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Full Length Research Paper

Cloning and expression of a tomato glutathione S- transferase (GST) in *Escherichia coli*

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Glutathione S- transferases (GSTs) fulfill a diverse range of functions in an organism. In a previous study, a putative glutathione S-transferase gene (*ShGSTU1*) from a wild-type tomato, *Solanum habrochaites* G1.1560, was identified to be a key gene in pathogen resistant response against powdery mildew in tomato. In this study, *ShGSTU1* was cloned into plasmid pET-28a, efficiently expressed in *Escherichia coli* upon isopropyl-β-D-1-thiogalactopyronoside (IPTG) induction, purified with Ni^{2+} affinity chromatography and biochemically characterized. The results show that the optimal conditions for the expression of recombinant ShGSTU1 in *E. coli* were growth under 37°C, and 4-h IPTG induction with 1 mM concentration. About 18.93 mg ShGSTU1 was recovered from 1 g wet bacteria. The recombinant ShGSTU1 exhibited enzymatic activity with specific activity 0.625 U/mg. These results might provide a significant foundation for the later research on the mechanism of ShGSTU1 in tomato resistance to powdery mildew.

Key words: Tomato, glutathione S-transferase, expression, purification, enzyme activity.

INTRODUCTION

Glutathione S- transferases (GSTs, EC 2.5.1.18) are soluble proteins, each of which is composed of two polypeptide subunits. Classically, GSTs catalyze the transfer of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) into a co-substrate state with a polar S- glutathionylated conjugate. GSTs in plants can be divided on the basis of sequence identity into the phi (GSTF), tau (GSTU), theta (GSTT), zeta (GSTZ), lambda (GSTL) and dehydroascorbate reductases (DHARs) classes, and most of plant GSTs belong to the phi and tau classes (Ghelfi et al., 2011; Chronopoulou et al., 2011a). GSTs have been evoked as a cellular-protection system against the toxic effects of GSH; this process is thought to be a key step and its product plays roles in biocatalysis and vacuolar import in plants (Hayes and Pulford, 1995; Kilili et al., 2004). GSTs also have roles in detoxifying oxidative-stress metabolites (Grotao et al., 2005; Monteiro et al., 2011), and essential roles in leukotriene biosynthesis (Sheehan et al., 2001). Moreover, GSTs are activated by stress tolerances including herbicide application (Edwards et al., 2000; Martins et al., 2011), pathogen attack (Mauch and Dudler, 1993; Dean et al., 2005), salt (Jha et al., 2011), dehydration (Bianchi et al., 2002; Ji et al., 2010), chilling (Lo Piero et al., 2005) and other stresses.

Tomato powdery mildew caused by *Odium neolycopersici* is a worldwide fungal disease, which can cause decrease in tomato production and quality. In a previous study of tomato and *O. neolycopersici* interaction, 887 diffferentially expressed transcript derived fragments (DE-TDFs) were obtained using a cDNA-amplified fragment length polymorphism method. Among them, 230 DE-TDFs were sequenced and some DE-TDFs were determined as candidate sequences of key

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Abbreviations: GSTs, Glutathione S- transferases; **IPTG,** isopropyl-β-D-1-thiogalactopyronoside.

genes in resistance response (Li et al., 2006, 2007, 2012). One candidate sequence, which was annotated to a putative GST, was upregulated by powdery mildew during resistance response. Virus induced gene silencing (VIGS) was used to analyze function of the candidate sequence, which was found to be required in *Ol-1* mediated tomato (*Solanum habrochaites* G1.1560) resistance to powdery mildew, demostrating it is a key gene of tomato resistance to *O. neolycopersici* (Pei et al., 2011). The code region sequence of the putative GST gene from *S. habrochaites* G1.1560 was obtained with genomic walking method based on the tomato whole genome sequence. The sequence was uploaded to GenBank with accession no. JF957860 (GenBank ID: JF957860). According to the nomenclature of GSTs (Edwards and Dixon, 2000), the putative GST gene was named *ShGSTU1*.

From a consideration of the way in which GSTs have adapted to fulfill a diverse range of functions, it is of interest to study the enzyme chemistry of the GSTs. In this paper, *ShGSTU1* gene was expressed in *Escherichia coli*. Purification and enzymatic activity analysis were further conducted, which will be of benefit in the functional and stuctructal analyses of this key gene in tomato resistance to *O. neolycopersici* and also provide useful insights into the role of *ShGSTU1* in pathogen attack.

MATERIALS AND METHODS

T4-DNA ligase, restriction endonucleases, polymerase chain reaction (PCR) purification kit and plasmid purification kit, RNAiso reagent and M-MLV RTase cDNA synthesis kit were obtained from TaKaRa (Dalian, China). A pET expression kit, including expression vector pET-28a, *E. coli* strains BL21 (DE3) and Ni–NTA His·Bind resin, were purchased from Novagen. Glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma. All other chemicals were of analytical grade.

Gene amplification

Total RNA was extracted from tomato leaves using RNAiso reagent. First-strand cDNA was synthesized using 1 µg total RNA and an M-MLV RTase cDNA Synthesis kit according to the manufacturer's protocol. The complete coding region of tomato *ShGSTU1* gene was amplified with primers: (forward) 5′-CGGGATCCCGAAAAATTGAGAACCA-3′ (B*am*HI site underlined) and (reverse) 5′-GCGTCGACTTGCCACACAAAATCTT-3′ (S*al*I site underlined) by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed by 1 cycle of 5 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 52°C and 1 min at 72°C, followed by 10 min at 72°C. The PCR products were purified with PCR purification kit under the manufacturer's protocol and a small aliquot was analyzed on 1% agarose gel electrophoresis.

Construction of expression vector

Both PCR product and pET-28a expression vector were digested with B*am*HI and S*al*I separately. Digested DNAs were purified by a PCR purification kit. The recovered DNA fragments were ligated in 20 µL solution containing 400 ng DNA fragments and equal moles of pET-28a, 66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 mM ATP and 5 U T4-DNA ligase at 16°C overnight to construct expression vector pET-ShGSTU1. The competent *E. coli* strain DH5α cells were transformed with the ligated mixtures. Single colony on the plates was inoculated in liquid Luria-Bertani (LB) media containing 100 µg/ml kanamycin. Expression vectors were isolated from the cultured cells and confirmed by DNA sequencing.

Expression of recombinant ShGSTU1

Expression vector (pET-ShGSTU1) was used to transform the competent *E. coli* strain BL21 (DE3) using standard $CaCl_2$ method. Single colony was inoculated in 10 ml LB (1% tryptone, 0.5% yeast extract, 0.8% NaCl, pH 7.0 to 7.5) medium containing 100 µg/ml kanamycin, and cultured overnight at 37°C with shaking (220 rpm). The overnight culture was diluted to 1 L fresh LB medium containing 100 µg/ml kanamycin and left to grow until 0.6 to 0.8 OD_{600}. Then 1 mM isopropyl-β-D-1-thiogalactopyronoside (IPTG) was added to induce pET-ShGSTU1 expression at 37°C for 4 h with shaking. Induced bacteria were harvested by centrifugation at 5000 g for 10 min at 4°C, and then the pellets were used directly for protein purification or stored at -20°C for future use.

Optimal conditions for the expression of recombinant ShGSTU1

To optimize the growth condition for the recombinant *E. coli* cells, growth temperature, concentration of IPTG and induction time were investigated. The gradients of growth temperatures were 25, 30, 37 and 42°C, IPTG concentrations were 0.02, 0.1, 1.0 and 2.0 mM, and the induction time periods were 2, 3, 4 and 5 h, respectively.

Denaturing Ni-NTA purification and re-folding

Induced bacteria of one liter LB medium were harvested by centrifugation at 5000 g for 10 min. The pellets were dissolved with 25 ml of 10 mM Tris-HCl, pH 8.0, 100 mM NaH_2PO_4, 300 mM NaCl and 6 M guanidine hydrochloride (GuHCl) for 50 min. The lysed mixture was centrifuged at 12000 g for 20 min, and the collected supernatant was loaded on to a 10 ml Ni-NTA column pre-equilibrated with 10 mM Tris-HCl, pH 8.0, 100 mM NaH_2PO_4, 300 mM NaCl and 6 M urea. The column was washed with 100 ml of 10 mM Tris-HCl, pH 8.0, 100 mM NaH_2PO_4, 300 mM NaCl and 6 M urea until no discharged protein was washed out from the column. The recombinant ShGSTU1 was then eluted with 20 ml buffer including 10 mM Tris-HCl, pH 8.0, 100 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole and 8 M urea. The flow velocity was at about 1 ml/min, and the eluted solutions were collected with individual collection tubes. Furthermore, the eluted solutions in collection tubes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, the protein-enriched eluted solutions were converged into a collection tube, and were renatured by dialyzing against 1000 ml buffer, including 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 5% glycerol and 5 mM β-ME for 16 h at 4°C with the gradient of urea from 4 M urea, 2 M urea to 0 M urea respectively. Then the renatured ShGSTU1 was dialyzed against storage buffer (20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT and 50% glycerol), and stored in small aliquots at -20°C.

Activity assay

ShGSTU1 activity was determined according to the method of

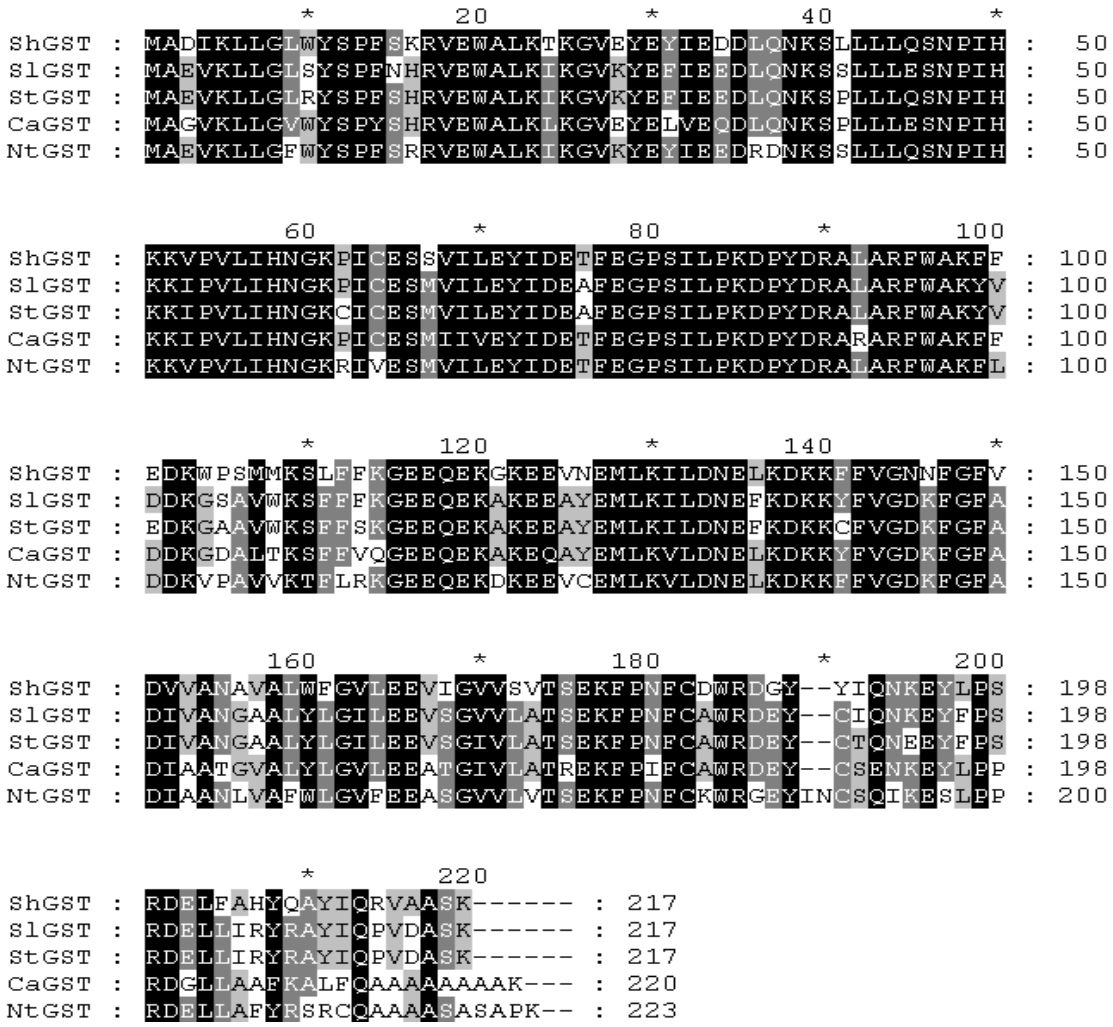

Figure 1. The amino acid sequences alignment of GSTs from different plants. ShGST, SlGST, StGST, CaGST and NtGST stand for the GST from *Solanum habrochaites* (GenBank ID: JF957860), *Solanum lycopersicum* (GeneBank ID: AK328577), *Solanum tuberosum* (GeneBank ID: AAA68430), *Capsicum annuum* (GeneBank ID: ACN60408) and *Nicotiana tabacum* (GeneBank ID: CAA39709), respectively.

Habig et al. (1974) with some modifications. ShGSTU1 was incubated with 100 mM K_2HPO_4-KH_2PO_4, pH 6.5, 1 mM CDNB and 2 mM GSH (reduced glutathione) for 1 min at 25°C, then the formation of GSH conjugate of CDNB was measured at 340 nm using spectrophotometer. One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 µmole product (extinction coefficient 9.6 mM^{-1} cm^{-1}). Specific activity is defined as the units of enzyme activity per mg of protein as measured with bovine serum albumin as standard. Protein concentrations were determined by the method of Bradford based on a standard curve with bovine serum albumin (Bradford, 1976).

RESULTS AND DISCUSSION

S. habrochaites GST

The best studied groups of tau GSTs seem to be involved in responses to different environmental stresses. The GST gene amplified from *S. habrochaites* involved in tomato resistance to powdery mildew was annotated to a putative tau class GST, which was named *ShGSTU1* (GeneBank ID: JF957860). The open reading frame (ORF) of the 651-bp cDNA encoded a polypeptide of 217 amino acids.

Amino acid alignment of ShGSTU1 with GSTs from other different plants *S. lycopersicum* (GeneBank ID: AK328577), *S. tuberosum* (GeneBank ID: AAA68430), *Capsicum annuum* (GeneBank ID: ACN60408) and *Nicotiana tabacum* (GeneBank ID: CAA39709) produced using GENEDOC software, the results show a high conservatism between ShGSTU1 and other plants GSTs (Figure 1).

Construction of recombinant ShGSTU1

ShGSTU1 was successfully amplified and ligated with

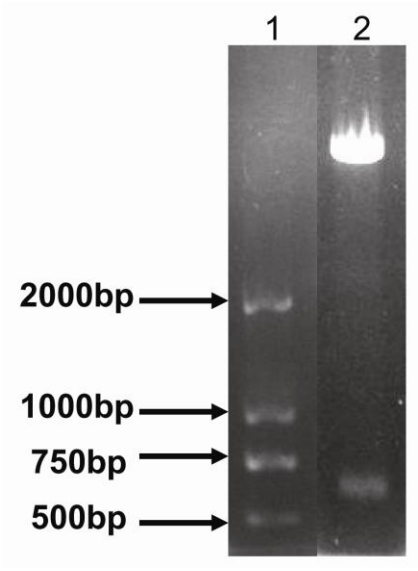

Figure 2. Restriction enzyme digestion identification of recombinant pET-ShGSTU1 vector. Lane 1, DL 2000 marker; lane 2, pET-ShGSTU1 vector digested by B*am*H/*Sal*I.

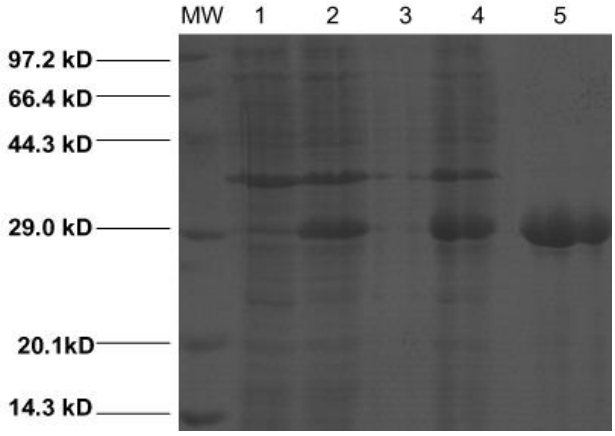

Figure 3. SDS-PAGE assay of different extracts and purified protein. MW, Low molecular weight protein marker; lane 1, un-induced total cell extracts carrying pET-ShGSTU1; lane 2, induced total cell extracts carrying pET-ShGSTU1; lane 3, ultrasonication supernatant of induced total soluble extracts; lane 4, ultrasonication precipitation of induced total dissoluble extracts; lane 5, purified ShGSTU1 with denaturing Ni-NTA.

pET-28a. The length of amplified gene was 734 bp, which was finally confirmed by digestion with double restriction endonucleases (Figure 2) and sequencing (data not shown).

Expression of recombinant ShGSTU1

The recombinant vector was expressed in *E. coli* BL21 (DE3) as previously described. Following the incubation, the reaction mixture was analyzed by SDS-PAGE, which revealed an accumulation of polypeptides with a molecular mass about 25 kDa, coinciding with predicted mass value 25298.06 Da based on gene sequence. The soluble fraction and dissoluble fraction were prepared through cell disruption with ultrasonication. As showed in the Figure 3, the expression level in dissoluble extracts was much higher than soluble extracts because most of the produced fusion proteins were in the insoluble form.

Optimal conditions for the expression of recombinant ShGSTU1

It was observed that the growth of the recombinant *E. coli* which grew under 37°C after 1 mM IPTG induction for 4 h resulted in the best ShGSTU1 expression compared with other conditions (Figure 4). Therefore, 4-h IPTG induction with 1 mM concentration, and growth under 37°C were the optimal conditions for expression of recombinant ShGSTU1.

Purification of recombinant ShGSTU1

The cells of 1 L culture grown under optimal growth and induction conditions were collected by centrifugation. The obtained cell pellets were dissolved with GuHCl. After centrifugation, the supernatant was loaded on a Ni-NTA column. The bound fusion proteins were eluted with 250 mM Imidazole solution and examined by SDS-PAGE (Figure 3). Although proteins recovered by denaturing conditions were inactive, renaturing by dialysis against decreasing concentration of urea could generate active proteins. As summarized in Table 1, using one step Ni-NTA column chromatography with denaturing protocol, we finally obtained about 18.93 mg of the purified ShGSTU1 from 1 g of wet induced bacteria. The specific activity of purified ShGSTU1 was 0.625 U/mg, which was in the range of 0.07 to 9.22 U/mg (nmol•mg^{-1}•min^{-1}) of tau class LeGSTUs reported by Kilili et al. (2004).

Characterization of recombinant ShGSTU1

The optimal pH for conjugation varies among the tested substrates and among some of the transferases acting on the same substrate (Habig et al., 1974). The optimum pH of ShGSTU1 for the conjugation of GSH with CDNB (for the catalytic reaction) was pH 6.5 to 7.5 (Figure 5). This was similar to the broad optimum pH found with transferase and 1,2-dichloro-4-nitrobenzene (Habig et al., 1974; Pabst et al., 1974). Thermal stability of ShGSTU1 was analyzed, showing that the optimal temperature of purified ShGSTU1 for the catalytic reaction was 30°C (Figure 6).

Plant GSTs are involved in protecting plants against both

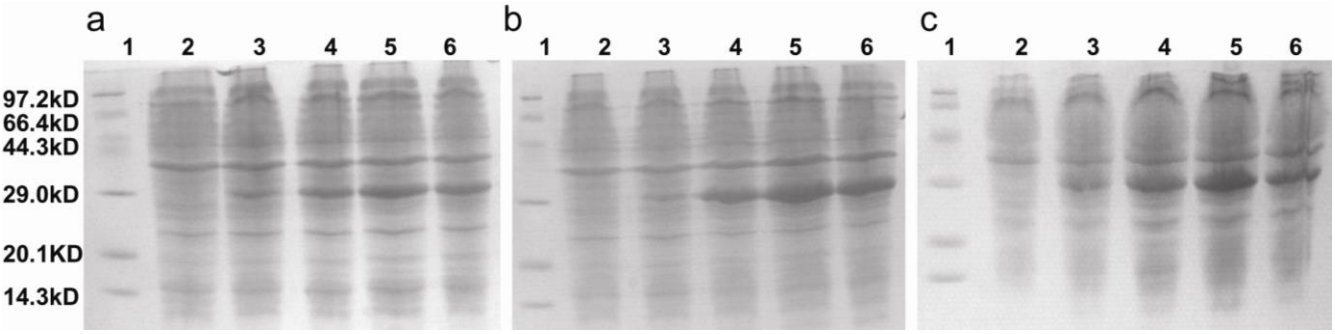

Figure 4. The optimal growth conditions for the expression of ShGSTU1 in *E. coli* BL21(DE3). (a) Different induction time of the expression of ShGSTU1. Lane 1, Molecular weight makers; lane 2, un-induced; lanes 3 to 6: 2, 3, 4 and 5 h after induction. (b) Different concentrations of IPTG of the expression of ShGSTU1. Lane 1, Molecular weight makers; lane 2, un-induced; lanes 3 to 6: 0.02, 0.1, 1.0 and 2.0 mM of IPTG. (c) Different induction temperature of the expression of ShGSTU1. Lane 1, Molecular weight makers; lane 2, un-induced; lanes 3 to 6: 25, 30, 37 and 42°C induction temperature.

Table 1. ShGSTU1 purified from 1 g of wet induced bacteria.

Step	Total protein (mg)	ShGSTU1 (mg)	Purity (%)	Yield (%)
Lysate	38.65	30.53	79	100
After Ni-NTA	20.15	18.93	94	62

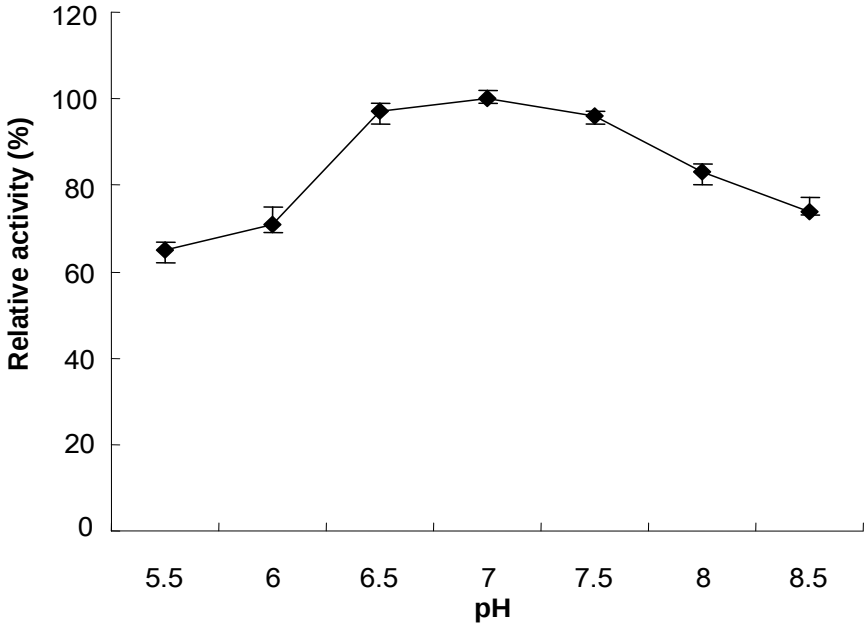

Figure 5. Effects of pH on the ShGSTU1 activity.

diverse biotic and abiotic stresses, including the following: herbicide detoxifying in *Phaseolus vulgaris* (Chronopoulou et al., 2011b), drought and salt tolerance in soybean (Ji et al., 2010) and various stresses in rice (Jain et al., 2010). So far, a large number of stress responsive GST genes have been cloned and identified from various plant species, which are important to understand the mechanisms of stress tolerance at molecular levels and produce novel germplasm with improved stress tolerance by genetic engineering.

In our previous study, a tau class GST named *ShGSTU1* from *S. habrochaites* G1.1560 was cloned,

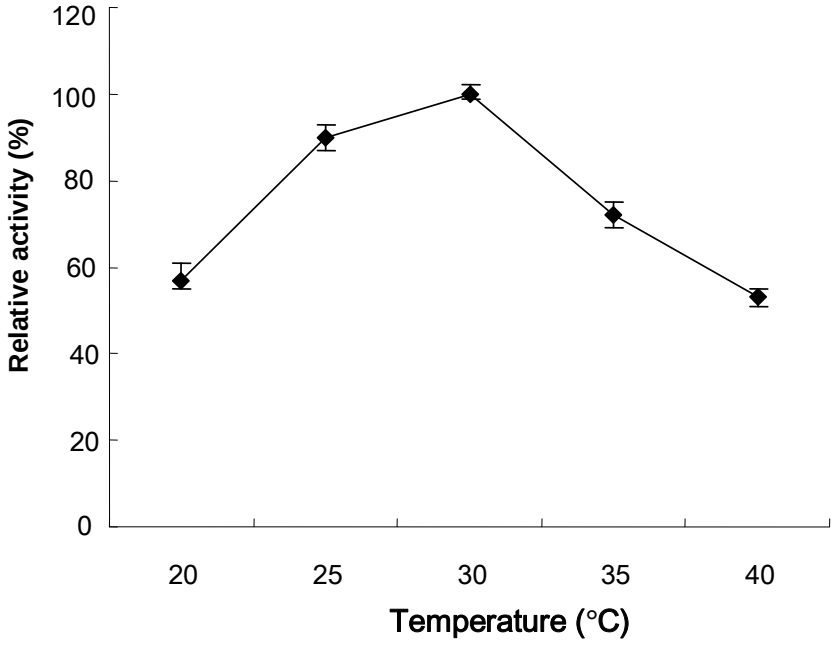

Figure 6. Effects of temperature on the ShGSTU1 activity.

which is required for resistance against *O. neolycopersici* in tomato associated with hypersensitive response (Pei et al., 2011). In order to identify functional characteristics of *ShGSTU1* related to resistance, the gene was successfully expressed in *E. coli* and purified by one step Ni-NTA column chromatography with denaturing protocol. The recovered recombinant protein *in vitro* was active with specific activity 0.625 U/mg, suggesting that the cloned *ShGSTU1* is a gene coding protein with GST function. The cloned and expressed ShGSTU1 not only provided a prerequisite for further biochemical, structural and functional study, but can also be a potential gene resource for genetic engineering to improve crop stress tolerance.

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