen 2001), using expression of the soybean *tubulin* gene for normalization. The expression of *GmGSTL1* in untreated samples was set to 1 for comparison.

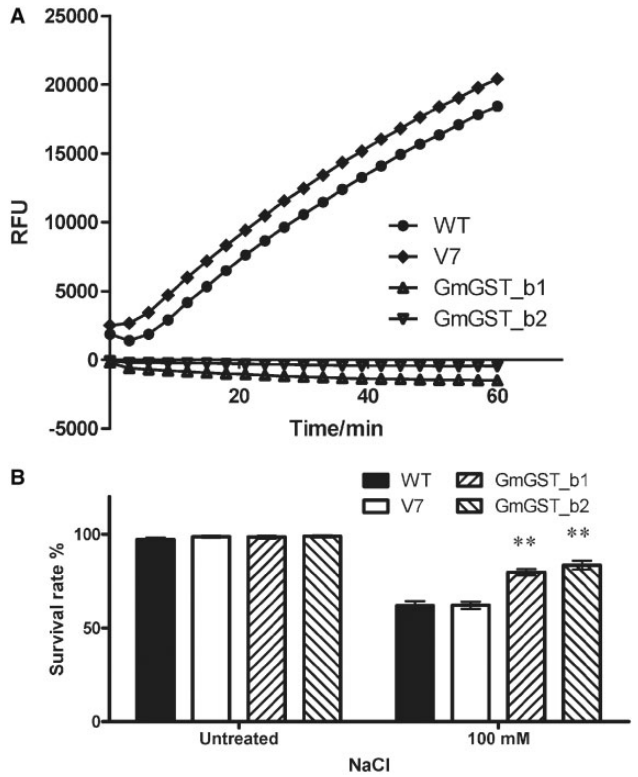

Fig. 2 Gain-of-function study using transgenic tobacco BY-2 cells. (A) Ectopic expression of *GmGSTL1* in BY-2 cells reduced ROS accumulation under salinity stress. Three-day-old culture was subjected to 100 mM NaCl treatment for 1 h. H_2DCFDA fluorescence was quantified in microplates. The increase in relative fluorescence units (RFU) was much more profound in control cell lines (WT, wild type Col-0; V7, empty vector transgenic control) than in two independent *GmGSTL1* transformants (GmGST_b1 and GmGST_b2); $n = 4$. (B) Ectopic expression of *GmGSTL1* in BY-2 cells enhanced cell survival under salinity stress. Three-day-old culture was subjected to 100 mM NaCl treatment for 24 h. Survival rates were monitored by trypan blue staining. Values represented mean percentage survival of at least 10 random views of a total cell count of >400. Error bar: standard error. **Denotes a significant mean difference at $P < 0.01$ when compared with the WT, using one-way ANOVA followed by the post-hoc Tukey's test.

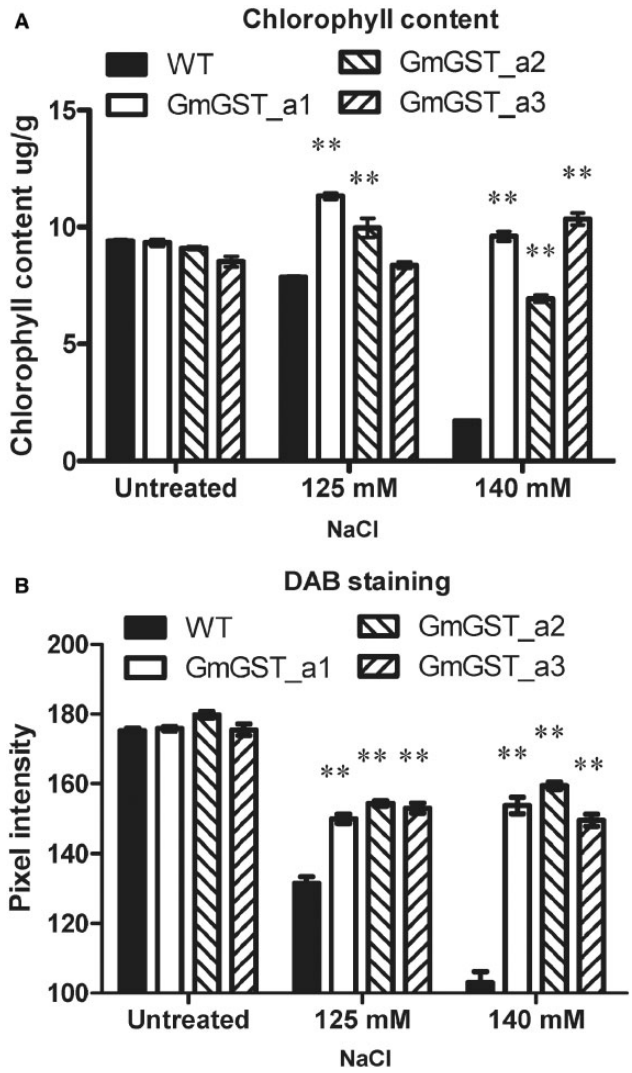

Fig. 3 Functional study using transgenic *A. thaliana*. Overexpression of *GmGSTL1* in *A. thaliana* reduced leaf chlorosis and ROS accumulation under salinity stress. Ten-day-old seedlings were subjected to NaCl treatment for 14 d. (A) The extent of leaf chlorosis was revealed by determining the Chl content. GmGST_a1, GmGST_a2 and GmGST_a3 are independent *GmGSTL1* transgenic lines. WT, untransformed wild type Col-0; $n = 3$ (each data point represented a pooled sample of at least four individual plants). (B) ROS accumulation was detected by histological staining with diaminobenzidine tetrahydrochloride (DAB). Signals were quantified by measuring the image pixel intensity using ImageJ (ver. 1.47). A higher intensity of pixels indicates less ROS accumulation; $n \geq 10$. Error bar: standard error **Denotes a significant difference at $P < 0.01$ when compared with the WT control, according to one-way ANOVA followed by the post-hoc Tukey's test.

while *GmGSTL1* transformants (GmGST_b1 and GmGST_b2) contained much lower levels of ROS (**Fig. 2A**). After the application of 100 mM NaCl for 1 d, cell death was observed. The survival rates in the WT and V7 lines were 65 ± 3% and 62 ± 1%, respectively, which were significantly lower than those of the transgenic lines, 80 ± 5% and 84 ± 6% for GmGST_b1 and GmGST_b2, respectively (**Fig. 2B**).

Ectopic expression of *GmGSTL1* in *A. thaliana* reduced leaf chlorosis and ROS accumulation under salinity stress

To provide further evidence for the protective function of *GmGSTL1* in planta, transgenic *A. thaliana* ectopically expressing *GmGST1* was constructed and the expression of the transgene was confirmed (**Supplementary Fig. S4A**). No abnormal phenotypes were observed in the transgenic plants under normal growth conditions. Under NaCl treatment, growth retardation was observed (**Supplementary Fig. S4B**) and ROS accumulation was revealed by histological staining

with diaminobenzidine tetrahydrochloride (DAB) (**Supplementary Fig. S4C**). Severe leaf chlorosis was observed and quantitative measurements confirmed that the Chl content in the WT was reduced (**Fig. 3A**). On the other hand, the *GmGSTL1* transgenic lines exhibited enhanced tolerance and higher Chl contents than the WT in general. Similar patterns of results were obtained when the NaCl concentration was raised to 140 mM (**Fig. 3A**). Quantitative measurement using DAB staining also showed that the *GmGSTL1* transgenic lines accumulated less ROS (**Fig. 3B**).

GmGSTL1 interacts with flavonoids

The enzymatic activity of GmGSTL1 was confirmed using the His-tagged GmGSTL1 recombinant protein produced in *Escherichia coli*. After His-tag purification, the specific GSH-dependent GST activity was measured (**Supplementary Fig. S5**). The specific enzyme activity of GmGSTL1 was found to be 63.52 ± 6.12 nmol mg^{-1} min^{-1}, which is of the same order of magnitude as the reported soybean homolog but up to three orders of magnitude lower than other reported GSTs (McGonigle et al. 2000). The relatively low GSH-dependent GST activity of the Lambda class GSTs suggests that they may play a functional role different from that of other high activity GSTs. We therefore extracted the total polyphenolic compounds from soybean leaves and searched for potential ligands that might bind to GmGSTL1. Owing to limited standards available, we only targeted the well-known phenolic antioxidants, including chlorogenic acid, rutin, quercetin and kaemferol, and also those flavonoids most abundant in soybean, including daidzin, glycitin, genistin, glycitein and daidzein (**Fig. 4A**). Details of authentic standards used in the experiment are summarized in **Table 1**. Two candidate flavonoids, namely kaemferol (EIC 285.041 ± 0.001, retention time 63.82) and quercetin (EIC 301.036 ± 0.001, retention time 60.50), were identified in the GmGSTL1-bound fraction (**Fig. 4B**). Estimation of the ligand amounts was also performed using standard curves (**Supplementary Fig. S6**).

Application of the flavonoid quercetin alleviated salinity-induced leaf chlorosis

Quercetin has one more -OH group and has approximately 4-fold higher antioxidation activity (based on TEAC value) than kaemferol (Heim et al. 2002). Therefore, we focused our further tests on quercetin and studied its possible protective roles against salinity stress in plants. Interestingly, addition of quercetin could reduce salinity stress-induced ROS accumulation in tobacco BY-2 cells (**Fig. 5**), and leaf chlorosis (**Fig. 6A**) as well as ROS accumulation in *A. thaliana* (**Fig. 6B**). Therefore, supplementation of exogenous quercetin could functionally improve plant tolerance toward salinity stress in a way similar to the ectopic expression of the *GmGSTL1* transgene.

Discussion

The antioxidation characteristics of GSTs have been extensively reviewed (Mittler et al. 2004, Dixon et al. 2010, Gill and Tuteja 2010). However, the molecular mechanism of the transferase activity in relation to plant stress tolerance remains largely unknown. In this study, we reported a salinity stress-induced Lambda class GST in soybean, and provided evidence for its physiological role in the protection against salinity stress. The mechanism of its function was further elucidated by the identification of its molecular interactants.

GmGSTL1 was one of the gene candidates in the salt tolerance locus that was up-regulated in response to salinity stress. The functional role of *GmGSTL1* was confirmed by heterologous systems, including the tobacco cell line model and *A. thaliana* in planta model. In both cases, the overexpression of *GmGSTL1* conferred functional protection and enhanced survival of the transformants under salt treatment.

In plants, GSTs were found to interact with secondary metabolites (Smith et al. 2003, Dixon and Edwards 2010, Dixon et al. 2011). For example, rutin and kaempferol-3-*O*-rutinoside, which were previously unknown natural products in wheat, were discovered as putative ligands binding to TaGSTL1 (Dixon and Edwards 2010). Instead of directly acting on ROS, it has been suggested that GSTLs help maintain the antioxidant flavonol pool by catalyzing the conversion of quinone intermediates to their active antioxidative forms (Dixon and Edwards 2010). On the other hand, it has also been proposed that GST protein itself, instead of GST activity, functions in flavonoid transport mediated by ATP-binding cassette transporters (Zhao and Dixon 2010, Petrussa et al. 2013). The GST–flavonoid complex protect flavonoids from oxidation and direct their transport to the vacuole (Mueller et al. 2000, Zhao and Dixon 2010). The *AtTT19* mutant, a GST mutant in *A. thaliana*, was defective in endoplasmic reticulum surface transport, and accumulation of vacuolar anthocyanins and proanthocyanins was reduced (Kitamura et al. 2004).

Phenolic compounds, when in the reduced form, serve as powerful antioxidants. However, the phenoxyl radicals produced after an antioxidative reaction are pro-oxidative (Bartwal et al. 2013). Therefore, the enzymatic removal of phenoxyl radicals to regenerate and maintain the pool of active phenolic antioxidants will be essential for homeostasis. In vitro experiments showed that TaGSTL1 could mediate the GSH-dependent reduction of derivatives to regenerate active quercetin, which acts as a proton donor to oxidative species. The oxidized quercetin derivatives then spontaneously react with water and GSH to form an adduct, which is recycled as a substrate for the GSTL enzyme activity (Dixon and Edwards 2010). Therefore, GSTL is the potential missing link between recycling and maintaining the antioxidative capacity provided by phenolic compounds. In this study, we provide physiological data supporting that *GmGSTL1* encodes a functional protein in scavenging ROS induced by salt stress using the cell model. Ectopic expression of the transgene also enhanced the survival

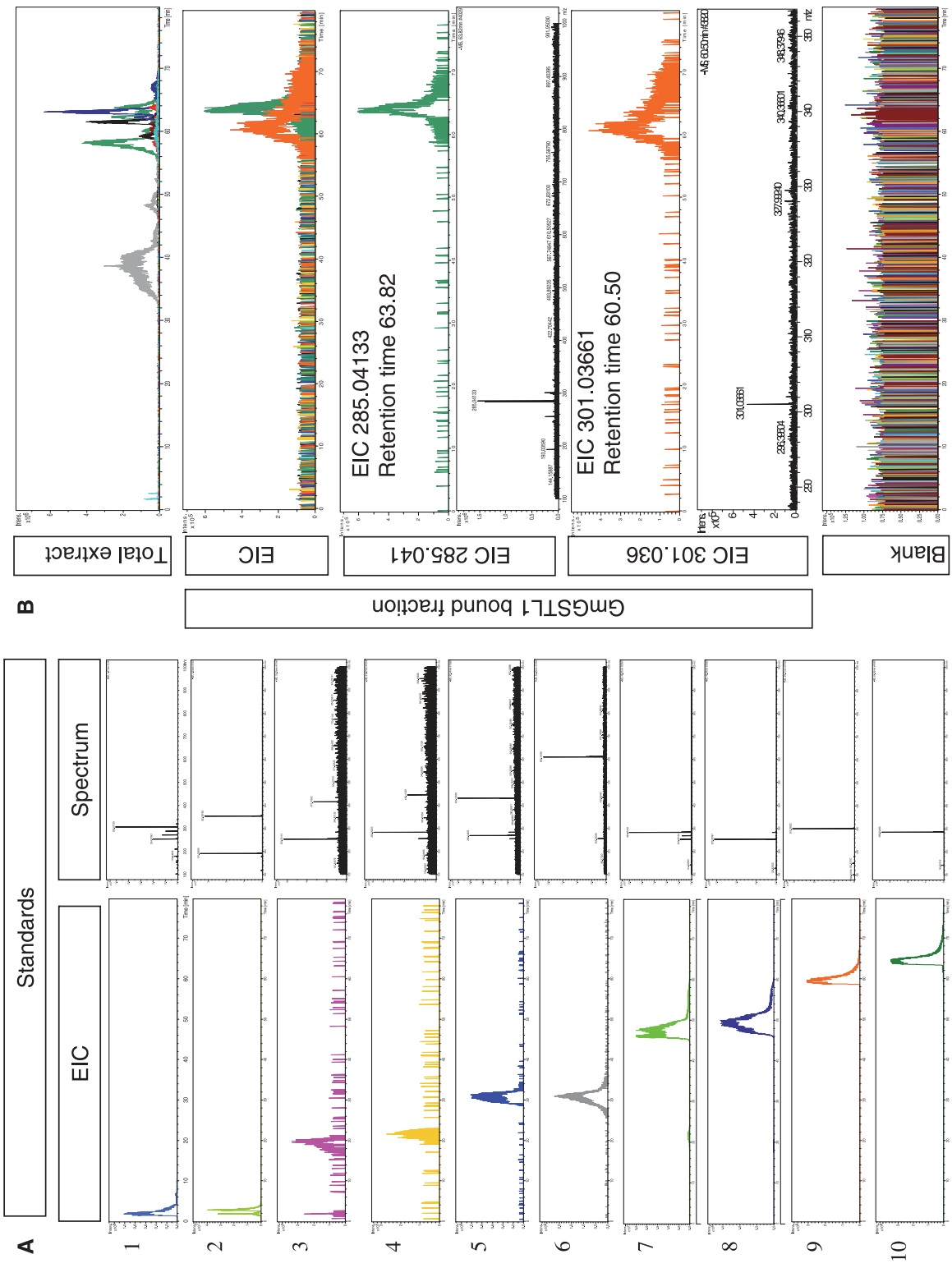

Fig. 4 Ligand identification and FTMS analysis. GmGSTL1 binds to polyphenolic metabolites. (A) Liquid chromatography profile and spectrum of available authentic standards. (B) Total phenolic compounds were extracted from soybean leaflets by solvent extraction followed by purification using an affinity column embedded with HP-20 resin. The recombinant protein of GmGSTL1 was immobilized on a His-Trap column and flushed with total phenolic extracts. Fractions of eluted ligands were pooled, concentrated and subjected to FTMS analysis. EIC: extracted ion chromatogram.

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Table 1 Authentic standards used in the FTMS experiment

No.	Chemical name	Chemical formula	Calculated *m/z* [M-H]-	Measured *m/z* [M-H]-	Error (p.p.m.)	Retention time (min)
1	GSH	C10H17N3O6S	306.075432	306.077290	6.07	1.94
2	Chlorogenic acid	C16H18O9	353.086709	353.087630	2.61	1.97
3	Daidzin	C21H20O9	415.102359	415.105220	6.89	19.59
4	Glycitin	C22H22O10	445.112923	445.113200	0.62	21.67
5	Genistin	C21H20O10	431.097273	431.100390	7.23	30.54
6	Rutin	C27H30O16	609.145011	609.144500	−0.84	30.98
7	Glycitein	C16H12O5	283.060100	283.061470	4.84	46.02
8	Daidzein	C15H10O4	253.049535	253.050970	5.67	49.09
9	Quercetin	C15H10O7	301.034279	301.036220	6.45	59.11
10	Kaemferol	C15H10O6	285.039364	285.040990	5.70	64.08

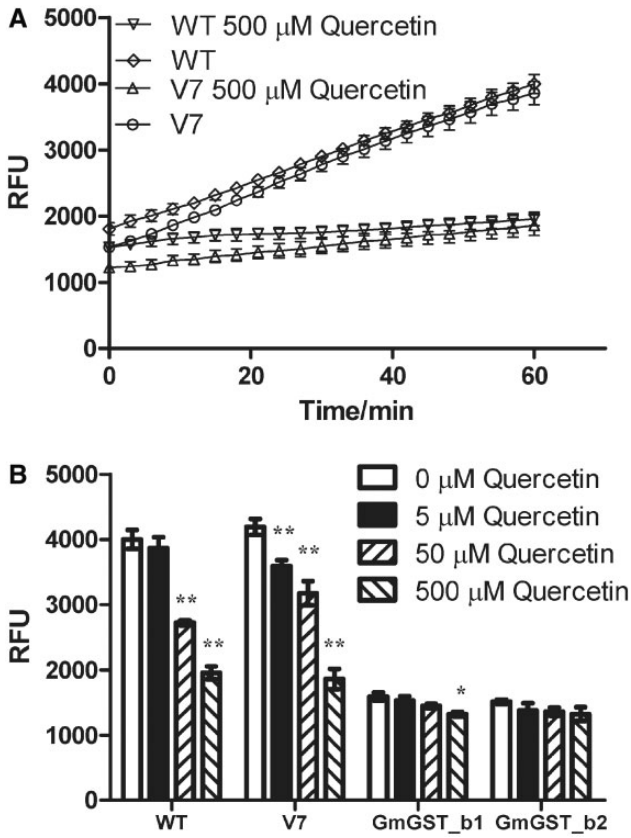

Fig. 5 Application of quercetin reduced salinity-induced ROS accumulation in BY-2 cells. Three-day-old culture was subjected to 100 mM NaCl treatment for 1 h. H_2DCFDA fluorescence was quantified in microplates. (A) Kinetics of H_2DCFDA fluorescence accumulation in the untransformed wild-type (WT) cells and empty vector transformation control (V7) with or without 500 μM quercetin. (B) End-point measurement after 60 min in the presence of different final concentrations of quercetin. Error bar: standard error; $n = 4$. * and ** denote a significant mean difference at $P < 0.05$ and $P < 0.01$, respectively, when compared with the control without quercetin supplementation, using one-way ANOVA followed by the post-hoc Tukey's test.

of cell lines and plants under salt stress. On the other hand, the exogenous supplementation of quercetin could also alleviate salinity stress in planta, suggesting that both GSTL and the phenolic interactant may play similar protective roles.

In conclusion, *GmGSTL1* encodes a Lambda class GST gene which was up-regulated in the soybean leaf under salinity stress. The GmGSTL1 protein interacts with secondary metabolites and both of them play a role in salinity stress tolerance. Both the ectopic expression of the gene and the supplementation of its molecular interactant, quercetin, could functionally improve plant tolerance towards salinity stress. Taken together, we have presented the molecular and physiological evidence supporting the involvement of a Lambda class GST in the salinity tolerance of plants.

Materials and Methods

Plant materials and growth conditions

Soybean seeds were germinated in vermiculite until the cotyledons were fully opened. The seedlings were then transferred to a hydroponic medium (0.5× Hoagland solution) and until the first trifoliate was fully opened. For salt treatment, the culture medium was replenished with fresh 0.5× Hoagland solution supplemented with 0.9% NaCl. Tobacco BY-2 cells, *A. thaliana* (ecotype Col-0) and their respective transformants were cultured following procedures previously reported (Li et al. 2008, H.M. Wang et al. 2012).

Expression analyses and molecular cloning

Total RNAs were isolated from plant materials by TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using a SuperScript III RT Kit (Life Technology). Expression analyses were performed using real-time PCR according to previous publications (Li et al. 2008, H.M. Wang et al. 2012). The primers for PCR experiments are listed in **Supplementary Table S1**. The full-length cDNA of

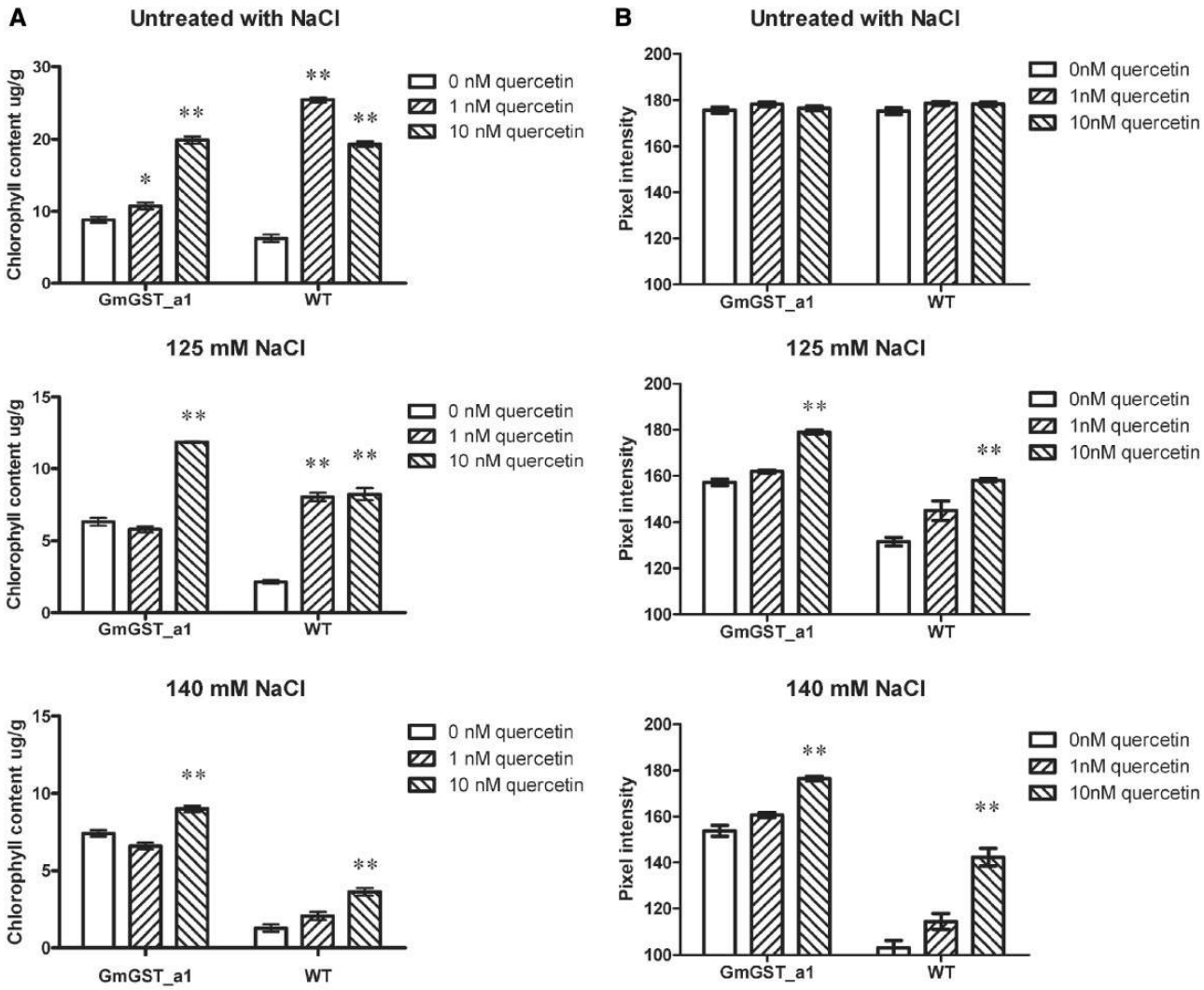

Fig. 6 Application of quercetin reduced salinity-induced leaf chlorosis and ROS accumulation. (A) Chl content and (B) ROS accumulation were measured when 10-day-old seedlings were subjected to NaCl treatment without or with 1 and 10 nM quercetin supplements for 14 d. Methodologies were described in **Fig. 3**. WT, untransformed wild-type Col-0; GmGST_a1, GmGSTL1 transgenic line; $n \geq 10$. Error bar: standard error. * and ** denote a significant difference at $P < 0.05$ and $P < 0.01$. respectively, when compared with the control without quercetin supplementation, using one-way ANOVA followed by the post-hoc Tukey's test.

GmGSTL1 was amplified using primers HMOL7721 (5′-GGg gatccATGGCAACTCCGAGTGTGTTA) and HMOL7722 (5′-GGctcgagTTAAGCCAAAAACTTTTTCTTGAAA), and cloned into the V7 binary vector (Brears et al. 1993). The full-length insert was also subcloned into the pET32a(+) expression vector and transformed into *E. coli* (DE3) for protein expression.

Generation of transgenic models

BY-2 transgenic cell lines and transgenic *A. thaliana* were constructed as previously described (Li et al. 2008, H.M. Wang et al. 2012). Briefly, constructs bearing the full-length *GmGSTL1* under the *Cauliflower mosaic virus* (CaMV) 35S constitutive promoter were transformed into *Agrobacterium tumefaciens* LBA4404 and GV3101, for tobacco BY-2 cell and *A. thaliana* transformation, respectively. Transgenic BY-2 cell lines stably expressing *GmGSTL1* and single-insertion (T_2 3 : 1 segregation ratio) homozygous (T_3 no segregation) *A. thaliana* lines were used in subsequent experiments.

Salt treatment of BY-2 cells

Three day freshly subcultured BY-2 cells were used. For ROS detection, cells were pre-stained with 10 μM H_2DCFDA for 30 min, followed by washing with fresh Murashige and Skoog medium (MS medium). NaCl was then added to give a final concentration of 100 mM. Fluorescence signals were captured using a confocal microscope (Olympus FV1000) or a microplate reader (Synergy H1; excitation, 485 nm; emission, 528 nm). For survival analyses, NaCl was added to the cultures to give a final concentration of 100 mM. The cultures were maintained at 28°C for 24 h. For

visualization and counting, trypan blue was mixed with cell aliquots in a 1 : 1 ratio and at least 10 random views were captured for each experiment. The survival rate was calculated as the percentage of live cells vs. the total number of cells. The experiment was performed twice with independent biological preparations.

Salt treatment of *A. thaliana*

Seeds were germinated on MS medium for 10 d until the root length of seedlings reached approximately 10 mm. The seedlings were transferred onto fresh MS (control) medium containing 125 mM/140 mM NaCl with or without quercetin supplements and grown for another 14 d.

Measurement of Chl content

Chl was extracted from 0.4 g of pooled plant samples by direct immersion into 100% dimethyl formamide, followed by incubation at 4°C overnight. The absorbances at 603, 64 and 664 nm were measured by a spectrophotometer (Synergy H1). The Chl content was calculated according to a published formula (Moran 1982). The experiment was performed twice with independent biological preparations.

Diaminobenzidine tetrahydrochloride (DAB) staining

Histological staining for ROS was performed based on Jambunathan (2010). Briefly, plant materials were immersed in DAB solution (1 mg ml^{-1} in 50 mM Tris-acetate, pH 5.0) for 24 h. The samples were then boiled in 96% ethanol and fixed in fixer solution (ethanol : lactic acid : glycerol = 3 : 1 : 1) prior to imaging.

Phylogenetic studies

For phylogenetic analyses, the sequences of known GST members from *A. thaliana* and mammalian GSTs were retrieved from Phytozome and NCBI, respectively. Multiple sequence alignments were performed by the ClustalW algorithm using MEGA (version 4.0). A phylogenetic tree was constructed using a Neighbor–Joining method (MEGA version 4.0) with default settings. Each protein was named with the abbreviation of the species followed by the Phytozome loci tag or GenBank accession number.

Protein purification and enzyme assay

The full-length cDNA of *GmGSTL1* was fused to a polyhistidine tag in the pET-32a(+) vector (Novagen, cat. no. 69017-3). The recombinant GmGSTL1-His fusion protein was expressed and purified by a HisTrap affinity column according to the manufacturer's protocol (GE Healthcare, 71-5027-68 AH). The identity of the fusion protein was determined using matrix-assisted laser-desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) analysis. A total of 13 trypsin-digested fragments were matched to the hypothesized sequence with an overall score of 244, confirming the succesful expression of the target protein.

For enzyme assays, buffer exchange was performed according to the instructions for HisTrap desalting column (GE Healthcare, 71-7154-00 AK). The protein was eluted with Dulbecco's phosphate-buffered saline (pH 7.0). GST activity was determined by measuring the absorbance of the 1-chloro-2,4-dinitrobenzene (CDNB) conjugate according to the Glutathione S-transferse Assay Kit (Sigma, CS0410). Absorbance at 340 nm was monitored for 20 min at 50 s intervals. Specific activity was calculated according to the formula: $[(\Delta A_{340})\ \text{min}^{-1} \times \text{V (ml)} \times \text{dil}]/[\varepsilon\text{mM} \times \text{Venz (ml)}] = \mu\text{mol ml}^{-1}\,\text{min}^{-1}$; where dil = the dilution factor of the original sample and ε = the extinction coefficient for the CDNB conjugate at 340 nm.

Ligand identification

The identification of novel ligands binding to GmGSTL1 was performed according to Dixon and Edwards (2010) with the following modifications. Total phenolic compounds were purified from soybean leaves. Samples were ground and then extracted in 80% methanol overnight with continuous shaking at ambient temperature. Large particles were removed by centrifugation at 5,000×*g* for 10 min . Hexane was added in a 1 : 1 ratio to remove lipid components. The aqueous layer was retained and applied to an affinity column embedded with HP-20 resin. The column was washed with milli-Q water, followed by the elution of bound phenolic compounds with absolute methanol. Extracted fractions were pooled and concentrated by Speedvac centrifugation.

The recombinant protein was first immobilized onto a HisTrap column in the equilibration buffer [50 mM Na_2HPO4, pH 7.4, 0.3 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 6 M GuHCl, 10 mM imidazole] and gradually flushed with the washing buffer (50 mM Na_2HPO4, pH 7.4, 0.5 M NaCl, 10 mM imidazole) to remove GuHCl. The total phenolic extract was then loaded onto the column in washing buffer, followed by elution with 50 mM imidazole. Eluted fractions were pooled and concentrated for subsequent analyses.

Fourier transform mass spectrometry (FTMS)

HPLC was optimized according to Chang and Wong (2004). The FTMS analysis was performed with the same HPLC profile as described, interfaced with a Bruker Daltonik mass spectrometer equipped with an electrospry ionization (ESI) source. NH_4HCO_3 was used as the ion carrier instead of formic acid, as mass spectrometry in the negative mode was reported to be more favorable for phenolic compound analyses (Chang and Wong 2004). Data acquisition and processing were performed using DataAnalysis Version 4.0 SP1 (Bruker Daltonik). The mass spectra were recorded in the range of *m*/*z* 100–1,000. Optimization of ionization conditions was based on the intensity of the mass signals of deprotonated molecules, and was performed using the ESI

tuning mix (Agilent Technologies, G2421-60001) for negative mass spectrometry experiments. Mass parameters were optimized as follows: capillary voltage, 4.0 kV; spray shield, 3.5 kV; neb gas flow, 2.0 l min^{-1}; dry gas flow, 4.0 l min^{-1}; dry temperature, 200°C. Standards and purified phenolic extracts described above were reconstituted in methanol/water (50 : 50, v/v). The standards (10 mg ml^{-1}) and sample extracts were filtered by a syringe filter with a 0.2 µm polyvinylidene fluoride (PVDF) membrane (Gelman Laboratory), and 10 µl was injected into the HPLC column for analysis.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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