

Full Length Research Paper

Functional characterization of a glutathione S-transferase gene from *Limonium bicolor* in response to several abiotic stresses

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In the present study, we characterized a glutathione S-transferase gene (*LbGST1*) in response to different abiotic stresses. Real time polymerase chain reaction (RT-PCR) indicated that the *LbGST1* can be differentially regulated by stress of NaCl, NaHCO₃ and low temperature, suggesting a role in plant stress response. To further investigate abiotic stress tolerance of *LbGST1*, the transgenic yeast harboring the *LbGST1* was generated under the control of inducible GAL promoter (pYES2 vector); yeast cells transformed with empty pYES2 were also generated as a control. Stress tolerance tests showed that *LbGST1* yeast transformants exhibited elevated tolerance to the stresses of thermal (53°C), alkali (NaHCO₃), ultraviolet radiation, salt (NaCl), drought (sorbitol) and freezing (-20°C) compared with the control transformants, suggesting that the *LbGST1* is tolerant to these abiotic stresses. Our results indicate that the *LbGST1* gene is resistant to a wider repertoire of stresses and may play a common role in plant acclimation to the examined stress conditions.

Key words: Glutathione S-transferases (GST), abiotic stress, expression analysis, *Limonium bicolor*.

INTRODUCTION

Plant glutathione S-transferase (GSTs) are mainly involved in stress responses including glutathione (GSH)-conjugation in the metabolic detoxification of herbicides and natural products and GSH-dependent peroxidase reactions that protect cell components from oxidative damage by scavenging toxic organic hydroperoxides. In addition, plant GSTs play a role in GSH-dependent thiol-transferase that safeguards protein function from oxidative damage, and are also involved in dehydroascorbate reductase (DHAR) that functions in redox homeostasis. Moreover, plant GSTs serves as ligands or binding proteins for phytohormones (including auxins and

cytokinins) or anthocyanins, thereby facilitating their transport and distribution in plants. Finally, plant GSTs play an indirect role in the regulation of apoptosis and in stress signaling pathways (Dixon et al., 2002a, b; Edwards et al., 2000; Frova, 2003). Therefore, GSTs are involved in stress tolerance mechanism of plants, and are required for the characterization of plant tolerance to stresses.

Previous studies have demonstrated that plant GSTs can be differentially regulated by different abiotic stress factors, such as herbicides (Cummins et al., 1999; Kunieda et al., 2005; Nutricati et al., 2006), hydrogen peroxide (H₂O₂) (Levine et al., 1994), dehydration (Kim et al., 2008; Kiyosue et al., 1993; Liu et al., 2007), ultraviolet (UV) light (Liu et al., 2007; Loyall et al., 2000), cold (Anderson and Davis, 2004; Kunieda et al., 2005; Nutricati et al., 2006; Seppänen et al., 2000), phosphate starvation (Ezaki et al., 1995), ozone exposure (Sharma and Davis, 1994), high temperature (Ezaki et al., 2004; Marrs, 1996; Nutricati et al., 2006), high salt and hormone treatments such as ethylene (Zhou and Goldsbrough, 1993), auxin (Takahashi and Nagata, 1992), methyl

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Abbreviations: GSTs, Glutathione S-transferase; GSH, glutathione; DHAR, dehydroascorbate reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene; RT-PCR, real time polymerase chain reaction; ORF, open reading frame; GPX, glutathione peroxidase; WT, wild type.

jasmonate, salicylic acid (Wagner et al., 2002) and abscisic acid (Xu et al., 2002). These studies suggested that plant GSTs play an important role in various stress responses. Therefore, cloning and characterization of plant GSTs may be helpful in knowing the stress tolerance mechanism and improvement of plant stress tolerance by molecular breeding approach.

The yeast *Saccharomyces cerevisiae* is an excellent model organism to produce heterologous proteins, and is suitable for investigating the mechanisms underlying stress tolerance (Han et al., 1999; Posas et al., 2000; Serrano and Montesinos, 2003). For instance, Jeong et al. (2000) transferred a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene into yeast, and yeast expressing *GAPDH* showed elevated resistance to cold, salt, heat and drought stresses. Mahalakshmi et al. (2006) transformed a serine-rich protein gene from *Porteresia coarctata* into *S. cerevisiae*, and the transgenic yeast displayed increased NaCl tolerance. Rausell et al. (2003) showed that expression of a sugar beet *eIF1A* gene increased the sodium and lithium salt tolerance of transgenic yeast. These results indicated that the yeast expression system is a desirable tool for determining stress tolerance of exogenous gene.

Limonium bicolor (Bunge) Kuntze (Plumbaginaceae), a halophytic flowering plant species, which can thrive in the salt soil of the Songnen plain in northeastern China, demonstrates a developed efficient salt tolerance system. Therefore, *L. bicolor* has potential utility as a source of genetic determinants for saline tolerance.

In the present study, we cloned a GST gene (*LbGST1*) from *L. bicolor*. Real time reverse transcription polymerase chain reaction (RT-PCR) was conducted to investigate its expression in response to different abiotic stresses. To characterize stress tolerance of *LbGST1*, it was transformed into yeast *S. cerevisiae*, and the tolerance to salt, drought, high temperature, freezing, alkali and UV was analyzed. Our results may provide useful insights into the role of *LbGST1* in abiotic stress tolerance.

MATERIALS AND METHODS

Gene and plant culture conditions

The *GST* gene from *L. bicolor*, *LbGST1* (GenBank number: FJ620899), was investigated in this study. *L. bicolor* seedlings were grown in a greenhouse under controlled conditions of 70 - 75% relative humidity, 14 h of light, an average temperature of 24°C and suitable watering during germination and seedling growth. Two-month-old seedlings were exposed to each of the following chemical and temperature treatments: 0.2 mol/l NaCl, 0.2 mol/l NaHCO₃, 20% (w/v) PEG6000 or low temperature (3°C) for 0 (control), 6, 12, 24, 48 and 72 h. After these treatments, leaves and roots from each sample (contains at least five seedlings) were harvested at each treatment time, and immediately frozen in liquid nitrogen for RNA isolation. These materials were used for RT-PCR analyses.

Quantitative RT-PCR

Total RNA (600 ng) was reverse transcribed into cDNA with oligo

(deoxythymidine) primers and six-mer random primers using PrimeScript™ RT reagent Kit (TaKaRa) in 10 µl reaction volume. The reverse transcription product was diluted to 100 µl and used as the RT-PCR template. Primers for *LbGST1* amplification were: G1, 5'-TTGGACGTGCATCCACAAGC-3' and G2, 5'-CGGCTGTTTTAG CCGTACTG-3'. The 18S rRNA (EU039827) and β-Tubulin (EH793552) genes were used as internal references. The primers for 18S rRNA amplification are 18S1: CCGTCTTAGTTGGTGGAG and 18S2: CTCGTTGAATACATCAGTGAG, and the primers for amplifying β-Tubulin are Tub1: 5'-GGTTGAGTGAGCAGTTCAC-3' and Tub2: 5'-GATAACCAGACCACACCTTAGC-3'. The reactions were conducted in a 20 µl volume containing 10 µl of SYBR Green Realtime PCR Master Mix (Toyobo), 0.5 µM of forward and reverse primers and 2 µl cDNA template. The amplification was completed with the following cycling parameters: 94°C for 30 s, 45 cycles at 94°C for 12 s, 58°C for 30 s, 72°C for 45 s and 1 s at 81°C for plate reading. A melting curve was generated for each sample at the end of each run to assess the purity of the amplified products. Each reaction was conducted in triplicate. Expression levels were calculated from the cycle threshold according to the delta-delta CT method (Livak and Schmittgen, 2001).

Construction of the yeast expression vector and yeast transformation

The full open reading frame (ORF) of *LbGST1* was cloned into pYES2 under the control of the inducible GAL1 promoter, and this vector was designated as pYES2-LbGST1. The pYES2-LbGST1 and empty pYES2 vector were transformed into yeast *S. cerevisiae* INVSc1 (His-, Leu-, Trp-, Ura-) using a lithium acetate method following the pYES2 protocol (pYES2 [Catalog no. V825-20], Invitrogen).

Analysis of *LbGST1* expression in *S. cerevisiae* at different induction time

To determine the expression of *LbGST1* gene in yeast *S. cerevisiae* during induction period, northern blot analysis was performed. Yeast transformed with *LbGST1* were cultured in an induction medium (SC-ura supplied with 2% galactose) for 3, 12, 24, 36, 48, 60 and 72 h at 30°C, and harvested for RNA isolation. The empty pYES2 transformants were cultured in the induction medium for 24 h at 30°C and harvested as control sample. Total RNA was isolated from each sample using trizol reagent (Invitrogen). RNA (20 µg) was dissolved in denaturing buffer (formamide: formaldehyde [37% solution]: 10 x Mops buffer, 500:162:100 [v/v/v]), heated (65°C, 15 min), fractionated on formaldehyde agarose gels, blotted on Hybond N⁺ membranes and fixed by UV cross-linked (254 nm, 8 min). Probe was labeled with DIG-dUTP (Roche) by PCR amplification of *LbGST1* ORF using specific primers. After prehybridization at 65°C for 2 h, the membrane was hybridized with probe for 18 h at 65°C. The washing and detection procedures were performed following the manual's instruction (Dig Northern starter kit instruction manual, Roche).

Characterizing the stress resistance of the *LbGST1* gene

Yeast transformants harboring pYES2-LbGST1 and its control (yeast transformed with empty pYES2) were grown in SC-ura medium containing 2% (W/V) glucose, and incubated for 24 h at 30°C. The cell densities were adjusted to OD₆₀₀ of 0.4 in 5 ml of induction medium, and incubated at 30°C for 24 h to induce *LbGST1* gene expression. After incubation, cell densities were adjusted to an approximate equal cell number for stress treatments. The following protocols were followed to induce abiotic stress: NaCl

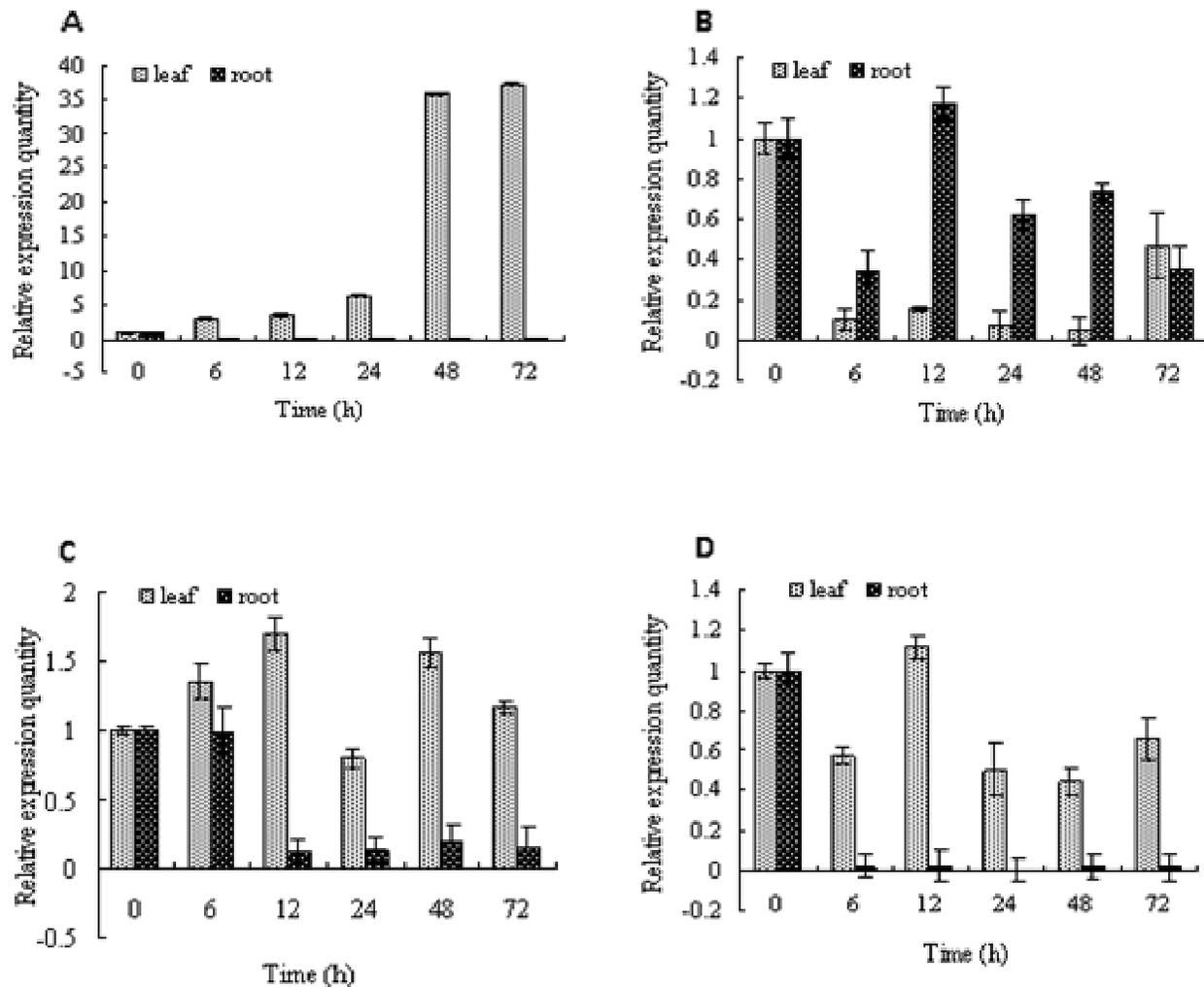


Figure 1. The time course expression patterns of the *LbGST1* gene in response to different abiotic stresses. A: Cold; B: NaCl; C: PEG6000; D: NaHCO₃.

stress, yeast cells were incubated in 5 mol/l NaCl solution at 4°C for 24 h; NaHCO₃ stress, yeast cells were incubated in 10% (w/v) NaHCO₃ solution at 30°C for 8 h; drought stress, yeast cells were incubated in 8 mol/l sorbitol solution for 24 h at room temperature; thermal stress, yeast were treated with 53°C for 1 to 2 h; for freezing stress, yeast were incubated in tubes placed in an ethanol bath at -20°C for 24 h. Following these treatments, stressed cells were dotted on SC-ura solid medium (supplied with 2% glucose) at dilutions of 1, 10, 100, 1000 and 10000-fold and incubated at 30°C. Yeast cells were also subjected to ultraviolet radiation stress by dotting on SC-ura solid medium and exposure to 254 nm wavelength for 6, 12, 18 and 24 s. After these treatments, the plates were subsequently placed at 30°C for 48 - 52 h.

RESULTS AND DISCUSSION

LbGST1 gene expression in leaves and roots of *L. bicolor*

In the present study, the expression of *LbGST1* in response

to NaCl, NaHCO₃, PEG6000 and freezing stress was investigated using RT-PCR (Figure 1). Previous studies have demonstrated that plant GST genes are responsive to various abiotic stresses, including dehydration, UV light, cold, drought, high salt and abscisic acid (Xu et al., 2002), indicating that plant GST genes play a role in abiotic stress response. Consistent with these results, our results demonstrated that the expression of *LbGST1* could be differentially regulated by abiotic stresses, indicating that it was a stress response gene. NaCl and NaHCO₃ treatments inhibited the expression of *LbGST1* in both leaves and roots during stress period (Figure 1B, D). However, the expression of *LbGST1* was highly induced in leaves by cold stress treatment (Figure 1A), but was strongly inhibited in roots, suggesting that *LbGST1* may play an important role in cold tolerance in leaves of *L. bicolor*. Polyethylene glycol (PEG) stress failed to differentially regulate *LbGST1* in leaves of *L. bicolor*; while the expression of *LbGST1* was highly



Figure 2. Analysis of *LbGST1* gene expression in yeast at different induction time using northern blot. Yeast transformants harboring *LbGST1* gene were induced by galactose for 3, 12, 24, 36, 48, 60 or 72 h; Con, yeast cells transformed with empty pYES2 and induced by galactose for 24 h.

inhibited in roots by PEG stress (Figure 1C). Interestingly, the *LbGST1* was highly repressed in roots tissues under all the test abiotic stresses, implying that it may mainly play its role in leaves under stress condition. The differential regulation of *LbGST1* genes by these abiotic stresses suggested that *LbGST1* activity was regulated in *L. bicolor* to adapt to the abiotic stress condition.

Expression analysis of exogenous *LbGST1* in yeast

To determine the expression of the exogenous *LbGST1* gene in yeast, northern blot analysis was performed. The yeast transformants harboring the *LbGST1* gene were induced by galactose at different treatment times, and an empty pYES2 was used as the negative control. The results showed that the *LbGST1* gene was expressed in yeast and can be induced by galactose, with an expression peak at induction time from 24 to 36 h (Figure 2). Therefore, the desirable time for *LbGST1* function analyses in yeast should be after induction from 24 to 36 h.

Abiotic stress tolerance analysis of the *LbGST1* gene

LbGST1 gene stress tolerance was determined by comparing the growth between *LbGST1* transformed yeast and empty pYES2 transformed yeast (as control). Our results showed that there was no difference in growth between the *LbGST1* transformed and control yeast under non-stress conditions (Figure 3A), indicating that the *LbGST1* transformed and control yeast had identical growth and survival rates. Therefore, comparing the growth between them under different stress conditions can reflect the stress tolerance of the exogenous gene.

Alkali is one of the common adverse environmental conditions in the world; therefore, study of plant adaptation to alkali stress is an important aspect for plant stress research. However, there is still no report on the GST genes being tolerant to alkali stress. In the present study, our results showed that the *LbGST1* transformed yeast cell grew better than the yeast harboring empty pYES2 under NaHCO_3 stress condition (Figure 3B), indicating that the *LbGST1* is tolerant to alkali stress. Therefore, the *LbGST1* play a role in alkali stress tolerance in *L. bicolor*.

Drought is a common environmental factor limiting crop

production globally. Cloning and identification of drought tolerant genes are important for agriculture production. Previous study (George et al., 2009) showed that overexpression of a GST gene from *Prosopis juliflora* (*PjGSTU1*), confer high drought tolerance to transgenic tobacco, suggesting that it is tolerant to drought stress. Consistent with this, our result also showed that the *LbGST1* transformed yeast showed elevated drought tolerance compared with control yeast under drought stress (Figure 3C), indicating that the *LbGST1* is tolerant to drought stress. Combined with the fact that *LbGST1* was highly induced in leaves of *L. bicolor* by drought stress, these results suggested that the *LbGST1* play a role in drought tolerance mechanism of *L. bicolor*.

Thermal stress is one of the environmental factors that plants usually encounter. In the present study, we investigate thermal tolerance of the *LbGST1*. Our result showed that the transgenic yeast expressing *LbGST1* grew better than control yeast under thermal stress (Figure 3D), suggesting that the *LbGST1* was also tolerant to thermal stress. Consistent with this result, Roxas et al. (2000) transformed a tobacco GST gene into tobacco plants. Compared with wild type (WT) tobacco, the transgenic plants overexpression of this GST gene improved the growth and caused inhibited levels of lipid peroxidation under thermal stress condition. These results indicated that GST genes are involved in thermal tolerance of plants.

Previous study showed that GST genes play a role in low temperature tolerance. For instance, a gene encoding an enzyme with both GST and glutathione peroxidase (GPX) activity was introduced into tobacco, and the tobacco overexpressing GST grew faster than WT seedlings when exposed to chilling stress. In this study, our result showed that the yeast cells expressing *LbGST1* grew better than the control yeast under freezing stress condition (-20°C) (Figure 3E), indicating that the *LbGST1* is tolerant to freezing stress.

UV radiation can cause plant serious damage and affect plant growth and development. UV radiation also gradually stops growth and sprouting of seed. Therefore, tolerance to ultraviolet radiation is important for plants. Liu and Li, (2002) isolated a UV inducible GST gene from *Arabidopsis* and transformed it into *Arabidopsis thaliana*. Their results revealed that overexpression of this UV inducible GST gene could increase tolerance of the transgenic plants to UV radiation. Consistent with this

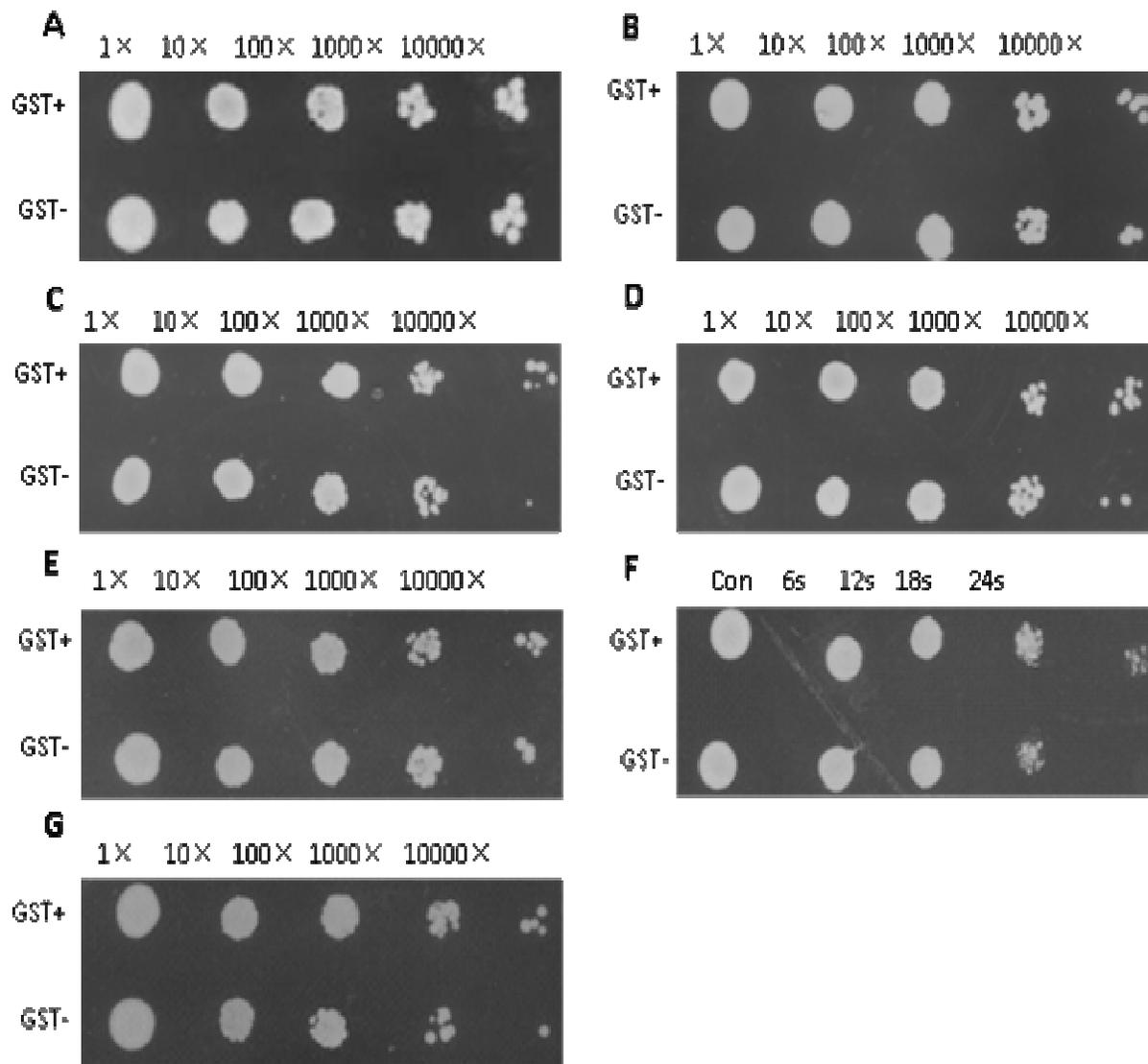


Figure 3. Stress tolerance analysis of the *LbGST1* gene in transgenic yeast cells. A: yeast cells without any stress treatment. B: NaHCO_3 tolerance analysis, yeast cells were treated with 10% (w/v) NaHCO_3 solution at 30°C for 8 h. C: Drought tolerance analysis, yeast cells were treated with 8 mol/l sorbitol solution for 24 h. D: Thermal tolerance analysis, yeast cells were incubated at 53°C for 1 h. E: Freezing tolerance analysis, yeast cells were placed in a water bath for 24 h at -20°C . F: Ultraviolet radiation resistance analysis, yeast cells were dotted on SC-Ura medium plate, then exposed to ultraviolet radiation (254 nm, $100 \mu\text{J}/\text{cm}^2$) for 6, 12, 18 and 24 s, respectively. G: NaCl tolerance analysis, yeast cells were incubated in 5 mol/l NaCl solution for 24 h at 4°C . GST+, *LbGST1* transformed yeast cells; GST-, yeast transformed with the empty pYES2; Con, untreated yeast cells; 1, 10, 100, 1000 and 10000x: cells were diluted by 10-, 100-, 1000- and 10000-fold and dotted.

result, our study also showed that the transgenic yeast expressing *LbGST1* displayed increased UV radiation tolerance compared with control yeast cells (Figure 3F). These results suggested that the *LbGST1* gene is involved in UV radiation resistance mechanism.

Salt stress is one of the major environmental stress that limit crop production worldwide. In the present study, we studied salt tolerance of *LbGST1*. The result revealed that the *LbGST1* transgenic yeast exhibited improved salt tolerance compared with control yeast (Figure 3G),

indicating that the *LbGST1* is tolerant to salt stress. Similarly, previous study also showed that the tobacco overexpressing GST could reduce oxidative damage compared with WT plants under salt stress condition, showing a role in plant salt tolerance (Roxas et al., 2000). These results suggested that *LbGST1* play a role in salt tolerance of *L. bicolor*.

In summary, our studies indicate that the *LbGST1* gene was tolerant to various abiotic stresses, suggesting that the *LbGST1* gene may contribute to the high ability of

adapting to stressful environments of *L. bicolor*. This study may be helpful in understanding the role of *GST* genes in abiotic stress, and has implications for the genetic engineering of plants with enhanced stress tolerance.

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