Heterologous expression of a Glyoxalase I gene from sugarcane confers tolerance to several environmental stresses in bacteria

Qibin Wu, Shiwu Gao, Yong-Bao Pan, Yachun Su, Michael P. Grisham, Jinlong Guo, Liping Xu, Youxiong Que

1 Fujian Agriculture and Forestry University, Key Laboratory of Sugarcane Biology and Genetic Breeding, Fuzhou, Fujian, China
2 USDA-ARS, Sugarcane Research Unit, Houma, LA, USA

Corresponding Authors: Liping Xu, Youxiong Que
Email address: xlpmail@126.com, queyouxiong@126.com

Glyoxalase I belongs to the glyoxalase system that detoxifies methylglyoxal (MG), a cytotoxic by-product produced mainly from triose phosphates. The concentration of MG increases rapidly under stress conditions. In this study, a novel glyoxalase I gene, designated as SoGloI was identified from sugarcane. SoGloI had a size of 1,091 bp with one open reading frame (ORF) of 885 bp encoding a protein of 294 amino acids. SoGloI was predicted as a Ni^{2+}-dependent GLOI protein with two typical glyoxalase domains at positions 28-149 and 159-283, respectively. SoGloI was cloned into an expression plasmid vector, and the His-tagged SoGloI protein produced in Escherichia coli was about 51 kDa. The recombinant E. coli cells expressing SoGloI compared to the control grew faster and tolerated higher concentrations of NaCl, CuCl_2, CdCl_2, or ZnSO_4. SoGloI ubiquitously expressed in various sugarcane tissues. The expression was up-regulated under the stress of NaCl, CuCl_2, CdCl_2, ZnSO_4, as well as under simulated pathogen stress conditions upon exposure to salicylic acid (SA), methyl jasmonate (MeJA), or abscisic acid (ABA). SoGloI activity steadily increased when sugarcane was subjected to NaCl, CuCl_2, CdCl_2, or ZnSO_4 stress. Sub-cellular observations indicated that the SoGloI protein was located in both cytosol and nucleus. These results suggest that the SoGloI gene may play an important role in sugarcane's response to various biotic and abiotic stresses.
Heterologous expression of a Glyoxalase I gene from sugarcane confers tolerance to several environmental stresses in bacteria

Qibin Wu¹, Shiwu Gao¹, Yong-Bao Pan², Yachun Su¹, Michael P. Grisham², Jinlong Guo¹, Liping Xu¹*, and Youxiong Que¹*

¹ Key Laboratory of Sugarcane Biology and Genetic Breeding, Fujian Agriculture and Forestry University, Ministry of Agriculture, Fuzhou 350002, China
² USDA-ARS, SEA, Sugarcane Research Unit, Houma, LA 70360, USA

*Correspondences: Youxiong Que and Liping Xu, Key Laboratory of Sugarcane Biology and Genetic Breeding, Fujian Agriculture and Forestry University, Ministry of Agriculture, No. 15 Shangxia Dian Road, Cangshan District, Fuzhou city, Fujian Province, 350000, P.R. China. E-mails: queyouxiong@126.com, xlpmail@126.com

Abstract

Glyoxalase I belongs to the glyoxalase system that detoxifies methylglyoxal (MG), a cytotoxic by-product produced mainly from triose phosphates. The concentration of MG increases rapidly under stress conditions. In this study, a novel glyoxalase I gene, designated as SoGloI was identified from sugarcane. SoGloI had a size of 1,091 bp with one open reading frame (ORF) of 885 bp encoding a protein of 294 amino acids. SoGloI was predicted as a Ni²⁺-dependent GloI protein with two typical glyoxalase domains at positions 28-149 and 159-283, respectively. SoGloI was cloned into an expression plasmid vector, and the Trx-His-S-tag SoGloI protein produced in Escherichia coli was about 51 kDa. The recombinant E. coli cells expressing SoGloI compared to the control grew faster and tolerated higher concentrations of NaCl, CuCl₂, CdCl₂, or ZnSO₄. SoGloI ubiquitously expressed in various sugarcane tissues. The expression was up-regulated under the treatments of NaCl, CuCl₂, CdCl₂, ZnSO₄ and abscisic acid (ABA), or under simulated biotic stress conditions upon exposure to salicylic acid (SA) and methyl jasmonate (MeJA). SoGloI activity steadily increased when sugarcane was subjected to NaCl, CuCl₂, CdCl₂, or ZnSO₄ treatments. Sub-cellular observations indicated that the SoGloI protein was located in both cytosol and nucleus. These results suggest that the SoGloI gene may play an important role in sugarcane's response to various biotic and abiotic stresses.
INTRODUCTION

Ubiquitously occurring in nature, the glyoxalase pathway involves a two-step catalytic reaction. In the first step, glyoxalase I (GLOI, lactoylglutathione lyase; EC 4.4.1.5) catalyzes the isomerization of hemithioacetal formed spontaneously between methylglyoxal (MG) and reduces glutathione (GSH) to S-D-lactoylglutathione (S-LG) (Thornalley, 1993). In the second step, S-LG is hydrolyzed by glyoxalase II (GLOII, hydroxyacylglutathione hydrolase; EC 3.1.2.6) to produce GSH and D-lactate (Yadav et al., 2005). Under normal physiological conditions, MG is produced primarily through glycolysis at the triose-phosphate step (Phillips & Thornalley, 1993), and to a much lesser extent, through catabolism of amino acids (threonine and glycine) and acetone (Yadav et al., 2005, 2007). Under abiotic stresses, however, the concentration of MG in plants can significantly increase by 2-6 folds (Yadav et al., 2005). The high-level accumulation of MG is toxic to cells, as it can react with DNA to form modified guanylate residues (Papoulis, Al-Abed & Bucala, 1995). MG also can react with proteins to form glycosylamine derivatives of arginine, lysine and hemithioacetal with cysteine residues (Lo et al., 1994). Apart from the direct effect of MG, its intermediate compound S-LG, a substrate for glyoxalase II, is also cytotoxic at higher concentrations by inhibiting DNA synthesis (Thornalley, 1996). Therefore, glutathione-based detoxification of harmful metabolites is one of the main roles of both glyoxalase enzymes (Thornalley, 1990).

The glyoxalase I enzyme is broadly categorized into Zn$^{2+}$-or Ni$^{2+}$-dependent class of metal activation. Previous studies have been showed that the Zn$^{2+}$-dependent GLOI enzymes are thought to be of eukaryotic origin (Frickel et al., 2001; Ridderström et al., 1996), while Ni$^{2+}$-dependent GLOI enzymes are thought to be of prokaryotic origin (Sukdeo et al., 2004). The coexistence of both Zn$^{2+}$-dependent and Ni$^{2+}$-dependent GLOI enzymes in Pseudomonas aeruginosa (Sukdeo et al., 2007) and the characterization of a Ni$^{2+}$-dependent GLOI enzyme from rice (Mustafiz et al., 2014) have led to the discouragement of the view that Zn$^{2+}$-dependent GLO I belongs to eukaryotes and Ni$^{2+}$-dependent GLOI exists only in prokaryotes (Jain et al., 2016). In plants, the metal specificity of each member of the GLOI family is an important determinant of its catalytic efficiency (Kaur et al., 2017; Mustafiz et al., 2014).

The glyoxalase system has been widely studied in animals and microbes (Price & Knight, 2009; Thornalley, 2003). Some studies have suggested that the glyoxalase system is associated with clinical disorders, such as cancer and diabetes (Price & Knight, 2009; Thornalley, 2003). To date, GLOI has been cloned and characterized from several plant species, including Oryza sativa (Kaur et al., 2017; Mustafiz et al., 2014; Usui et al., 2001; Zeng et al., 2016), Glycine max (Skipsey et al., 2000), Zea mays (Chen et al., 2004), Triticum aestivum (Lin et al., 2010), Triticum medicata (Johansen, Svendsen & Rasmussen, 2000), Brassica juncea (Veena, Reddy &
Sopory, 1999), Solanum lycopersicum (Espartero, Sanchez-Aguayo & Pardo, 1995), Allium cepa (Hossain & Fujita, 2009), and Beta vulgaris M14 (Wu et al., 2013a). Studies have shown that GLOI activity is up-regulated in response to light and phytohormones (Chakravary & Sopory, 1988; Deswal & Sopory, 1999; Sethi, Guha-Mukherjee & Basu, 1988). Moreover, enzyme activity and transcription of GLOI are up-regulated in response to abiotic and biotic stresses, such as NaCl, heavy metals, mannitol, MG, abscisic acid or pathogen attack (Espartero, Sanchez-Aguayo & Pardo, 1995; Ghosh and Islam, 2016; Jain et al., 2016; Kaur et al., 2017; Lin et al., 2010; Mustafiz et al., 2011; Mustafiz et al., 2014; Singla-Pareek, Reddy & Sopory, 2003; Singla-Pareek et al., 2006; Veena, Reddy & Sopory, 1999). Other GLOI genes such as Gly I, GLX1, BvM14-glyoxalase I, TaGly I, OsGLYI-11.2, OsGly I, and OsGlyI-8 isolated from B. juncea (Veena, Reddy & Sopory, 1999), S. lycopersicum (Espartero, Sanchez-Aguayo & Pardo, 1995), B. vulgaris M14 (Wu et al., 2013a), T. aestivum (Lin et al., 2010), and rice (Kaur et al., 2017; Mustafiz et al., 2014; Zeng et al., 2016) respectively, have been associated with significant tolerance to salt stress. Singla-Pareek et al. (2006) have shown that Gly I from B. Juncea enhances the tolerance to heavy metals, such as zinc, cadmium or lead. Lin et al. (2010) also have shown that TaGly I was up-regulated under ZnCl₂ treatment. Mustafiz et al. (2011) reported a high level of stress inducibility of Ni²⁺-dependent Glxl encoding AtGLYI3 and AtGLYI6 in response to salt, drought, wounding, cold and heat treatments in both Arabidopsis thaliana roots and shoots, whereas Zn²⁺-dependent Glxl encoding AtGLYI2 was found to be heat inducible. Transgenic tobacco plants over-expressing GlyI were highly tolerant to MG, salt, or zinc and were able to grow, flower, and produce viable seeds (Singla-Pareek, Reddy & Sopory, 2003; Singla-Pareek et al., 2006; Veena, Reddy & Sopory, 1999). Zeng et al. (2016) reported that OsGly I was markedly up-regulated in response to NaCl, ZnCl₂ and mannitol in rice seedlings. Compared to the wild-type plants, the OsGly I-overexpression transgenic rice lines showed increased glyoxalase enzyme activity, decreased MG level, improved tolerance to NaCl, ZnCl₂ and mannitol, and produced higher rates of seed setting and higher yields. Another Ni²⁺-dependent Glxl gene, OsGLYI-11.2, was found to be highly stress inducible amongst the rice glyoxalase family (Mustafiz et al., 2014). Similarly, Ni²⁺-dependent Glxl genes from Arabidopsis were found to be more responsive to various stress treatments compared to Zn²⁺-dependent Glxl genes (Mustafiz et al., 2011). All these findings suggest that the GLOI genes may play an important role in response to stresses.

Sugarcane (Saccharum spp. hybrids) is a cash crop mainly used for sugar, biofuel and other food industries such as industrial alcohol in tropical and subtropical regions. It is one of the world's largest crops. According to FAO, sugarcane was cultivated in 101 countries on about 26.1 million hectares of land in 2012 (Que et al., 2014). However, the yields of sugarcane are often influenced by many diseases and various environmental stresses, such as smut, rust, ratoon stunting disease (RSD), salt, heavy metal and drought. Sugarcane is reportedly susceptible to salt and shows toxicity symptoms, low sprout emergence, nutritional imbalance, and overall biomass reduction (Akhtar, Wahid & Rasul, 2003; Plaut, Meinzer & Federman, 2000; Wahid, Rao &
Rasul, 1997). Though sugarcane plants can overcome a short period of water deficit during the late, sucrose accumulating growth stage, an extended period of drought can cause a significant loss in cane and sugar yields (Begcy et al., 2012). RSD causes significant yield losses, 12% – 37% under normal conditions and up to 60% under drought conditions. Moreover, it may also lead to variety deterioration (Bailey et al., 1997; James 1996; Que et al., 2008). Sugarcane smut also causes serious losses in cane and sugar yields (Hoy et al., 1986; Padmanaban, Alexander & Shanmugan, 1998; Que et al., 2012).

In our previous study, we constructed a sugarcane cDNA library from Sporisorium scitamineum-infected buds (Wu et al., 2013b). An expressed sequence tag of 613 bp (GenBank Accession Number: CA140600.1) had a high similarity to the GLOI gene of Zea mays (GenBank Accession Number: EU966885.1) (Wu et al., 2013b). To study stress response of GLOI in sugarcane, we cloned the entire sugarcane Glyoxalase I gene, designated as SoGloI. We determined the sub-cellular location of the SoGloI’s protein using tobacco protoplasts and investigated growth patterns of Escherichia coli Rosetta cells producing the SoGloI recombinant protein in response to salt and heavy metal ion stresses. We also assessed SoGloI expression and glyoxalase I enzyme in sugarcane in response to simulated biotic and abiotic stresses. The results provided valuable information for the improvement of stress resistance in sugarcane.

MATERIALS & METHODS

Plant material
Sugarcane genotype YCE 05-179 was used in this study. Plants were maintained in a genetic nursery at the Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture, Fujian Agriculture and Forestry University, Fuzhou, China. In addition, tissue culture-derived young, healthy plantlets of YCE 05-179 were also involved in the study.

Cloning of SoGloI gene
A BLAST search of the sugarcane database (taxid: 286192; taxid: 4547; taxid: 128810; taxid: 62335) of GenBank (http://www.ncbi.nlm.nih.gov/) was conducted with the sugarcane EST, CA140600.1 (Wu et al., 2013b). Several highly homologous sugarcane ESTs were obtained and aligned. A 1,446 bp cDNA of sugarcane Glo I gene, designated as SoGloI, was assembled using the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php). SoGloI cDNA sequence was amplified in a 25 μL reaction mixture on ABI Veriti96 PCR with primers G-F and G-R (Table 1) under a thermal cycling program: 94°C, 4 min; 35 cycles of (94°C, 1 min; 54°C, 1 min; 72°C, 1.5 min); and 72°C, 10 min. Reaction mixture contained 2.5 μL 10× PCR buffer (plus Mg²⁺), 2.5 μL dNTPs (2.5 mM), 1.0 μL first-strand cDNA, 1.0 μL each of forward and reverse primers (10 μM) and 0.125 μL Ex-Taq enzyme (5 U·μL⁻¹) (Takara, China). Amplified SoGloI cDNA product was separated through 1% agarose gel electrophoresis, gel purified using Omega Gel Extraction Kit (Omega, China), and cloned into the PMD 18-T vector (Takara, China).
Putative recombinants were confirmed by PCR, of which six were sequenced (Shenggong Co., Ltd., China).

**Bioinformatics analysis**

The ORF of the full-length *SoGloI* was predicted using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Molecular weight and isoelectric point was calculated using Compute pl/Mw tool (http://www.expasy.ch/tools/pi_tool.html). Protein domain was predicted using the SMART Program (http://smart.embl-heidelberg.de/). Protein hydrophilicity was analyzed by Protscale Program (http://www.expasy.ch/tools/protscale.html). Prediction of signal peptides and analysis of trans-membrane helix domain were conducted using the SignalP and TMHMM-2.0 Programs (http://www.cbs.dtu.dk/services/). A multiple protein-sequence alignment was carried out using the DNAMAN® Program (Guo et al., 2012; Liu et al., 2010; Su et al., 2013).

**Expression of *SoGloI* in field-grown sugarcane plants**

Five tissue samples, white young roots, leaf (+1), leaf sheath (+1), buds (6th-8th from the base), and internodes (6th and 7th from the base) were collected from 7- to 8-month-old plants in the field nursery. All samples except buds were cut into small pieces, wrapped up within tinfoil, and immediately flash-frozen in liquid nitrogen. The frozen samples were stored in a –80°C freezer until RNA extraction. Total RNA samples were isolated from the frozen buds using the TRIzol® kit (Invitrogen, USA) according to the manufacturer’s instructions. RNA samples were dissolved in diethylpyrocarbonate-treated H₂O. RNA concentration was quantified by measuring absorbance at OD₂₆₀ and OD₂₈₀ using Synergy H1 Microplate Reader Multi-Mode (Bio-Tek, USA). RNA quality was also assessed by 1.0% denaturing agarose gel electrophoreses.

First-strand cDNA was synthesized from 1 μg RNA in 20 μL reaction mixture by reverse transcription PCR (RT-PCR) using the Prime-Script® 1st Strand cDNA Synthesis Kit (Takara, China) on ABI Veriti96 PCR (ABI, USA). For real-time quantitative PCR (RT-qPCR), a total of 1 μg RNA in 20 μL reaction mixtures was used for the first-strand cDNA synthesis using the Prime-Script® RT Reagent Perfect Real Time Kit (Takara, China) on ABI Veriti96 PCR. *GAPDH* (CA254672) gene was used as the internal control. The primers of GAPDH-QF/GAPDH-QR were listed in Table 1 (Iskandar et al., 2004; Que et al., 2009).

**SoGloI** expression in greenhouse-grown sugarcane plantlets under different stress treatments

In the greenhouse, tissue culture-derived 4-month old healthy plantlets were rinsed with agar medium and transplanted into every other column of 46 mL-wells in two 96-well (8 x 12) plastic trays, containing only distilled water. The water was changed every morning. After 10 days, the plantlets were transplanted to 100-mL flat-bottomed glass tubes and subjected to seven different stress treatments in three replicates. Three plantlets of each treatment was treated with the
following solutions: 100 μM methyl jasmonate (MeJA, dissolved with 0.1 % (v/v) ethanol and 0.05 % (v/v) Tween-20); 5.0 mM salicylic acid (SA, dissolved with 0.05 % (v/v) Tween-20); 100 μM abscisic acid (ABA) for 6, 12, and 24 h (Li et al., 2009; Su et al., 2013); 250 mM NaCl; 500 μM CdCl₂; 100 μM CuCl₂; or 100 μM ZnSO₄ for 12, 24 and 48 h (Damaj et al., 2010; Guo et al., 2012; Que et al., 2009), respectively. Plantlets grown in distilled water were used as the control.

SoGloI expression in response to MeJA, SA, ABA (0, 6, 12, 24 hour-post-treatment or hpt) and NaCl, CdCl₂, CuCl₂, ZnSO₄ (0, 12, 24, 48 hpt) was analyzed by RT-qPCR on a 7500 RT-qPCR system (ABI, USA). The primers of 25S-QF/25S-QR were listed in Table 1. The SoGloI-specific primer pair of G-QF/G-QR was designed by Primer Premier 5.0 software (Premier Biosoft International, CA) (Table 1). RT-qPCR was carried out with FastStart Universal SYBR Green Master (ROX) (Roche, China) in a 25 μL volume containing 12.5 μL FastStart Universal SYBR Green PCR Master (ROX), 0.5 μM of each primer and 1.0 μL template (100× diluted cDNA). RT-qPCR with sterile dH₂O as template was the control. The RT-qPCR thermal cycle program included 2 min at 50°C; 10 min at 95°C; and 40 cycles of (15 s at 94°C and 1 min at 60°C). The reactions were repeated three times for each sample. The 2⁻ddCt method was used to calculate relative gene expression levels (Livak & Schmittgen, 2001).

Expression of pET32a-SoGloI in E. coli Rosetta Cells (DE3)

A SoGloI gene fragment was amplified from the cDNA clone with primers G-32aF/G-32aR (Table 1). The SoGloI PCR fragment was digested with EcoRI and XhoI (NEB, USA) and subsequently sub-cloned into the EcoRI–XhoI sites of pET32a (+) (Guo et al., 2012) to produce pET32a-SoGloI. After sequence verification, the pET32a-SoGloI was transformed into Rosetta Cells (DE3) (Tiangen BioTech Co. Ltd., China). Expression of pET32a-SoGloI was induced in 1 mM isopropyl β-D-thiogalactoside (IPTG) for 8 h at 37°C and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 0, 2, 4, and 8 h. Non-transformed (blank) and the vector pET32a-transformed Rosetta cells (control) were used as the controls.

Spot assays were performed to assess the response of pET32a-SoGloI transformed E. coli cells to NaCl, CdCl₂, CuCl₂ or ZnSO₄ treatments. When the E. coli culture mixture reached OD₆₆₀ = 0.6, 1 mM IPTG was added into the LB medium and the culture mixture was incubated for 12 h at 28°C. Then the cultures were first diluted to 0.6 (OD₆₆₀), and further diluted to two levels (10⁻³ and 10⁻⁴) (Guo et al., 2012). Thereafter, 10 μL each of the diluted cultures was spotted on LB plates containing 170 μg·mL⁻¹ chloramphenicol and 80 μg·mL⁻¹ ampicillin, along with each test chemical. The concentrations of the chemicals used were NaCl at 250, 500 and 750 mM, CdCl₂ at 250, 500 and 750 μM, CuCl₂ at 250, 500 and 750 μM, and ZnSO₄ at 250, 500 and 750 μM, respectively (Guo et al., 2012; Su et al., 2013). All plates were incubated overnight at 37°C.

Assay of sugarcane glyoxalase I enzyme activity
Entire flash-frozen 4-month old plantlets (100 mg wet weight) were pulverized in liquid N\textsubscript{2} in a mortar. Protein was extracted with an extraction buffer containing 0.1 M potassium phosphate buffer (PPB, pH 7.5), 50% (v/v) glycerol, 16 mM MgSO\textsubscript{4}, 0.2 mM Phenylmethanesulfonyl fluoride (PMSF) and 0.2% (v/v) polyvinylpyrrolidone (PVP40). The extract was centrifuged twice at 13,000 rpm at 4°C for 30 min to obtain the crude protein extract from the supernatant (Zeng et al., 2016). The supernatant was used as the cytosolic extract for the assessment of glyoxalase activity, and protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. SoGloI activity assay was carried out following Hossain et al. (2009) and Hasanuzzaman et al. (2011). Briefly, the assay mixture contained 100 mM K-phosphate buffer (PPB, pH 7.0), 15 mM magnesium sulfate, 1.7 mM GSH, and 3.5 mM MG in a final volume of 0.8 mL. Thioester formation was measured by the increase in absorbance at 240 nm for 1 min. The enzyme activity was calculated using an extinction coefficient (\varepsilon) of 3.37 mM\textsuperscript{-1} \cdot cm\textsuperscript{-1}.

**Sub-cellular localization**

The SoGloI gene was sub-cloned into the XcmI/BamHI restriction sites of pCXSN to construct a fusion protein expression vector of 35S::SoGloI::GFP. The GFP-containing pCXSN vector was a gift of Songbiao Chen, Institute of Biotechnology, Fujian Academy of Agricultural Sciences. The pCXSN-SoGloI recombinant plasmids were transformed into Agrobacterium tumefaciens cells, strain GV 3101 (Chen et al., 2006). The transgenic GV 3101 cells were inoculated into LB medium containing kanamycin (50 µg·mL\textsuperscript{-1}) and rifampicin (34 µg·mL\textsuperscript{-1}). The culture was incubated overnight at 28°C with shaking at 200 rpm. The culture was then centrifuged at 5,000 x g to harvest the Agrobacterium cells followed by, re-suspension in 10 mM MgCl\textsubscript{2} and 10 mM fatty acid methyl ester sulfonate (MES). The concentration of the bacterial suspension was measured and adjusted to \textit{OD}\textsubscript{600} = 0.6 with Murashige and Skoog (MS) liquid medium supplemented with 200 mM acetosyringone. The resulting culture was incubated at 28°C for 3 h (Yang et al., 2014). Then, 1 mL of the bacterial culture was infiltrated into 4-week old tobacco leaves with disposable syringes. The injection sites were marked. Injected plants were incubated under a 12 h-light/12 h-dark cycle at 28°C for three days (Su et al., 2013). Then, the protoplasts were isolated from well-expanded leaves following the rice protoplast isolation protocol of Chen et al. (2006). Briefly, the leaves were cut into 1-mm strips and placed in a dish containing 12 mL of K3 medium (3 mM MES, 7 mM CaCl\textsubscript{2}, 0.35 M mannitol, 0.7 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.35 M sorbitol, 20 mM KCl, pH 5.6) supplemented with 0.4 M sucrose, 1.5% cellulase R-10 (Yakult Honsha, Japan) and 0.3% macerozyme R-10 (Yakult). The leaf tissue was vacuum-infiltrated for 30 min at 20 mm Hg and digested at room temperature with gentle shaking for 4 h to produce protoplasts. Then, the K3 medium was replaced with 12 mL of W5 solution (2 mM MES, 154 mM NaCl, 125 mM CaCl\textsubscript{2}, 5 mM KCl, pH 5.8). The protoplasts were collected by centrifugation at 300 x g for 4 min at 4°C and re-suspended in 1 mL WI solution (4 mM MES, 0.5 M mannitol,
252 20 mM KCl, pH 5.7). The sub-cellular location of the *SoGloI* gene was observed using fluorescence microscopy (Ci-L, Nikon, Japan).

### RESULTS

**Sequence analysis of *SoGloI***

The full-length of *SoGloI* (GenBank Accession Number: KC857628) was 1,091 bp with one ORF of 885 bp (Fig. S1). The deduced *SoGloI* protein had 294 amino acids with a predicted molecular mass of 32.9 kDa and a pI value of 5.45. Two glyoxalase domains were found, located at 28–149 and 159–283, respectively (Fig. S2). The ProtScale predicted that *SoGloI* was a hydrophilic protein (Fig. S3). TMHMM Server v.2.0 and SignalP 4.1 Server program predicted that *SoGloI* was not a trans-membrane protein (Fig. S4, S5).

In order to predict the metal dependency of *SoGloI* protein, a multiple sequence alignment of *SoGloI* with other known GloI proteins from *Oryza sativa* (OsglyI-11.2 and O. Zn-GloI), *Arabidopsis thaliana* (AtGLYI2, AtGLYI3 and AtGLYI6), *Pseudomonas aeruginosa* (GloA1, GloA2 and GloA3), *Glycine max* (G. Zn-GloI and G. Ni-GloI) and *Escherichia coli* (GlxI) was done using DNAMAN® (Fig 1). *SoGloI* shared 87.8%, 69.7%, 57.3%, and 72.0% amino acid sequence identities with OsglyI-11.2, AtGLYI3, AtGLYI6, and G. Ni-GloI, respectively (Fig. 1). Extended amino acid sequences (the letters A, B and C in Fig. 1) of OsglyI-11.2, AtGLYI3, AtGLYI6, GloA1, GloA2, G. Ni-GloI, and GlxI were missing in *SoGloI*, a characteristic of Ni$^{2+}$-dependent GloI. Contrarily, these extended amino acid sequences were present in AtGLYI2, GloA3, O. Zn-GloI and G. Zn-GloI, which were Zn$^{2+}$-dependent GloI.

**Expression of *SoGloI* in E. coli***

Upon IPTG induction, the recombinant *SoGloI* gene expressed well in Rosetta cells (Fig. 2, Lanes 5-8) to yield *SoGloI* protein that was 51 kDa in size and carried a Trx-His-S-tag of 18.3 kDa. Moreover, gradually increased amounts of *SoGloI* protein were also observed when the IPTG induction was extended from 2 h to 8 h.

In the spot assays, *SoGloI*-expressing Rosetta cells (Fig. 3A-E, Row a) grew more rapidly on LB agar plates containing NaCl, CuCl$\textsubscript{2}$, CdCl$\textsubscript{2}$ or ZnSO$\textsubscript{4}$ than control Rosetta cells (pET 32a) (Fig. 3A-E, Row b). Compared to control cells, *SoGloI*-expressing cells tolerated salt up to 250 mM (Fig. 3B) as well as heavy metal ions up to 750 μM (Fig. 3C-E). Consistently, *SoGloI*-expressing cells also grew faster than the control cells in LB liquid media containing 250 mM NaCl, 750 μM CuCl$\textsubscript{2}$, 750 μM CdCl$\textsubscript{2}$ or 750 μM ZnSO$\textsubscript{4}$ (Fig. 4). These results may demonstrate that the recombinant *SoGloI* protein enhanced the growth of Rosetta cells under stress conditions.

**Expression patterns of *SoGloI* in sugarcane tissues***

RT-qPCR was conducted to detect both tissue-specific and stress-related expression of *SoGloI*. The *SoGloI* gene was ubiquitously expressed in five tissues of 7- to 8-month old plants collected...
from the field. The highest level was detected in buds, followed by leaves, roots, leaf sheaths, and internodes (Fig. 5A).

SoGloI expression patterns in healthy 4-month old plantlets under NaCl, CuCl₂, CdCl₂, ZnSO₄, SA, MeJA, and ABA treatments were shown in Fig. 5B and 5C. Under NaCl, CuCl₂, CdCl₂, and ZnSO₄ treatments, SoGloI expression was up-regulated steadily from 0 to 48 hpt. The peak level of SoGloI expression was about 3.1-, 2.9- 2.8- and 1.9-fold of the level in control, respectively (Fig. 5B). In contrast, under SA and MeJA treatments, SoGloI expression decreased after peaking at 6 hpt. The maximum level of SoGloI expression was detected at 6 hpt, which was about 2.6- and 2.1-fold of the level in control, respectively (Fig. 5C). Similarly, the peak level of SoGloI expression was detected at 12 hpt under ABA treatments, which was 2.4-fold of the level in control (Fig. 5C). Thus, SoGloI gene has been found to provide tolerance to multiple abiotic stresses.

Glyoxalase I activity in sugarcane under NaCl, CuCl₂, CdCl₂ or ZnSO₄ treatment

As shown in Fig. 5D, under NaCl, CuCl₂, CdCl₂, and ZnSO₄ treatments, the glyoxalase I activity was increased steadily from 0 to 48 hpt. Under a 250 mM NaCl treatment, the glyoxalase I activity was about 1.8-, 2.2-, and 2.3-fold at 12, 24, and 48 hpt comparing to control, respectively. At 48 hpt, the level of glyoxalase I activity reached 0.3230 μmol·min⁻¹·mg⁻¹. Under a 750 μM CuCl₂ treatment, the level of glyoxalase I activity was about 2.0-, 2.7-, and 3.0-fold of the level in control, with 0.4128 μmol·min⁻¹·mg⁻¹ protein produced at 48 hpt. Similarly, under a 750 μM CdCl₂ treatment, the glyoxalase I activity was 2.1-, 3.1- and 4.2-fold comparing to control, with a highest activity of 0.5730 μmol·min⁻¹·mg⁻¹ protein at 48 hpt. Under 750 μM ZnSO₄ treatment, the glyoxalase I activity at 12, 24, and 48 hpt was about 1.3-, 2.6-, and 3.1-fold of the level at 0 hpt, and 0.2883 μmol·min⁻¹·mg⁻¹ protein produced at 48 hpt. Thus, glyoxalase I activity was increased in varying degrees under salt and heavy metal ions stress conditions.

Determination of subcellular localization of ScGloI

To further understand the function of SoGloI gene, its subcellular localization was determined. The SoGloI gene was inserted into a plant expression vector pCXSN between the 35S promoter and GFP. The recombinant pCXSN-SoGloI-GFP construct was then introduced into tobacco leaves through Agrobacterium-mediated transformation. As shown in Fig. 6B, green fluorescence signals were observable in the cytosol and nucleus of both pCXSN-SoGloI-GFP and the pCXSN-GFP transformed tobacco protoplasts.

DISCUSSION

Glyoxalase I functions to detoxify the potent cytotoxic compound MG (Thornalley, 1993). In response to stress conditions, cells undergo active metabolism to produce more MG through
leakages in the glycolysis and TCA cycle (Umea et al., 1994). GlyI, the first enzyme of the glyoxalase system, plays a critical role in controlling MG levels and cytotoxicity (Wu et al., 2013a). The GloI gene has been cloned and characterized from several plant species. However, the glyoxalase I gene was never cloned and characterized in sugarcane. In the present study, a length GloI gene, designated as SoGloI, was isolated from a smut-resistant sugarcane cultivar YCE 05-179.

The GloI enzyme requires Ni^{2+} or/and Zn^{2+} for its catalytic activity (Sukdeo et al., 2004). Sukdeo et al. (2007) reported that Pseudomonas aeruginosa, a gamma proteobacteria, encodes both Ni^{2+} and Zn^{2+} forms of the enzyme; GloA1, GloA2 (both Ni binding), and GloA3 (Zn binding). Jain et al. (2016) also found three active GLYI enzymes (AtGLYI2, AtGLYI3 and AtGLYI6) belonging to different metal activation classes coexisting in Arabidopsis thaliana. AtGLYI2 was found to be Zn^{2+}-dependent, whereas AtGLYI3 and AtGLYI6 were Ni^{2+}-dependent. Ni^{2+}-dependent GloI is present as a two-domain protein in all eukaryotes. Among the early branching eukaryotes, algae appears to be the first to encode this gene (Kaur et al., 2013).

In this study, a sugarcane SoGloI gene was found to encode two glyoxalase domains as well (Fig. S2). Besides, the multiple protein sequence alignment of SoGloI with those from other species indicated that SoGloI was a Ni^{2+}-dependent enzyme (Fig. 1). The result was similar to OsGLYI-11.2 (Mustafiz et al. 2014), who’s expression was substrate inducible. However, unlike other eukaryotic Zn^{2+}-dependent glyoxalases, OsGLYI-11.2 is a Ni^{2+}-dependent monomeric enzyme.

Plant glyoxalase system in different tissues plays an important role at various vegetative and reproductive stages (Mustafiz et al., 2011). GLOI gene is required for cell division and proliferation; a higher enzyme activity has been found in rapidly dividing cells of cell suspensions, seedlings, and root tips (Lin et al., 2010; Wu et al., 2013a). In this study, SoGloI was constitutively expressed in various tissues of sugarcane genotype YCE 05-179, with the highest level in buds, followed by leaves, roots, leaf sheaths, and internodes (Fig. 5A).

To date, only a few reports have shown that GLOI gene is associated with disease resistance in plants. For instance, a maize Glx-I gene enhances the host defense against Aspergillus flavus through the detoxification of MG, a major product of A. flavus (Chen et al., 2004). The expression of wheat TaGly I is up-regulated 2.3-fold upon infection by Fusarium graminearum (Lin et al., 2010). In our previous study, SoGloI expression was up-regulated during infection with S. scitamineum, the pathogen of sugarcane smut (Wu et al., 2013b). In the present study, we used SA and MeJA to simulate biotic stress. Consistently, under SA and MeJA treatments, the SoGloI expression peaked at 6 hpt, when its activity reached 2.6- and 2.1-fold higher than that of the control, respectively (Fig. 5C). These results suggest that SoGloI expression can increase significantly under pathogenic stresses; however, the exact role of SoGloI in pathogenic resistance process needs to be further investigated.

Glyoxalase I genes also have been implicated to enhance plant tolerance to salt stress. The expression of Gly, a glyoxalase I gene of B. juncea, is up-regulated after exposure to a high concentration of salt (Veena, Reddy & Sopory, 1999). The mRNA and polypeptide levels of
GLXI, a glyoxalase I gene of tomato, increased by two to three folds in roots, internodes and leaves when the plants were treated with 10 g/L NaCl (Espartero, Sanchez-Aguayo & Pardo, 1995). The expression of two other glyoxalase I genes, BvM14-glyoxalase I of sugar beet (Wu et al., 2013a) and TaGly I of wheat (Lin et al., 2010), also significantly enhanced hosts’ tolerance to salt stress. In this study, SoGloI-expressing Rosetta cells grown on agar plates tolerated high concentrations of NaCl up to 250 mM (Fig. 3B) and grew faster in LB liquid medium containing 250 mM NaCl (Fig. 4A). SoGloI expression was increased steadily from 0 to 48 hpt in sugarcane under salt stress (Fig. 5B). Under salt stress, glyoxalase I activity also elevated (Fig. 5D). Taken together, the results indicate the expression level of SoGloI can be significantly up-regulated under salt stress; however, more research is needed to reveal the underlying mechanism.

Zinc (Zn^{2+}), a micronutrient, is necessary for plant growth, but an excessive amount of Zn^{2+} can inhibit plant growth (Sun et al., 2006; Zarcinas et al., 2004). A few studies have demonstrated that plant GloI genes enhance host tolerance to Zn^{2+}. Singla-Pareek et al. (2006) showed that GlyI from B. juncea enhanced host Zn^{2+} tolerance to toxic levels in the transgenic tobacco. The expression of TaGly I, a glyoxalase I gene of T. aestivum, is induced continuously under 20 mM ZnCl_2 treatment. Compared to control, the increase in TaGly I expression is nearly 1.5-fold at 24 h (Lin et al., 2010). In the present study, SoGloI-expressing E. coli Rosetta cells were able to tolerate high concentrations of ZnSO_4 up to 750 μM (Fig. 3E) and also grew faster in LB liquid medium containing 750 μM ZnSO_4 (Fig. 4D).

Consistently, under ZnSO_4 stress, the SoGloI expression in sugarcane was up-regulated steadily from 0 to 48 hpt, when its level and enzyme activity were 1.9-fold and 3.1-fold higher than that of the control (Fig. 5B, 5D). These results showed that SoGloI gene can enhance tolerance to excessive zinc stress even in a heterologous host system. Over-expression of glyoxalase I has been shown to confer tolerance to other heavy metals, such as cadmium or lead (Singla-Pareek et al., 2006). The level of expression and activity of SoGloI in E. coli (Fig. 3D, 4C) under CdCl_2 treatment (Fig. 5B, 5D) also supported this notion about tolerance to cadmium. Our work further showed that SoGloI expression and its enzyme activity were increased significantly under CuCl_2 treatment (Fig. 5B, 5D). All these findings suggest that SoGloI may be a good candidate gene for engineering to develop heavy metal resistant sugarcane cultivars.

As is known, sugarcane is a polyploidy and aneuploidy crop (Scortecci et al., 2012), in which low transformation efficiency remains one of the major limiting factors on transgenic sugarcane production (DalBianco et al., 2012; Gómez-Merino, Trejo-Téllez & Sentíes-Herrera, 2014; Scortecci et al., 2012). This has also limited the functional analysis of isolated sugarcane genes; nonetheless, a model plant species (Arabidopsis thaliana, Nicotiana benthamiana or Brachypodium distachyon) with a shorter life cycle and simpler genome can be explored as an alternative host for transforming and assessing the functional properties of isolated sugarcane genes, such as SoGloI.
CONCLUSIONS

This is the first report on the cloning and characterization of glyoxalase I (SoGloI) gene in sugarcane. We isolated and characterized SoGloI gene and demonstrated the enzyme activity of glyoxalase I protein. We found that SoGloI expression and SoGloI enzymatic activity were elevated significantly when sugarcane tissues were subject to simulated biotic and abiotic stress conditions, such as high concentrations of salt or heavy metal ions. The findings have opened up a new research avenue for sugarcane to grow in polluted or salty environments via genetic engineering and breeding of SoGloI to enhance host resistance.

SUPPLEMENTARY MATERIALS

Figure S1 Nucleotide acid sequences and deduced amino acid sequences of SoGloI obtained by RT-PCR.

Figure S2 Domain architecture analysis of SoGloI protein.

Figure S3 Hydrophobicity prediction of SoGloI protein.

Figure S4 Signal peptides prediction of SoGloI protein.

Figure S5 Trans-membrane helix domain prediction of SoGloI protein.

REFERENCES
Akhtar S, Wahid A, Rasul E. 2003. Emergence, growth and nutrient composition of sugarcane sprouts under NaCl salinity. *Biologia Plantarum* 46:113-116 DOI 10.1023/a:1022326604192.

Bailey RA, Bechet GR. 1997. Further evidence of the effects of ratoon stunting disease on production under irrigated and rainfed conditions. *In Proceedings of the South African Sugar Technologists' Association* 71:97-101.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Begcy K, Mariano ED, Gentile A, Lembke CG, Zingaretti SM, Souza GM, Menossi M. 2012. A novel stress-induced sugarcane gene confers tolerance to drought, salt and oxidative stress in transgenic tobacco plants. *PloS One* 7:e44697 DOI 10.1371/journal.pone.0044697.

Chakravarty TN, Sopory SK. 1988. Blue light stimulation of cell proliferation and glyoxalase I activity in callus cultures of *Amaranthus paniculatus*. *Plant Science* 132:63-69 DOI 10.1016/S0168-9452(97)00264-1.

Chen SB, Tao LZ, Zeng LR, Vega-Sanchez ME, Umemura K, Wang GL. 2006. A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. *Molecular Plant Pathology* 7:417-427 DOI 10.1111/j.1364-3703.2006.00346.x.

Chen ZY, Brown RL, Damann KE, Cleveland TE. 2004. Identification of a maize kernel stress-related protein and its effect on aflatoxin accumulation. *Phytopathology* 94:938-945 DOI 10.1094/PHYTO.2004.94.9.938.

Clelland JD, Thornalley PJ. 1991. S-2-Hydroxyacyl glutathione-derivatives: enzymatic preparation, purification and characterization. *Journal of the Chemical Society, Perkin Transactions 1* 12:3009-3015 DOI 10.1039/P19910003009.

Dal-Bianco M, Carneiro MS, Hotta CT, Chapola RG, Hoffmann HP, Garcia AAF, Souza GM. 2012. Sugarcane improvement: how far can we go? *Current Opinion Biotechnology* 23:265-270 DOI 10.1016/j.copbio.2011.09.002.

Damaj MB, Kumpatla SP, Emani C, Beremand PD, Reddy AS, Rathore KS, Buenrostro-Nava MT, Curtis IS, Thomas TL, Mirkov TE. 2010. Sugarcane DIRIGENT and O-methyltransferase promoters confer stem-regulated gene expression in diverse monocots. *Planta* 231:1439-1458 DOI 10.1007/s00425-010-1138-5.

Deswal R, Sopory SK. 1999. Glyoxalase I from *Brassica juncea* is a calmodulin stimulated protein. *Biochimica et Biophysica Acta* 1450:460-467 DOI 10.1016/S0167-4889(99)00047-6.

Espartero J, Sanchez-Aguayo I, Pardo JM. 1995. Molecular characterization of glyoxalase I from a higher plant: up-regulation by stress. *Plant Molecular Biology* 29:1223-1233 DOI 10.1007/BF000020464.

Fricket EM, Jemth P, Widersten M, Mannervik B. 2001. Yeast glyoxalase I is a monomeric enzyme with two active sites. *J. Biol. Chem.* 276:1845-1849. DOI 10.1074/jbc.M005760200.
Ghosh A and Islam T. 2016. Genome-wide analysis and expression profiling of glyoxalase gene families in soybean (Glycine max) indicate their development and abiotic stress specific response. BMC Plant Biol. 16: 87. DOI 10.1186/s12870-016-0773-9.

Gómez-Merino FC, Trejo-Téllez LI, Sentíes-Herrera HE. 2014. Sugarcane as a novel biofactory: potentials and challenges. In Biosystems Engineering: Biofactories for Food Production in the Century XXI. Springer International Publishing. 129-149.

Guo JL, Xu LP, Fang JP, Su YC, Fu HY, Que YX, Xu JS. 2012. A novel dirigent protein gene with highly stem-specific expression from sugarcane, response to drought, salt and oxidative stresses. Plant Cell Report 31:1801-1812 DOI 10.1007/s00299-012-1293-1.

Hasanuzzaman M, Hossain MA, Fujita M. 2011. Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings. Plant Biotechnol. Rep. 5:353-365 DOI 10.1007/s11816-011-0189-9.

Hossain MA, Fujita M. 2009. Purification of glyoxalase I from onion bulbs and molecular cloning of its cDNA. Bioscience, Biotechnology & Biochemistry. 73:2007-2013 DOI 10.1271/bbb.90194.

Hoy JW, Hollier CA, Fontenot DB, Grelen LB. 1986. Incidence of sugarcane smut in Louisiana and its effects on yield. Plant Disease. 70:59–60.

Iskandar HM, Simpson RS, Casu RE, Bonnett GD, Maclean DJ, Manners JM. 2004. Comparison of reference genes for quantitative real-time polymerase chain reaction analysis of gene expression in sugarcane. Plant Molecular Biology Reporter. 22:325-337 DOI 10.1007/BF02772676.

Jain M, Batth R, Kumari S, Mustafiz A. 2016. Arabidopsis thaliana Contains both Ni^{2+} and Zn^{2+} dependent glyoxalase I enzymes and ectopic expression of the latter contributes more towards abiotic stress tolerance in E. coli. PLoS One 11(7): e0159348. DOI 10.1371/journal.pone.0159348.

James G. 1996. A review of ratoon stunting disease. International Sugar Journal 98: 532-545.

Johansen KS, Svendsen I, Rasmussen SK. 2000. Purification and cloning of the two domains glyoxalase I from wheat bran. Plant Science 155:11-20 DOI 10.1016/S0168-9452(99)00250-2.

Kao KN, Michayluk MR. 1975. Nutritional requirements for growth of Vicia hajastana cells and protoplasts at a very low population density in liquid media. Planta 126:105-110 DOI 10.1007/BF00380613.

Kurz A, Rabbani N, Walter M, Bonin M, Thornalley P, Auburger G, Gispert S. 2011. Alpha-synuclein deficiency leads to increased glyoxalase I expression and glycation stress. Cellular & Molecular Life Sciences 68:721-733 DOI 10.1007/s00018-010-0483-7.

Kaur C, Vishnoi A, Ariyadasa TU, Bhattacharya A, Singla-Pareek SL, Sopory SK. 2013. Episodes of horizontal gene-transfer and gene-fusion led to co-existence of different metal-ion specific glyoxalase I. Scientific Reports. 3: 3076. DOI 10.1038/srep03076.

Kaur C, Tripathi AK, Nutan KK, Sharma S, Ghosh A, Tripathi JK, Pareek A, Singla-Pareek SL, Sopory SK. 2017. A nuclear-localized rice glyoxalase I enzyme, OsGLYI-8, functions in the detoxification of methylglyoxal in the nucleus. Plant J. 89(3): 565-576. DOI 10.1111/tpj.13407.
Li DM, Stachelin C, Wang WT, Peng SL. 2009. Molecular cloning and characterization of a chitinase-homologous gene from *Mikania micrantha* infected by *Cuscuta campestris*. *Plant Molecular Biology Reporter* 28:90-101 DOI 10.1007/s11105-009-0125-0.

Lin FY, Xu JH, Shi JR, Li HW, Lin B. 2010. Molecular cloning and characterization of a novel glyoxalase I gene TaGly I in wheat (*Triticum aestivum* L.). *Molecular Biology Reports* 37:729-735 DOI 10.1007/s11033-009-9578-3.

Liu B, Xue XD, Cui SP, Zhang XY, Han QM, Zhu L, Liang XF, Huang LL, Chen XM. Kang ZS. 2010. Cloning and characterization of a wheat β-1, 3-glucanase gene induced by the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici*. *Molecular Biology Reports* 37:1045-1052 DOI 10.1007/s11033-009-9823-9.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCt} method. *Methods* 25:402-408 DOI 10.1006/meth.2001.1262.

Lo T, Westwood M, McLellan A, Selwood T, Thornalley P. 1994. Binding and modification of proteins by methylglyoxal under physiological conditions: a kinetic and mechanistic study with N-acetylarginine, N-acetylcysteine, and N-acetyl-lysine and bovine serum albumin. *Journal of Biological Chemistry* 269:32299-32305.

Mustafiz A, Singh AK, Pareek A, Sopory SK, Singla-Pareek SL. 2011. Genome-wide analysis of rice and *Arabidopsis* identifies two glyoxalase genes that are highly expressed in abiotic stress. *Functional & Integrative Genomics* 11:293-305 DOI 10.1007/s10142-010-0203-2.

Mustafiz A, Ghosh A, Tripathi AK, Kaur C, Ganguly AK, Bhavesh NS, Tripathi JK, Pareek A, Sopory SK, Singla-Pareek SL. 2014. A unique Ni^{2+}-dependent and methylglyoxal-inducible rice glyoxalase i possess a single active site and functions in abiotic stress response. *Plant J.* 78: 951-963. DOI 10.1111/tpj.12521.

Padmanaban P, Alexander KC, Shanmugan N. 1998. Effect of smut on growth and yield parameters of sugarcane. *Indian Phytopathology* 41:367-369.

Papoulis A, Al-Abed Y, Bucala R. 1995. Identification of N2-(1-carboxyethyl) guanine (CEG) as a guanine advanced glycosylation end product. *Biochemistry* 34:648-655 DOI 10.1021/bi00002a032.

Phillips SA, Thornalley PJ. 1993. The formation of methylglyoxal from triose phosphates. *European Journal of Biochemistry* 212: 101-105 DOI 10.1111/j.1432-1033.1993.tb17638.x.

Plaut Z, Meinzer FC, Federman E. 2000. Leaf development, transpiration and ion uptake and distribution in sugarcane cultivars grown under salinity. *Plant and Soil* 218:59-69 DOI 10.1023/a:1014996229436.

Price CL, Knight SC. 2009. Methylglyoxal: possible link between hyperglycaemia and immune suppression? *Trends in Endocrinology & Metabolism* 20:312-317 DOI 10.1016/j.tem.2009.03.010.

Que YX, Xu JS, Xu LP, Gao SJ, Chen RK. 2008. PCR detection for *Leifsonia xyli* subsp. *Xyli*, pathogen of the sugarcane ratoon stuntng disease. *Fujian Journal of Agricultural Sciences* 23:364-367.

Que YX, Xu LP, Xu JS, Zhang JS, Zhang MQ, Chen RK. 2009a. Selection of control genes in real-time qPCR analysis of gene expression in sugarcane. *Chinese Journal of Tropical Crops* 30:274-278.
Que YX, Yang ZX, Xu LP, Chen RK. 2009b. Isolation and identification of differentially expressed genes in sugarcane infected by Ustilago scitaminea. Acta Agronomica Sinica 35:452-458.

Que YX, Xu LP, Lin JW, Chen RK, Grisham MP. 2012. Molecular variation of Sporisorium scitamineum in Mainland China revealed by RAPD and SRAP markers. Plant Disease 96:1519-1525 DOI 10.1094/PDIS-08-11-0663-RE.

Que YX, Xu LP, Wu QB, Liu YF, Ling H, Liu YH, Zhang YY, Guo JL, Su YC, Chen JB, Wang SS, Zhang CG. 2014. Genome sequencing of Sporisorium scitamineum provides insights into the pathogenic mechanisms of sugarcane smut. BMC Genomics 15:996 DOI 10.1186/1471-2164-15-996.

Ridderström M, Mannervik B. 1996. Optimized heterologous expression of the human zinc enzyme glyoxalase I. Biochem J. 314: 463-4677. PMID: 8670058.

Scortecci KC, Creste S, Calsa TJ, Xavier MA, Landell MGA, Figueira A, Benedito VA. 2012. Challenges, opportunities and recent advances in sugarcane breeding. In Plant Breeding. In Tech 267-296.

Sethi U, Basu A, Guha-Mukherjee S 1988. Control of cell proliferation and differentiation by regulating polyamine biosynthesis in cultures of Brassica and its correlation with glyoxalase activity. Plant Science 56:167-175 DOI 10.1016/0168-9452(88)90031-3.

Singla-Pareek SL, Reddy MK, Sopory SK. 2003. Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. Proceedings of the National Academy of Sciences 100:14672-14677.

Singla-Pareek SL, Yadav SK, Pareek A, Reddy, MK, Sopory SK. 2006. Transgenic tobacco over-expressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. Plant Physiology 140:613-623 DOI 10.1104/pp.105.073734.

Skipsey M, Andrews CJ, Townson JK, Jepson I, Edwards R. 2000. Cloning and characterization of glyoxalase I from soybean. Archives of Biochemistry & Biophysics 374:261-268 DOI 10.1006/abbi.1999.1596.

Su YC, Xu LP, Xue BT, Wu QB, Guo JL, Wu LG, Que YX. 2013. Molecular cloning and characterization of two pathogenesis-related \( \beta-1,3 \)-glucanase genes \( \text{ScGluA1} \) and \( \text{ScGluD1} \) from sugarcane infected by Sporisorium scitaminea. Plant Cell Reports 32:1503-1519 DOI 10.1007/s00299-013-1463-9.

Sukdeo N, Clugston SL, Daub E, Honek JF. 2004. Distinct classes of glyoxalase I: metal specificity of the Yersinia pestis, \( \text{Pseudomonas aeruginosa} \) and \( \text{Neisseria meningitidis} \) enzymes. Biochem J. 384:111-117. DOI 10.1042/BJ20041006.

Sukdeo N, Honek JF. 2007. Pseudomonas aeruginosa contains multiple glyoxalase I-encoding genes from both metal activation classes. Biochim. Biophys. Acta-Proteins Proteomics 1774:756-763.

Sun YF, Xie ZM, Li J, Xu JM, Chen ZL, Naidu R. 2006. Assessment of toxicity of heavy metal contaminated soils by the toxicity characteristic leaching procedure. Environmental Geochemistry & Health 28:73-78 DOI 10.1007/s10653-005-9014-0.
Thornalley PJ. 1990. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochemical Journal* 269:1-11.

Thornalley PJ. 1993. The glyoxalase system in health and disease. *Molecular Aspects of Medicine* 14:287-371 DOI 10.1016/0098-2997(93)90002-U.

Thornalley PJ. 1996. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification—a role in pathogenesis and antiproliferative chemotherapy. *General Pharmacology: The Vascular System* 27:565-573 DOI 10.1016/0306-3623(95)02054-3.

Thornalley PJ. 2003. Protecting the genome: defense against nucleotide glycation and emerging role of glyoxalase I over-expression in multidrug resistance in cancer chemotherapy. *Biochemical Society Transactions* 31:1372-1377 DOI 10.1042/bst0311372.

Tuomainen M, Ahonen V, Ka‘renlampi SO, Schat H, Paasela T, Švanys A, Tuohimetsä S, Peräniemi S, Tervahauta. 2003. Characterization of the glyoxalase 1 gene TcGLX1 in the metal hyperaccumulator plant. *Thlaspi caerulescens*. *Planta* 233:1173-1184 DOI 10.1007/s00425-011-1370-7.

Usui Y, Nakase M, Hotta H, Urisu A, Aoki N, Kitajima K, Matsuda T. 2001. A 33-kDa allergen from rice (*Oryza sativa L. Japonica*) cDNA cloning, expression, and identification as a novel glyoxalase I. *Journal of Biological Chemistry* 276:11376-11381 DOI 10.1074/jbc.M010337200.

Veena, Reddy VS, Sopory SK. 1999. Glyoxalase I from *Brassica juncea*: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. *The Plant Journal* 17:385-395 DOI 10.1046/j.1365-313X.1999.00390.x.

Wahid A, Rao A, Rasul E. 1997. Identification of salt tolerance traits in sugarcane lines. *Field Crops Research* 54:9-17 DOI 10.1016/S0378-4290(97)00038-5.

Wu C, Ma CQ, Pan Y, Gong SL, Zhao CX, Chen SX, Li HY. 2013a. Sugar beet M14 glyoxalase I gene can enhance plant tolerance to abiotic stresses. *Journal of Plant Research* 126:415-425 DOI 10.1007/s10265-012-0532-4.

Wu QB, Xu LP, Guo JL, Su YC, Que YX. 2013b. Transcriptome profile analysis of sugarcane responses to *Sporisorium scitaminea* infection using Solexa sequencing technology. *BioMed Research International* DOI10.1155/2013/298920.

Yadav SK, Singla-Pareek SL, Ray M, Reddy MK, Sopory SK. 2005. Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. *Biochemical & Biophysical Research Communications* 337:61-67 DOI 10.1016/j.bbrc.2005.08.263.

Yadav SK, Singla-Pareek SL, Reddy MK, Sopory SK. 2005. Methylglyoxal detoxification by glyoxalase system: a survival strategy during environmental stresses. *Physiology & Molecular Biology of Plants* 11:1-11.

Yadav SK, Singla-Pareek SL, Kumar M, Pareek A, Saxena M, Sopory SK. 2007. Characterization and functional validation of glyoxalase II from rice. *Protein expression & purification* 51:126-132. DOI 10.1016/j.pep.2006.07.007.

Yang YT, Fu ZW, Su YC, Zhang X, Li GY, Guo JL, Que YX, Xu LP. 2014. A cytosolic glucose-6-phosphate dehydrogenase gene, *ScG6PDH*, plays a positive role in response to various abiotic stresses in sugarcane. *Scientific Reports* 4:7090. DOI 10.1038/srep07090.
Zeng ZM, Xiong FJ, Yu XH, Gong XP, Luo JT, Jiang YD, Kuang KC, Gao BJ, Niu XL, Liu YS. 2016. Overexpression of a glyoxalase gene, OsGly I, improves abiotic stress tolerance and grain yield in rice (Oryza sativa L.). *Plant Physiology and Biochemistry*, 109: 62-71. DOI: 10.1016/j.plaphy.2016.09.006.

Zarcinas BA, Pongsakul P, McLaughlin MJ, Cozens G. 2004. Heavy metals in soils and crops in Southeast Asia 2. Thailand. *Environmental Geochemistry & Health*. 26:359-371. DOI 10.1007/s10653-005-4670-7.

### Table 1 Primers used in the present study

| Primer  | Sequence (5'-3')                  | Application                      |
|---------|----------------------------------|----------------------------------|
| G-F     | AGCCAGAAGAAAGGGAGC                | RT-PCR                           |
| G-R     | GTTTCCGCCTTGATGAAC                |                                   |
| G-32aF  | CGGAATTTCATGGCAACTGGTAGTGAAAG     | Prokaryotic expression vector     |
| G-32aR  | CCCTCGAGTCAGTGAAAGTTCCCTGAG       | construction                      |
| G-QF    | TGGACCGACAATCAAATACTACAC          |                                   |
| G-QR    | GTATGACATTGGGAACGGGCTTTTG         | RT-qPCR                           |
| 25S-QF  | GCAGCCAAGCGTTTATAGC               |                                   |
| 25S-QR  | CCTATTGGTGCGTGAAACATCC            |                                   |
| Primer   | Sequence                          |
|----------|-----------------------------------|
| GAPDH-QF | CACGGCCACTGGAAGCA                 |
| GAPDH-QR | TCCTCAGGGTTCTGATGCC               |
| PCX-GFP-F| ATGGTGAGCAAGGCGAGGAG              |
| GLO-LAP1-R| ACTACCAGTTGCACATTTGTACAGC        |
| GLO-LAP2-F| GCTGTACAAGATGGCAACTGGTAGT        |
| GLO-R   | TCAGTGAAGTTCCCTGAGGAAGTCG        |

Fusion PCR primers for subcellular localization
Figure 1 Multiple protein sequence alignment of SoGloI protein with glyoxalase I proteins from other species. The species name and accession numbers are as follows: Oryza sativa (OsGlyI-11.2 and O. Zn-GloI with Acc. No. Os08g09250 and EEC78918.1), Arabidopsis thaliana (AtGLY12, AtGLY13 and AtGLY16 with Acc. No. NP_172291.1, NP_172648.1, and NP_176896.1 respectively), Pseudomonas aeruginosa (GloA1, GloA2 and GloA3 with Acc. No. AAG06912, AAG04099 and AAG08496 respectively), Glycine max (G. Zn-GloI and G. Ni-GloI with Acc. No. XP_003539194.1 and XP_003528689.1) and Escherichia coli (GlxI with Acc. No. AAC27133). The letters A, B and C represent extended sequences found in Zn$^{2+}$-dependent GLOI, but absent in Ni$^{2+}$-dependent GLOI.
Figure 2 Heterologous expression of recombinant SoGloI in *E. coli* Rosetta cells (red arrow). Lane designation: M, protein size makers with 30, 40, 55, and 70 kDa indicated; 1, blank; 2, control without IPTG induction; 3, control with induction for 8 h; 4, pET 32a-*SoGloI* without induction; 5 to 8, pET32a-*SoGloI* with induction for 2, 4, 6 and 8 h, respectively.
Figure 3 Growth of pET32a-SoGloI-expressing (row a) and pET 32a (row b) E. coli Rosetta cells on LB agar plates with various concentrations of salt or heavy metal ions indicated above the figures. Panel 3A, Control; Panels 3B, NaCl; 3C, CuCl$_2$; 3D, CdCl$_2$, and 3E, ZnSO$_4$. 
Figure 4 Growth assessments of SoGloI overexpressing *E. coli* cells under salt and heavy metal treatments.

Panel 4A, under 250 mM NaCl treatment from 0 h to 24 h; Panel 4B, under 750 μM CuCl₂ treatment from 0 h to 28 h; Panel 4C, under 750 μM CdCl₂ treatment from 0 h to 26 h; Panel 4D, under 750 μM ZnSO₄ treatment from 0 h to 28 h.

All data were measured every two hours. All data points are mean ± SE (n = 3).
Figure 5 Expression patterns of *SoGloI* in sugarcane.

Panel 5A, *SoGloI* expression in different sugarcane tissues collected in the field; Panel 5B, *SoGloI* expression in greenhouse-grown sugarcane plantlets under NaCl, CuCl$_2$, CdCl$_2$, and ZnSO$_4$ treatments; Panel 5C, *SoGloI* expression in greenhouse-grown sugarcane plantlets under SA, MeJA, and ABA treatments; Panel 5D, glyoxalase I activity under NaCl, CuCl$_2$, CdCl$_2$, and ZnSO$_4$ treatments.

Different letters indicate a significant difference at 5% level (p ≤ 0.05). Each value represents the average of three biological repeats ± SE (n = 3).
Figure 6 Determination of subcellular localization of ScGloI in tobacco (Nicotiana benthamiana) protoplasts under a fluorescence microscopy. The emission wavelength is 515 nm and the excitation wavelength is between 470 nm and 490 nm.