Research Article

Phytoremediation of Pb-contaminated soil by *Salicornia iranica*: key physiological and molecular mechanisms involved in Pb detoxification†

Elina Kaviani¹, Ali Niazi¹*, Zohreh Heydarian¹, Ali Moghadam¹, Reza Ghasemi-Fasaei², and Tayebe Abdollahzadeh¹

¹ Institute of Biotechnology, College of Agriculture, Shiraz University, Iran
² Department of soil science, College of Agriculture, Shiraz University, Iran

Correspondence: Dr. A. Niazi, Institute of Biotechnology, College of Agriculture, Shiraz University, Iran
E-mail: niazi@shirazu.ac.ir

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as an ‘Accepted Article’, doi: [10.1002/clen.201500964]

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
Received: 6 December 2015; Revised: 17 February 2016; Accepted: 2 January 2017
Abstract
The heavy metal lead (Pb) is one of the most important environmental pollutants and abiotic stresses with harmful effects on all living beings including plants. In this study *Salicornia iranica* was used in an attempt to phytoremediate Pb-contaminated soil. Possible mechanisms of plant tolerance against Pb pollution and its detoxification were studied through expression analysis of *Glutathione-S-transferase* (*GST*) and measurement of involved key physiological components. Interestingly, promoter analysis of *GST* showed that there are some essential cis-acting elements responsive to gibberellin, abscisic acid, salicylic acid and myeloblastosis viral oncogene homolog (MYB) family binding sites involved in Pb detoxification. The concentration of chlorophyll a and b and carotenoids significantly decreased in plants grown on Pb-contaminated soil for 90 days and in 90-day-old plants treated for 24 and 48 h (at a concentration of 1000 mg/kg Pb). The result of free proline measurement showed that it significantly increased in shoot tissue. The Pb absorption and its concentration increased in shoot tissue. In addition, the *GST* expression significantly increased as a result of the 100 mg/kg Pb treatment, but not at the higher concentration of 1000 mg/kg Pb. It was also observed that Pb accumulation did not influence plant growth parameters such as shoot and root lengths. Therefore, *S. iranica* is able to accumulate Pb in shoot tissue, and it may be useful in phytoremediation of Pb-polluted soil.

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; ef1, elongation factor; FW, fresh weight; GSH, glutathione; GST, glutathione-s-transferase; GSTU, tau-class GST; MYB, myeloblastosis viral oncogene homolog; PCR, polymerase chain reaction; SiGSTU, *Salicornia iranica* GSTU

Keywords: Bioremediation, Biotechnology, Pollution, Plants

1 Introduction
Industrial activity including excavating and manufacturing, and synthetic compounds, have led to an enormous increase in the amounts of heavy metals released into the atmosphere, water, and soil [1--3]. Pb ranks fifth after Fe, Cu, Al, and Zn in industrial production of metals, for example, in the United States; about half of the Pb has been used for the manufacture of Pb storage batteries [4]. Other applications include the production of solder, bearing, plumbing, cable cover, ammunition, pigment, and caulking [4, 5]. In nature, Pb may be found in different compositions [4], but Pb$^{+2}$ is the most common form [5, 6]. Natural levels of lead in soil range between 50 and 400 ppm (US EPA). One major source of soil contamination is the aerial release of Pb from the combustion of petrol containing tetraethyl lead; this contributes substantially to the content of Pb in soils in urban areas [4]. According to the Environmental Protection Agency, one million children in the USA are currently affected by Pb contamination.
Pb poisoning can permanently affect a child’s development. Pb is not only a health hazard for humans [7] but also in high concentrations is harmful for plant growth, changes the metabolic process and causes plant death [8–10].

However, some plant species can cope with the negative effects of heavy metals and could be considered for use in phytoremediation [11, 12]. The heavy-metal hyper-accumulator plants have the ability to extract elements from the soil and concentrate them in the easily harvested plant stems, shoots, and leaves. These plant tissues can be collected, reduced in volume, and stored for later use [13].

Plants that have to cope with heavy metals pollution need to change their structure and the metabolism of specific components such as organic acids, phytochelatin, metallothioneins, heat shock proteins, special amino acids, and organic osmolytes like proline in order to deal with the poisonous metals [14]. These plants possess specific genes that regulate the amount of metals taken up from the soil by roots and deposited at other locations within the plant. These genes control processes that can increase the solubility of metals in the soil as well as the transport proteins that move metals into root cells. From there, the metals enter the plant’s vascular system for further transport to other parts of the plant and are ultimately deposited in leaf cells. While acting as phytoremediator, the unique plants must be able to tolerate and survive high levels of heavy metals in soil [15]. The use of the halophyte species for phytoextraction purposes has been recommended because these plants are naturally present in environments characterized by an excess of toxic ions [8].

Glutathione-S-transferases (GST, EC 2.5.1.18) are ubiquitous enzymes that play a key role in cellular detoxification of heavy metals [16]. In different plant species this gene family consists of 25–60 members which can be grouped into six classes (phi, tau, zeta, theta, lambda and dehydroascorbate reductase enzymes) on the basis of sequence identity, gene organization and active site residues of the protein [17, 18]. Tau and phi classes in comparison with other GST classes are more abundant in plants [19].

Jha et al. have isolated a tau class GST (GSTU) from Salicornia brachiata [17]. This gene was induced by different abiotic stresses, and seems to play an important role in protecting plants against oxidative damage. Salicornia is a halophyte that can be considered as a phytoremediation candidate because of its high ability to absorb and accumulate Cd$^{2+}$, Ni$^{2+}$ and As$^{3+}$ in leaf tissues [20].

Therefore, the ability of S. iranica for Pb phytoremediation and the possible role of GST enzyme in this procedure were considered by studying the glutathione transferase tau (SiGSTU) gene expression profile in soil contaminated with different Pb concentrations. In addition, the effects of Pb absorption on the physiological processes in Salicornia were studied.

2 Material and method

2.1 Plant material and growth condition

Salicornia iranica seeds were collected from Maharloo Lake, Shiraz, Fars Province. The lake covers an area of 600 km$^2$ and is located 27 km southeast of Shiraz. In the first study the seeds were planted in soil contaminated with Pb at start time and grown for 90 days, while in the second study they were planted in soil to which the Pb was added 90 days after culture. Pots were filled with 1 kg of mixed sand and lay, 1:1. The first study had two parts: In the first
part, four levels of Pb nitrate (10, 100, 500, 1000 mg/kg as Pb [NO3]2 in solution) were used to contaminate soil at start time for measuring Pb concentration and growth parameters. In the second part, plants that were treated with 1000 mg/kg Pb were used for RNA isolation and plants that were treated with 100 and 1000 mg/kg of Pb were used for proline, carotenoid, and chlorophyll measurement after 90 days. In the second study, plants grown for 90 days were treated with 100 and 1000 mg/kg Pb after 3, 24 and 48 h, and used to isolate RNAs and measure proline, carotenoids, and chlorophylls. The results of a previous study [21] had already shown that the amount of Pb in soil and plant under Pb-untreated conditions was lower than the detection limit of the atomic absorption spectrophotometer. Therefore, the level of 10 mg Pb/kg was considered as the lowest Pb level [21]. To maintain moisture in the pots, plants were watered as needed. Pots were irrigated every three days with distilled water. Plants were irrigated with Hoagland nutrient solution every two weeks. Pots were placed in a greenhouse under 16 h light and 8 h dark and 25°C with 40% humidity. The length of the shoots and the roots, and the number of the branches were measured within 40, 80 and 180 days of starting the culture.

2.2 Pb concentration in plant tissue
1 g of dried ashes of shoot tissue was dissolved in 2 M HCl. Pb concentration was measured by atomic absorption spectrophotometry (AA-670 Shimadzu, Japan). Pb uptake index was calculated by multiplying Pb concentration by dry weight of plant [21].

2.3 Expression analysis of SiGSTU in S. iranica
Primers were designed based on the sequence of S. brachiate GST (accession number: EB485111) and efl (elongation factor) (accession number: KC131466) using Allele ID 6 software (Table 1). efl (elongation factor as the workhorses of protein synthesis on the ribosome) was used as internal control.

2.4 RNA extraction, DNase treatment, and cDNA synthesis
Total RNA was extracted from green tissues of 90-day-old plants using the lithium chloride method [22]. In order to eliminate genomic contamination, the extracted RNA was treated with DNase I (Fermentas, Germany) and tested on 1% agarose gel. cDNA samples were synthesized by reverse transcriptase II (Fermentas, Germany) in accordance with the manufacturer’s protocol.

2.5 Quantitative real-time polymerase chain reaction (PCR)
Real-time PCR reaction was performed using SYBR green premix EXTAQII kit (Takara, Malaysia). The relative expression was calculated from the following Eq. (1) [23].

Relative expression = 2^(-ΔΔCt)

ΔCt = Ct target – Ct housekeeping gene

ΔΔCt = ΔCt in treatment time – ΔCt in time 0

(1)

where time 0 is before applying the treatment and Ct is the cycle number at which the fluorescence signal crosses the threshold.
2.6 Measurement of proline, carotenoids, and chlorophylls

Extraction and determination of proline were performed [24]. 1 g of fresh green tissue was used for proline extraction using acidic ninhydrin reagent (2.5 g ninhydrin/100 mL of a solution containing glacial acetic acid, distilled water and 85% ortho-phosphoric acid, 6:3:1). Readings were taken at a wavelength of 546 nm. The proline concentration was determined by standard curve and calculated on a fresh-weight basis (mmol proline/g FW) [24].

In order to calculate chlorophyll and carotenoid content, 100 mg of fresh green material was ground with 10 mL cold 80% acetone and centrifuged at 4000 rpm for 10 min at 4°C, until the residue became colorless. At the last step, 80% acetone was added up to 10 mL. Absorbance was read at 645, 663 and 480 nm with a spectrophotometer (SP-3000 plus-Optima).

Then, chlorophyll and carotenoid content were calculated in accordance with manufacturer’s protocol from the following Eqs. (2) to (4) [25].

\[
\text{Chlorophyll a (mg/mL) = } 0.0127 A_{663} - 0.00269 A_{645} \\
\text{Chlorophyll b (mg/mL) = } 0.0229 A_{645} - 0.00468 A_{663} \\
\text{Carotenoid = } A_{480} + 0.114 A_{663} - 0.638 A_{645}
\]

where \( A \) is the absorbance and index numbers indicate the wavelength at which absorption was measured.

2.7 Promoter analysis of GST

For promoter analysis of GST, the Arabidopsis genome sequence was used because it is completely sequenced and is a genome model for plants, whereas the genome of Salicornia is not completely sequenced. To identify the promoter region of the GST (accession number: EB485111) in Arabidopsis deposited at the NCBI, CDS of GST was searched against the genomic data of Arabidopsis by using the Phytozome database (www.phytozome.net/). After identifying the gene on the chromosome using the BLAST-N algorithm, the region 2000 bp upstream of the transcriptional start point (ATG) of Arabidopsis GST was considered as the promoter. The upstream region of the GST encoding a genomic fragment was analyzed using the Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/) database to predict their key cis-acting elements and the precise location of these elements [26].

2.8 Statistical analysis

The experimental design of the greenhouse experiment was a complete-block design with two blocks consisting of four Pb concentrations and control. Five biological and two experimental replicates were used for each treatment. The data were subjected to an analysis of variance by using STATISTICA 6.0 software and the statistical significance of the results was analyzed by the F-test \((P \leq 0.05)\). The raw data were imported into Microsoft Excel 2013 for drawing the graphs.

3 Results

3.1 Pb absorption in S. iranica shoot tissue

The lowest amount of Pb uptake by plants was observed in the application of the 100 mg/kg solution and the highest one with the 1000 mg/kg solution (Fig. 1). The results showed that Pb accumulation significantly increased \((P \leq 0.05)\) at 100, 500, and 1000 mg/kg Pb-contaminated soil at 90 days after treatment compared with control (1.2-, 1.7-, 2.6-...
and 2.8-fold respectively) (Fig. 1).

3.2 Expression analysis of SiGSTU under Pb treatment
The expression of GST in 100 mg/kg Pb-contaminated soil was significantly higher than control at 3 and 24 h after treatment (11.5- and 2.8-fold respectively), but dropped at 48 h after treatment on 90-day cultivated plants (Fig. 2a). Surprisingly, plants grown in treated soil with 1000 mg/kg Pb showed a decrease in the GST expression at 24 and 48 h after treatment on 90-day cultivated plants. This effect was observed in plants that were grown for 90 days in 1000 mg/kg Pb-contaminated soil (Fig. 2b). On the other hand, three months after stress, GST was dramatically suppressed.

3.3 Measurement of growth parameters after Pb treatment
Pb treatment did not show any significant effect on shoot or root growth of S. iranica plants during periods of 40, 80 and 180 days after planting in soil that was contaminated at start time (Fig. 3a and b).

3.4 Effect of Pb on the accumulation of photosynthetic pigments
To investigate the effect of heavy metal contamination on plants, photosynthetic pigments were measured at 3, 24, and 48 h after treatment with 100 and 1000 mg/kg Pb on 90-day cultivated plants and on plants that were grown for 90 days in 100 and 1000 mg/kg Pb-contaminated soil. The content of the chlorophylls a and b and carotenoids decreased significantly in 1000 mg/kg Pb at 24 and 48 h and also at 90 days after treatment (Figs. 4 and 5). In 100 mg/kg Pb, the content of chlorophyll b at 48 h and also at 90 days after treatment and carotenoids at 24 and 48 h after treatment decreased significantly (Figs. 4b and d and 5a). It should be noted that S. iranica plants did not show any signs of necrosis under Pb stress, either in the short or in the long-term Pb treatment. However, the chlorophyll content decreased.

3.5 Proline content during Pb treatment
The proline content of the plants increased significantly in the short term at 3, 24, and 48 h and, in the long term, 90 days after 100 and 1000 mg/kg Pb treatment (Fig. 6). The proline as an osmotic adjustment increased significantly in 100 and in 1000 mg/kg Pb.

3.6 Promoter analysis results
The GST promoter analysis pointed to the existence of the elements in response to phytohormones such as gibberellin (which are plant hormones essential for seed germination, stem elongation, leaf expansion, trichome development, pollen maturation and the induction of flowering in plants), abscisic acid (which functions in many plant developmental processes including bud dormancy as a plant hormone), salicylic acid (which is a beta hydroxy acid occurring as a natural compound in plants) and MYB gene family binding site (a very common transcription factor responsive to many biotic and abiotic stresses) (Table 2).
4 Discussion

In this study, *S. iranica* illustrated the ability to accumulate Pb in its shoots. With increasing Pb levels, metal densities and absorption rates in the plant tissues increased (Fig. 1). These results were consistent with the results of a previous study where *S. europaea* was introduced as a new candidate in order to phytoremediate saline soil polluted with cadmium [27]. In addition, *S. brachiate* was treated with heavy metals like Cd$^{2+}$, Ni$^{2+}$, and As$^{3+}$ in the presence of 200 mM sodium chloride. The results showed that the above-mentioned plants can be used as good sources for phytoremediation in saline soil areas [20]. Hamzenejad Taghlidabad et al. found during experiments on *S. europaea, Chenopodium album*, and *Arriplex verucifera* that *S. europaea* showed a considerable accumulation of soil cadmium and Pb under unsuitable conditions of the saline-sodic soil [28].

According to promoter analysis GST (Table 2) and previous research, biotic and abiotic stress, phytohormones such as auxins, ethylene, cytokinin and abscisic acid, heavy metals, GSH and hydrogen peroxide are considered to be inductive factors that regulate GST activity differentially [18]. ABRE is a major cis-acting element in the ABA-responsive gene expression because the ABA pervasive phytohormone is involved in many developmental processes and plays a key role in adaptation to various stresses. The MYB regulatory element is an early responsive element to osmotic stress and ABA induction [26]. Previous studies have demonstrated that GST expression was up-regulated in the first hours after various stresses. In fact, GST is a novel early stress responsive gene [17, 29, 30]. According to these results, changes of GST expression in the first hour after Pb stress show that it is an early responsive gene (Fig. 2). GST can also play a role as a non-catalytic carrier of cytokines and auxins or anthocyanins and can thus help hormone homeostasis or vacuolar anthocyanin accumulation. GST may also function in cellular redox homeostasis as stress signaling proteins or regulators [31].

A potential defense system, especially in halophytes, one based on GST, was developed to tolerate continuously changing weather and other environmental conditions [17]. In this study, 100 mg/kg Pb-contaminated soil induced GST expression in *S. iranica*, but at a level of 1000 mg/kg Pb-contaminated soil GST expression did not increase (Fig. 2). However, the highest rate of absorption and concentration in the Salicornia shoot tissue was observed at 1000 mg/kg Pb-contaminated soil (Fig. 1) and the plants did not show a difference in growth compared with the control (Fig. 3). It can be concluded that plants which showed resistance to toxic levels of Pb (1000 mg/kg Pb in soil) used other mechanisms such as organic acid production and secretion, phytochelatin, metallothioneins, heat shock proteins, amino acids, and proline [14]. These results were consistent with previous research in which heavy metals were found to induce soybean Gmgst1. Toxic chemical ZnSO$_4$ (2.5 or 5 mM), cobalt and nickel chloride (250 µM and 2.5 mM) markedly induced osgstu4 and osgstu3 in rice roots within 2 h of exposure [29]. In the presence of HgCl$_2$ (0.2 mM), GST expression increased in mustard leaves [30]. By sbGST gene transfer from *S. brachiata* to tobacco plant different patterns of SbGST gene expression in stresses such as cold, drought, abscisic acid, salicylic and sodium chloride were observed [17].

Free proline increase in shoot tissue in 1000 mg/kg Pb-contaminated soil shows that it is one of the defense mechanisms for plants at this level of pollution (Fig. 6). Proline accumulation reduces protein membrane damage. In addition, proline as an osmotic adjustment factor regulates cell pH and oxidation reactions, and is considered as a source for reduced forms of carbon and nitrogen in cells [32].
According to previous research, proline accumulation under heavy metal stress in different tissues has been reported in a number of higher plants. For example, cadmium inducing proline accumulation has been reported in radish, barley, wheat, bean, spinach, cabbage, while nickel has been found to affect wheat, rice, spinach, cabbage, soybeans. To this list may be added copper affecting sunflower, rice, and bean, Pb affecting bean, and cobalt affecting cabbage [33–39].

Heavy metals have deleterious effects on the content and functionality of the photosynthetic pigments [40]. In particular, Pb has inhibitory effects on enzymatic functions in chlorophyll biosynthesis such as γ-aminolevulinic acid dehydrogenase and protochlorophyllide reductase. Heavy metal interaction with the sulfhydryl group of enzymes is one of the main inhibitory mechanisms [35, 41–43]. In addition, another inhibitory mechanism in chlorophyll biosynthesis by heavy metals is chlorophyll biodegradation. The other effect of heavy metals on chlorophyll biosynthesis is substitution of the central chlorophyll magnesium that reduces the chlorophyll function in capturing light and Pb to reduce photosynthesis [44]. In general, several results have shown that heavy metals could reduce chlorophyll. Thus cadmium has an effect on the chlorophyll rate in wheat [33], Pb2+, Cd2+ and Hg2+ influence maize [45], and Ni2+, As3+ and Cd2+ affect the chlorophyll rate in S. brachiata [20]. Carotenoids display a protective role against oxidative stress. These pigments play a significant role in detoxification of heavy metals and decrease toxic effects of free radicals [46]. In this research, Pb significantly decreased the chlorophyll a and b rate and carotenoid in comparison to control (Figs. 4 and 5). However, no particular signs of necrosis in S. iranica plants, leading to cell death, were observed under Pb treatment [20]. In this study, Pb of 100 mg/kg concentration in soil induced GST expression in S. iranica but at 1000 mg/kg GST expression did not increase. However, the highest rate of absorption and concentration in Salicornia shoot tissue was observed at 1000 mg/kg Pb in soil and the plants did not show a difference in growth compared with the control. Therefore, plants that showed tolerance to toxic levels of 1000 mg/kg Pb possibly use other mechanisms. Because of the ability of the plant to tolerate and accumulate pollutants, S. iranica has the potential to tolerate Pb accumulation and can be useful in phytoremediation of Pb-polluted soils.

5 Concluding remarks

The focus of this study was an investigation into the process of phytoremediation for Pb-containing soil by S. iranica and molecular mechanisms involved in Pb detoxification. Salicornia iranica illustrated an ability to accumulate Pb in the shoots. By increasing the Pb level, metal density and absorption rate increased in the plant's shoot tissue. Therefore, S. iranica might be considered for phytoremediation of Pb-polluted soils. The results demonstrated that the GST enzyme had a key role in cellular detoxification of Pb at a level of 100 mg/kg in soil but not at the level of 1000 mg/kg. The highest absorption rate and concentration in Salicornia shoot tissues were observed at 1000 mg/kg Pb in soil, as the plants did not show a difference in growth compared with the control. Therefore, we could conclude that Salicornia tolerates high toxic levels of Pb using other mechanisms. In addition, the increase in free proline in shoot tissue in 1000 mg/kg Pb-contaminated soil showed that it may be one of the defense mechanisms against Pb pollution. Finally, after Pb accumulation, the synthesis rate of chlorophyll a and b and carotenoid decreased significantly in comparison to the control. However, S. iranica showed tolerance to Pb
pollution and there were no visible Pb stress necrosis spots identifiable in stressed plants. A successful estimation of Pb uptake by *S. iranica* at remediation regions would be useful in phytoremediation of toxic metals.

**ACKNOWLEDGEMENTS**

The authors would like to thank the Institute of Biotechnology and Soil Science Department for supporting this research in the College of Agriculture (Shiraz University).

The authors have declared no conflict of interest.

**6 References**


Figure 1. Pb concentration in Salicornia shoots under Pb stress (100, 500, and 1000 mg/kg Pb) 90 days after treatment. Control soils treated with 10 mg/kg Pb. Results are the mean of three replications ± SD.
**Figure 2.** *GST* relative expression in *S. iranica.* a) 3, 24, and 48 h after treatment with 100 or 1000 mg/kg Pb on 90-day cultivated plants. y-Axis shows the *GST* relative expression and x-axis shows time (h) after stress. b) 90-day old plants grown in treated soil at start time with 1000 mg/kg Pb-contaminated soil. y-Axis shows the *GST* relative expression and x-axis shows applied mg/kg Pb-contaminated soil. Results are the mean of three replications ± SD.

**Figure 3.** The effect of different Pb concentrations (100, 500, and 1000 mg/kg) on a) shoot length 40, 80 or 180 days after treatment, and b) root length 180 days after treatment. Results are the mean of 15 replications ± SD.
Figure 4. Effect of 100 and 1000 mg/kg Pb in soil 3, 24, and 48 h after treatment of 90-day cultivated plants on a) chlorophyll a content, and b) chlorophyll b content, and long-term effect of both Pb concentrations on the content of c) chlorophyll a, and d) chlorophyll b in plants grown 90 days after treatment. Results are the mean of six replications ± SD.
Figure 5. a) Short-term and b) long-term effect of 100, 1000 mg/kg Pb in soil on carotenoid content of plant shoots’ tissue. In the short-term treatment, plants were 90 days old and were treated for 3, 24, and 48 h. In the long-term treatment, plants were treated with Pb for 90 days. Results are the mean of six replications ± SD.

Figure 6. a) Short-term and b) long-term effect of 100 and 1000 mg/kg Pb in soil on proline content of plant shoots’ tissue. In the short-term treatment plants were 90 days old and were treated for 3, 24, and 48 h. In the long-term treatment, plants were treated with Pb for 90 days. Results are the mean of six replications ± SD.
### Table 1. Sequences of primers used for qRT-PCR amplification and the resulting product sizes

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Name</th>
<th>Sequence of primer</th>
<th>Ta (°C)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB485111</td>
<td>GST-F</td>
<td>CGGATTTCTTGACATTTGCTTGG</td>
<td>54.6</td>
<td>133 pb</td>
</tr>
<tr>
<td>EB485111</td>
<td>GST-R</td>
<td>AGCATCATCAGCAGACAGACCTC</td>
<td>54.6</td>
<td>133 bp</td>
</tr>
<tr>
<td>KC131466</td>
<td>efl-F</td>
<td>TCG GAACTGTGCTCTGGG</td>
<td>54.6</td>
<td>127 bp</td>
</tr>
<tr>
<td>KC131466</td>
<td>efl-R</td>
<td>TCTGGTAGAGACCTCGTATGC</td>
<td>54.6</td>
<td>127 bp</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; Ta, temperature annealing.

### Table 2. Summary of key cis-acting elements, their functions and their number in GST promoter.

<table>
<thead>
<tr>
<th>Site motif</th>
<th>Sequence</th>
<th>Function</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GARE</td>
<td>AAACAGA</td>
<td>Gibberellin-responsive element</td>
<td>1</td>
</tr>
<tr>
<td>ABRE</td>
<td>(GA)CACGTG(GC)</td>
<td>Involved in ABA responsiveness</td>
<td>3</td>
</tr>
<tr>
<td>TCA</td>
<td>(GAGAAGAATA)/(CAGAAAAGA)</td>
<td>Salicylic acid</td>
<td>2</td>
</tr>
<tr>
<td>MRE</td>
<td>AACCTAA</td>
<td>MYB binding site</td>
<td>1</td>
</tr>
<tr>
<td>CCAAT</td>
<td>CAACGG</td>
<td>MYBHv1 binding site</td>
<td>1</td>
</tr>
</tbody>
</table>