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*Full Length Research Paper*

# Cloning of a gene encoding glycosyltransferase from *Pueraria lobata* (Wild.) Ohwi and its expression in *Pichia pastoris*

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**The key enzyme of puerarin biosynthesis in *Pueraria lobata* (Willd.) Ohwi was unclear but may involve glycosylation. To investigate the regulation of puerarin biosynthesis, a putative UDP-dependent glycosyltransferase (UGT) gene, PlUGT1 was isolated from *P. lobata* root, which contained abundant puerarin. PlUGT1 encoded 480 deduced amino acid residues with a conserved UDP-glucose-binding domain, which has 61 to 84% similarity to homologues from other plant species. SDS polyacrylamide gel electrophoresis and western blotting results showed that, fusion protein migrated as a single protein band with a molecular weight of 55 kDa. A yeast expression vector pPICZA-PlUGT1 was constructed and was transformed into *Pichia pastoris* strain GS115. Several recombinants containing multi-copy expression cassettes were obtained on the zeocin-YPD plate and confirmed by southern dot blotting. The yield of PlUGT1 attained 0.05 g/l when recombinant cells were cultured at pH 5.5, 30°C and induced with 0.5% methanol for 72 h. The expression of PlUGT1 protein correlates positively with the copy numbers of PlUGT1 in transformed yeast cells. These results suggest that, the PlUGT1 protein can be expressed efficiently in the *P. pastoris* expression system and may supply a new economic and convenient way for the production of PlUGT1 protein.**

**Key words:** *Pueraria lobata* (Willd.) Ohwi, glycosyltransferase, cloning, expression, *Pichia pastoris.*

## INTRODUCTION

*Pueraria lobata* (Willd.) Ohwi (Fabaceae) is one of the oldest medicinal plants in traditional Chinese medicine. Kudzu roots (Radix pueraria from *P. lobota*), called Gegen in Chinese, is a rich source of isoflavone glucosides and has recently become commercially available in Western dietary supplements that have been marketed primarily for women health. The studies on pharmacology and clinical practice have shown that the active constituents in the extract are isoflavones. Puerarin (daidzein 8-C-glucoside), daidzin (daidzein 7-O-glucoside) and daidzein are the main components, of which puerarin is the predominant constituent (Harada and Ueno, 1975; Chen et al., 2001). The latter has been shown, experimentally and clinically to have extensive pharmacological action, for treatment of diabetes (Hsu et al., 2003), metabolic syndrome (Peng et al., 2009) and cardiovascular diseases (CVD), for example, hypertension (Song et al., 1988), atherosclerosis (Xiao et al., 2004), dyslipidemia (Duan et al., 2000) and myocardial infarction. The Kudzu root extract could also be an interesting candidate as an alternative for hormone replacement therapy (Zhang et al., 2005).

Puerarin and daidzin are both glucosides that may be synthesized by glucosylation of aglycones. Indeed, all reactions involving the transfer of a UDP-activated sugar moiety onto plant secondary metabolites are catalyzed by a ubiquitous family of enzymes called glycosyltransferases (GTs). These enzymes are encoded by large multigene families sometimes comprising several hundred genes (Gachon et al., 2005). According to the CAZY database

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(http://www.cazy.org/GlycosylTransferases.html), GTs can be classified into 92 families using nucleotide diphospho-sugar, nucleotide monophospho-sugars and sugar phosphates (EC 2.4.1.x) (Campbell et al., 1997; Coutinho et al., 2003). Family 1 GTs use uridine 5'-diphospho sugars as the sugar donors. Plants contain a number of UDP glycosyltransferases (UGTs) (Jones and Vogt, 2001). UGTs are so diverse that *Arabidopsis* has at least 120 UGTs, half of which are theoretically involved in plant secondary metabolism (*Arabidopsis* Genome Initiative 2000; Li et al., 2001; Ross et al., 2001); 165 have been identified in *Medicago truncatula* (Paquette et al., 2003; Achnine et al., 2005) and a total of 193 were found in rice (Ko et al., 2006).

UDP glycosyltransferases in higher plants are defined by the presence of a carboxy-terminal consensus sequence. The UGTs signature was thought to be the binding site of the nucleotide-activated sugar. Recently, the three-dimensional crystal structures of three members of plant family 1 GTs were determined. These plant UGTs crystal structures reveal the detailed interactions between the enzyme and its donor substrate as well as the roles of the UGT signature motif in substrate recognition and the catalytic activity of the enzyme (Li et al., 2007; Offen et al., 2006; Shao et al., 2005). A plant secondary product GT consensus sequence (called PSPG) motif was proposed for GTs involved in secondary metabolism. This motif was later found in all cloned secondary metabolism UGTs from plant species (Gachon et al., 2005).

Many reports concentrated on increasing puerarin production by cell culture or hairy root culture (Liu et al., 2002; Li and Zhang, 2006; Chen and Li, 2007) and pharmacological effects (Jiang, 2006), but few studies have been done on the biosynthetic pathway of puerarin, as well as the enzyme regulation of its metabolic processes. Inoue and Fujita (1974, 1977) reported that, the conversion from carboxyl-$^{14}$C labeled isoliquiritigenin to puerarin is more effective than the conversion from liquiritigenin. Biosynthesis of puerarin might be completed through *C*-glycosyltransferase, which transfers a sugar moiety to isoliquiritigenin with formation of a *C*-glycosidic linkage. *C*-glycosyltransferase may be one of the key enzymes in the biosynthesis of puerarin. Our previous study has demonstrated that, the *C*-glycosyltransferase activity could be detected in the enzyme extracted from Gegen (Chen et al., 2010). We have been interested in flavonoid metabolism in *P. lobata* and an *in vitro* characterization of flavonoid UGTs would be important before proceeding with *in vivo* study.

The methylotrophic yeast *Pichia pastoris* has been researched as an efficient host for the production of foreign proteins (Cregg et al., 1987). This organism has the potential for high expression levels (Sreekrishna et al., 1989; Hou et al., 2007), efficient secretion (Tschopp et al., 1987) and can grow to very high cell densities (>130 g dry cell weight per liter) (Cereghino and Cregg, 2000). We investigated the expression of PlUGT1, cloned from *P. lobata* and attempted to determine its potentials for large-scale PlUGT1 production.

## MATERIALS AND METHODS

### Plant material

*P. lobata* (Willd.) Ohwi was collected from Shukeng village in the mountainside in Meizhou city of Guangdong Province in China. Roots, stems and leaves were all immediately frozen in liquid $N_2$.

### Chemicals, enzymes and bacterial strains

Zeocin was purchased from Whiga (Guangzhou, China). The protein marker, Anti-Myc antibody and goat anti-mouse IgG-HRP were purchased from Tiangen Biotech (Beijing, China). Enzymes (*Taq*DNA polymerase, restriction endonucleases), 5'-Full RACE Kit, agarose gel DNA purification kit version 2.0 and DNA ligation kit version 2.1 were purchased from TaKaRa (Dalian, China). The methylotrophic yeast *P. pastoris* strain GS115 (His$^-$) and plasmid pPICZA were purchased from Invitogen (Carlsbad, CA, USA). *Escherichia coli* (*E. coli*) strain DH5α was used for transformation and propagation of the recombinant plasmids. All other materials are analytical grades and were purchased from the Sangon Bioengineering CO., Ltd. (Shanghai, China).

### Reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

For amplification of a specific GT fragment from *P. lobata*, degenerate primers (DF1, 5'-TTC/T GTA/C/G/T ACI CAC/T TGC/T GGI TGG AA-3'; DR2, 5'-TCC ATA/C/G/T AGI CTI CGI CAA/C/G/T GCC/T TTC/T TCA/G/T AT-3') were designed based on the conserved regions of PSPG box of the corresponding genes from other plants. Total RNAs were extracted from the root of *P. lobata* with Trizol reagent (Invitrogen, USA). cDNA was synthesized from total RNA (2 µg) using SuperScriptTM III RNase H$^-$ reverse transcriptase (Invitrogen) and an oligo dT18 primer. The cDNA was then used as template for PCR amplification using the degenerate primers. Conditions for RT were as follows: 65°C for 5 min, followed by 50°C for 50 min, then 70°C for 15 min. PCR amplification was performed as follows: 94°C for 5 min, 30 cycles of 94°C for 30 s, 47°C for 30 s and 72°C for 30 s and a final elongation step of 72°C for 10 min. Subsequent PCR fragment was sequenced and compared with the earlier-mentioned GTs sequences. After the product had been confirmed as a GTs homolog, its missing 5' and 3' ends were obtained by RACE-PCR performance using the 5'-Full RACE Kit and the GeneRacer kit (Invitrogen), respectively, according to the manufacturer's instructions (TaKaRa). The gene-specific primers for 5'-RACE were 5GSP1 (5'-TGC AGC ATG TAT CCA TCC AAA CAC GCC -3') (nested) and 5GSP2 (5'-AAC TGG TCC ACT AGC AGC TTC GCG TTC -3') (outer); the gene-specific primers for 3'-RACE were 3GSP1 (5'-AGT ACA CGA ACG CGA AGC TGC TAG -3') (outer) and 3GSP2 (5'-AGC TCG CAG AGG GAA TTG GAT GCG -3') (inner).

### Cloning, DNA sequencing and sequence analysis

In all cloning experiments, PCR fragments were gel-purified with an agarose gel DNA purification kit (TaKaRa) and were ligated into the pMD 18-T vector (TaKaRa). Plasmids were isolated and were sequenced from both strands. Sequence analysis was performed by using EditSeq software (DNASTAR). Computer analysis of the DNA and amino acid sequences was carried out using the BLAST program at the National Center for Biotechnology Information

Services. Multiple alignment of the amino acid sequences was carried out using the Clustal W program in DNASTAR software. A phylogenetic tree was constructed by the neighbor-joining algorithm using the DNAMAN software package, based on calculations from pairwise amino acid sequence distances for protein analyses derived from the multiple alignment formats. The data set was subjected to 1000 bootstrap replications.

**Construction of the *P. pastoris* expression vector pPICZA-PlUGT1**

A fragment containing the open reading frame was PCR amplified using the primers PlUGT1-F1 (5'-AAA GAA TTC ATG TCC ACC GCA AGA ACT C-3') and PlUGT1-R1 (5'-TTT TCT AGA CAA GAA GTG CTT TCC ACA C-3'), into which the restriction enzyme sites *Eco*R I (PlUGT1-F) and *Xba* I (PlUGT1-R) were introduced (underlined). The fragment including PlUGT1 cDNA digested with *Eco*R I and *Xba* I was sub cloned into the *Eco*R I and *Xba* I sites of the binary vector pPICZA driven by the AOX1 promoter that allows methanol-inducible and high-level expression in *P. pastoris*. The *Sh ble* gene encoding zeocin was used for the selection marker gene. Recombinant plasmids (pPICZA-PlUGT1) were identified by restriction analysis of purified plasmid DNA and used for sequencing.

**Transformation of *P. pastoris* and screening for multicopy transformants by zeocin selection**

Approximately 1 µg of linear (*Bst*X I-digested) pPICZA-PlUGT1 DNA was used for the transformation into *P. pastoris* GS115, via electroporation. Electroporation was essentially performed according to the method of Xu et al. (2006), with cells pulsed in 0.2 cm electroporation cuvettes at 1.5 KV using a BioRad MicroPulser with controller. One ml of cold 1 M sorbitol was then added to the cuvettes immediately after pulsing and the transformants were recovered on minimal dextrose (MD) agar (1.34% YNB, 4×10-5% biotin and 2% dextrose) plates (Rose et al., 1990). The plates were incubated for 3 to 5 days at 30°C. Single colonies from MD plate were carefully removed under sterile conditions and patched to YPD-zeocin medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar and 100 or 1000 or 4000 µg/ml zeocin) and incubated for 3 to 5 days at 30°C in order to select multicopy transformants. Some clones grew on 4000 µg/ml zeocin YPD plates, which indicated that, multicopy expression cassettes were inserted into the GS115 genomic DNA, and were selected to get an engineering strain, which would express fusion protein PlUGT1 at a high level.

**Southern dot blotting analysis**

A 100 ng DNA fragment of PlUGT1 was amplified from expression vector pPICZA-PlUGT1 with primers PlUGT1F and PlUGT1R and treated to become a DNA probe according to the kit instructions. Genomic DNA of the *P. pastoris* transformants after 48 h methanol induction was isolated by the method described previously. Then, southern dot blotting was performed according to the instructions of the Electrochemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection Systems Kit (Amersham, Uppsala, Sweden). Plasmid pPICZA-PlUGT1 and strain GS115/pPICZA, from GS115 transformed with the vector pPICZA, genomic DNA was used as positive and negative controls, respectively.

**Optimization of PlUGT1 protein expression conditions**

Some factors were tested, such as inductive duration time, methanol concentration, temperature and pH value. The optimum condition for PlUGT1 protein expression was achieved by single factor. Single colonies which contains multi-copy expression cassettes were inoculated in 25 ml of buffered glycerol-complex medium (BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4\times10^{-5}$% biotin and 1% glycerol) grown at 28°C in a shaking incubator (220 rpm) until culture reaches an optical density of 4 at 600 nm ($OD_{600}$). The cells were then pelleted and resuspended in 100 ml buffered methanol-complex medium (BMMY: 1% yeast extract, 2% peptone, 100mM potassium phosphate, pH 6.0, 1.34% YNB, $4\times10^{-5}$% biotin and 0.5% methanol) in a 500 ml shaking flask at 28°C and induced to express PlUGT1 protein under various conditions.

**The optimal induction methanol concentration**

*P. pastoris* cells were cultured at 100 ml BMMY induction medium with different methanol concentrations (v/v) (0.3, 0.5, and 1%) at 28°C. The same amount of methanol was added every 24 h. The fermentation broth was collected after induction for 96 h. The cells were harvest by centrifugation at 10000 g for 5 min at 4°C and the total intracellular protein was determined by the Bradford method (Bradford, 1976).

**The optimal induction time**

To determine this, *P. pastoris* cells were cultured as before, but induced with the optimal induction methanol concentration. 2 ml of the expression culture after induction for 0, 24, 48, 72 and 96 h was used to analyze protein expression levels and the optimal induction time was determined.

**The optimal culture temperature**

*P. pastoris* cells were cultured at different temperatures (26, 28 and 30°C) and induced with the optimal methanol concentration for the optimal induction time. Then, the protein concentration was monitored and the optimal culture temperature was obtained.

**The optimal pH value**

Different optimal initial pH value (4.5, 5.0, 5.5, 6.0 and 6.5) were also observed when cells grew at optimal methanol concentration, culture temperature and induction time. The other culture conditions were the same as before. Intracellular protein of cells in different fermentation broths was examined as before. All experiments were performed at least in triplicate.

**Protein purification**

*P. pastoris* cells were grown at the predetermined optimal conditions and were harvested by centrifugation at 10000 g for 5 min at 4°C and then washed once with deionized water after induction. Five ml ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol) was added to the cell pellets and resuspended. An equal volume of acid-washed glass beads (Sigma, 0.5 mm) compared to cell pellets was added and homogenization was carried out in eight 30 s bursts, separated by 30 s of cooling. The protein in the clear supernatant obtained by centrifugation at maximum speed for 10 min at 4°C was applied to an affinity Ni-NTA column. Purification of His-tagged PlUGT1 was performed according to the QIAexpressionist™ protein purification procedure and PlUGT1 fusion protein was desorbed

with 0.05 M sodium phosphate buffer containing each of the following concentrations of His (5, 10, 20, 400 mM ). The samples were stored at -20°C for SDS-PAGE and western blot analysis.

#### SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

SDS-PAGE was performed as described by Laemmli (1970) and Jung et al. (2007). The protein was stained with Coomassie Brilliant Blue R-250. After SDS-PAGE, the proteins in the gel were transferred electrophoretically to a membrane of polyvinylidene difluoride (PVDF, milipore, billerica, MA). Immunodetection was achieved by mouse monoclonal antibody against *c-myc* epitope as primary antibody and peroxidase-conjugated goat anti-mouse IgG as secondary antibody. Anti-Myc antibody was used at a 1:3000 dilution. Following incubation with the antibody, the filter membranes were washed three times with Tris-buffered saline (TBS) (20 mM Tris-HCl buffer, pH 7.6, 137 mM NaCl containing 0.1% Tween 20), followed by two more washes with the same buffer. The antigenantibody complex was detected with secondary antibody using an ECL western blotting analysis system (Amersham Pharmacia Biotech) and exposed on Kodak X-Omat AR film (Kodak, Rochester, NY, U.S.A.) for 20 min at room temperature.

#### Statistical analysis

The results were expressed as the mean ± S.D of data obtained from triplicate experiments. The statistical significance was estimated using a Student's *t*-test for unpaired observations. Values with $p < 0.05$ were considered significant.

## RESULTS

### Isolation and sequence analysis of the PlUGT1 cDNA

Several plant UGTs were chosen to design nested primers for amplification of a specific GT fragment from *P. lobata* (Figure 1). Using the RACE method, a putative UGT cDNA was successfully isolated from *P. lobata* and termed as *P. lobata* UDP glycosyltransferase 1 (*PlUGT1*) (GeneBank accession No. EU889119). The full-length of *PlUGT1* cDNA is 1636 bp and contains a 52 bp 5' untranslated region (5'UTR), a 186 bp 3' UTR and a 1398 bp open reading frame (ORF), encoding a deduced protein of 465 amino acid residues with a predicted molecular mass of 52.2 kDa and pI 6.93 (data not shown). When compared with several other plant UGTs, deduced amino acid sequence of PlUGT1, it shows a considerable homology throughout the entire coding region, including the conserved UDP-binding domain of 44 amino acid residues, called PSPG-box, located in the C-terminal region (Figure 1) (Hundle et al., 1992). This region has 61 to 84% identity in amino acid sequences to previously reported glycosyltransferases, indicating that PlUGT1 retained the generic features of the glycosyltransferase family. Within this PSPG-box, a peptide sequence of HCGWNS was located at the 367$^{th}$ amino acid, which has been detected in 95% of all glycosyltransferases (Figure 1) (Vogt and Jones, 2000). The amino acid sequence of PlUGT1 revealed 45% identity with *Arabidopsis thaliana* UGT89B1 (Lim et al., 2002), 43% with *Stevia rebaudiana* UGT89B2 (Richman et al., 2005), 34% with *Nicotiana tabacum* TOGT1 (Fraissinet-Tachet et al., 1998), 32% with *A. thaliana* UGT73B3 and *Solanum lycopersicum* TWI1 (Lim et al., 2002; O'Donnell et al., 1998), 30% identity with *Beta vulgaris* UGT73A4 and *A. thaliana* UGT73B1 (Hughes et al., 1994; Isayenkova et al., 2006), 29% identity with *Rhodiola sachalinensis* UGT73B6 (Ma et al., 2007) and 26 to 27% identity with *M. truncatula* UGT71G1 and GT99D (Achnine et al., 2005; Modolo et al., 2007). Phylogenetic analysis of the UDP-glucosyltransferase genes from plants also showed that PlUGT1 is located adjacent to *Vigna angularis* AdGt-4, *O. sativa* Os01g0650400, *A. thaliana* UGT89B1 and *S. rebaudiana* UGT89B2 (Figure 2).

When the genomic polymerase chain reaction (PCR) fragment was amplified by using the primer pairs PlUGT1-F1 (5′ -ATG TCC ACC GCA AGA ACT CAC GTG CTC -3′) and PlUGT1-R1 (5′ CT AAG TTA GTG CAG CAT GTA TCC ATC -3′), which were synthesized based on the N and C terminals of the *PlUGT1* coding region, the generated fragments were identical in length to the coding sequence of *PlUGT1*. This indicated that, the *P. lobata PlUGT1* lacked introns in the genome (data not shown).

### Confirmation of multi-copy transformants

The methylotrophic yeast *P. pastoris* and the integrating vector pPICZA were selected as an expression system to produce a biologically active glycosyltransferase enzyme. The PCR fragment encoding the mature PlUGT1 protein was cloned into the pPICZA vector to yield the expression plasmid pPICZA-PlUGT1 (Figure 3) and confirmed by restriction digestion and sequenced. The pPICZA-PlUGT1 plasmid was digested with *Bst*X I to generate a DNA fragment that was targeted for transplacement into the AOX1 locus of *P. pastoris*. Transformation by electroporation of the GS115 ($his^-$) strain with this DNA yielded approximately 103 $His^+$ transformants. A screening step to find the clones containing multicopy expression cassettes was performed on YPD plates containing each of the following concentrations of zeocin (100, 1000, 4000 µg/ml) (Figure 4a, b, c). Several zeocin resistance colonies were obtained on YPD plates containing 4000 µg/ml zeocin (Figure 4c). This indicates that, they contained multiple copies of the gene in the expression vector. In order to confirm these recombinants with high copies (HC) numbers gene, southern dot blotting was carried out. As shown in Figure 4d, ten *P. pastoris* engineering strain recombinants, named HC1 to HC10, displayed a positive hybridization signal, indicating that, the fusion gene was integrated into GS115 genomic DNA. The hybridization signal of HC4 was stronger than that of other strains and possibly the number of expression cassettes integrated into GS115 genomic DNA were more than that of other recombinants.

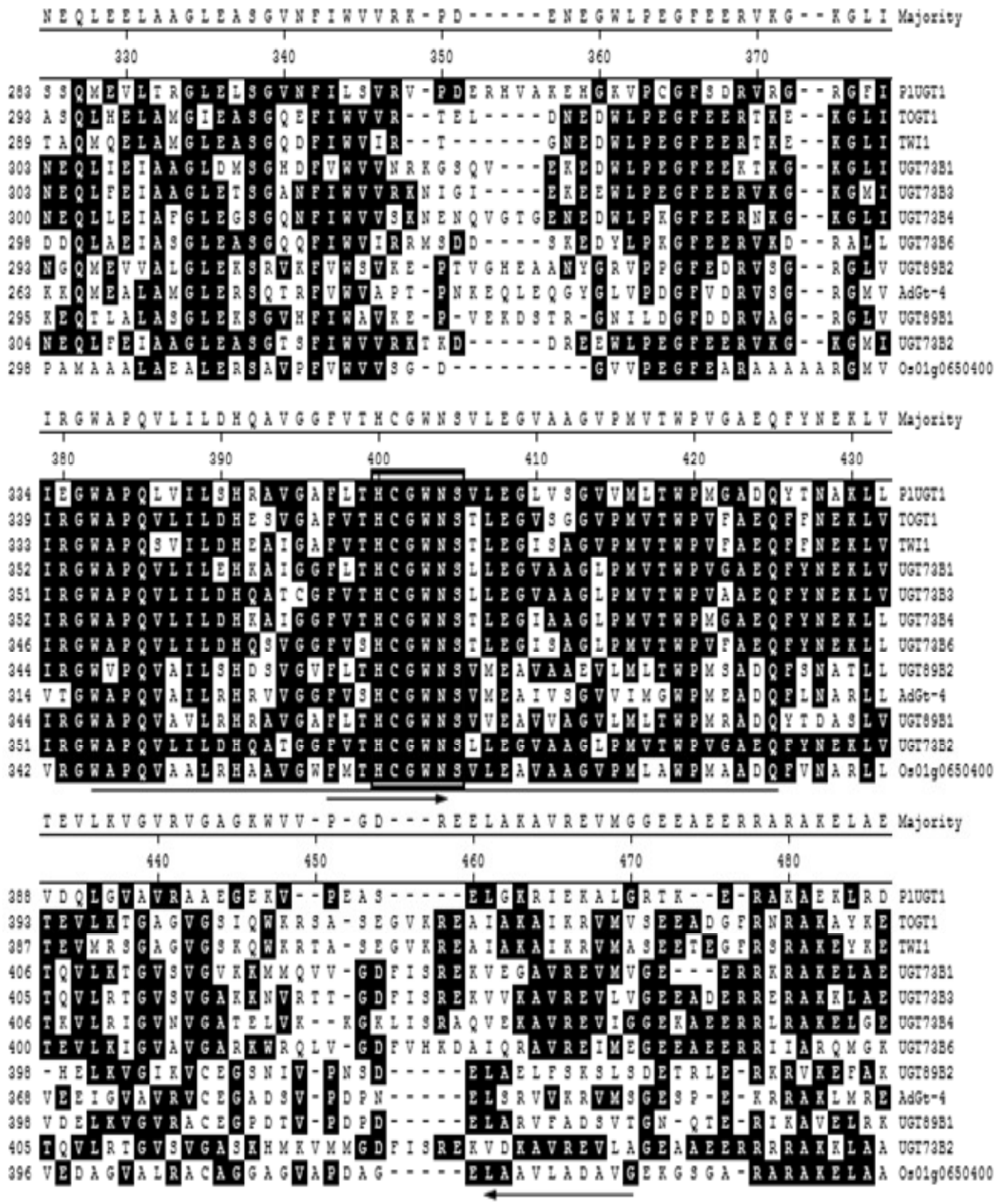


**Figure 1.** Amino acid sequence comparison of 12 glycosyltransferases of plant origin. Multiple sequence alignment was calculated with the DNAStar package. Black shading shows amino acid identities; underlining indicates the signature sequence (PSPG box) of glycosyltransferases. The conserved domain of UGT is boxed. Arrows indicate the primer sites used in this study for amplification of a specific GT fragment. The species and GenBank accession numbers of proteins are: PlUGT1 (*P. lobata,* ACJ72158), TOGT1 (*N. tabacum*, AF346431), TWI1 (*S. lycopersicum*, CAA59450), UGT73B1 (NP_567955), UGT73B2 (NP_567954), UGT73B3 (AL161584), UGT73B4 (NP_179151) and UGT89B1 (NP_177529) in *A. thaliana*, UGT73B6 (*R. sachalinensis*, AY547304), AdGt-4 (*V. angularis*, BAB86922), UGT89B2 (*S. rebaudiana*, AAR06921), Os01g0650400 (*O. sativa*, NP_001054090).

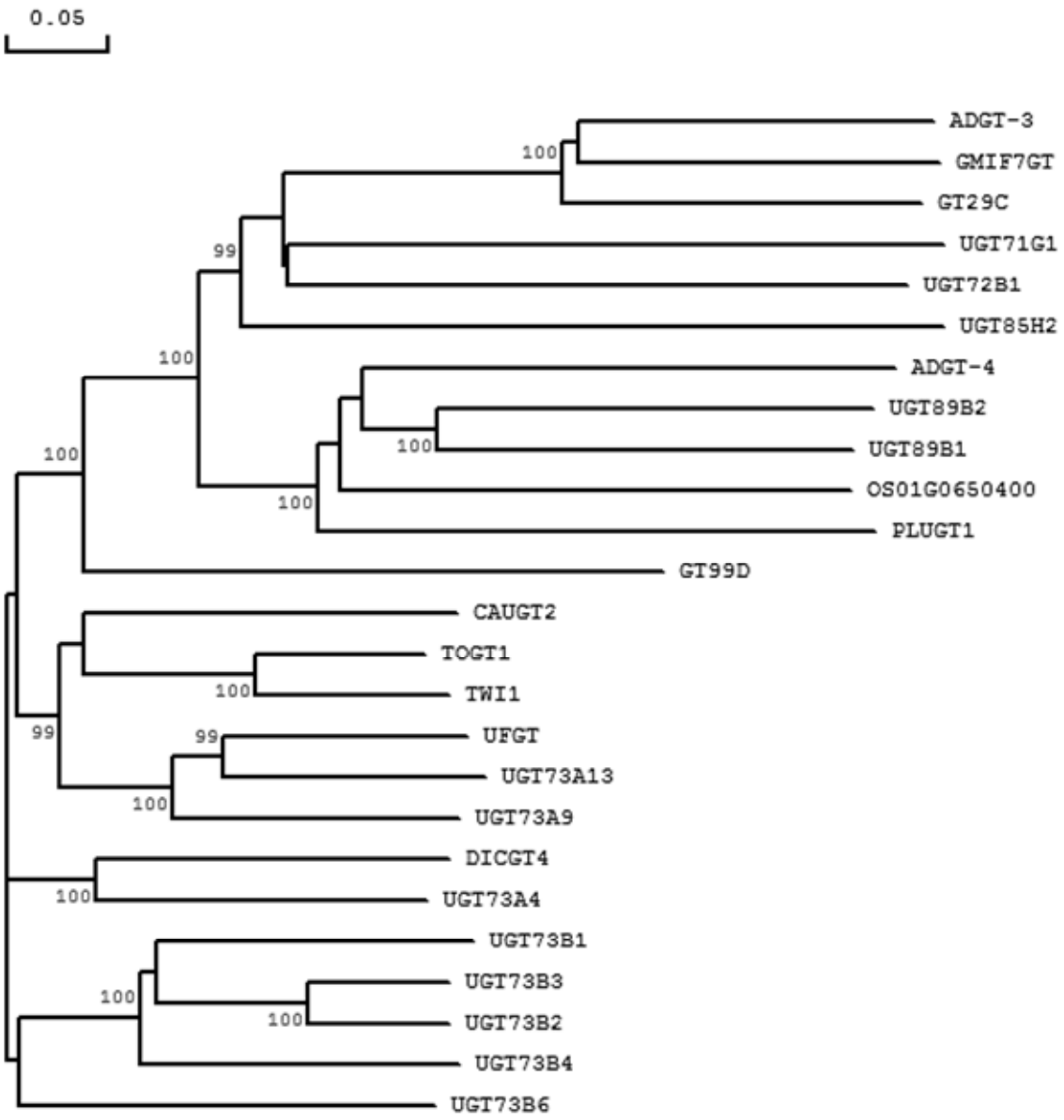


**Figure 2.** Phylogenetic tree based on the deduced amino-acid sequence of plant secondary product glucosyltransferase. Multiple sequence alignments were generated by using the DNAMAN package and the phylogenetic tree was constructed by the neighbor-joining method. The horizontal branch lengths are proportional to the genetic distance and the numbers at each point indicate bootstrap values. The data set was subjected to 1000 bootstrap replications. The above proteins include: Os01g0650400 (NP_001054090) in *O. sativa*; UGT73B1 (NP_567955), UGT73B2 (NP_567954), UGT73B3 (NP_567953), UGT73B4 (NP_179151), UGT72B1 (NP_192016) and UGT89B1 (NP_177529) in *A. thaliana*; ADGT-3 (BAB86921) and ADGT-4 (BAB86922) in *V. angularis*; GT29C (ABI94022), GT99D (DQ875465), UGT71G1 (AAW56092) and UGT85H2 (2PQ6_A) in *M. truncatula*; UGT73A4 (AAS94329) in *B. vulgaris*; UGT73A9 (BAG31950) in *A. majus*; UGT73A13 (BAG31952) in *P. frutescens*; CAUGT2 (AB159213) in *C. roseus*; DICGT4 (AB191248) in *D. caryophyllus*; GMIF7GT (BAF64416) in *G. max*; TOGT1 (AF346431) in *N. tabacum*; UGT73B6 (AY547304) in *R. sachalinensis*; UFGT (AB031274) in *S. baicalensis*; TWI1 (CAA59450) in *S. lycopersicum*; UGT89B2 (AAR06921) in *S. rebaudiana*; PlUGT1 (ACJ72158) in *P. lobata*.

## PlUGT1 protein expressed in *P. pastoris*

The result of the optimal induction methanol concentration is shown in Figure 5A. From methanol concentrations 0.3 to 1.0%, the expression level did not change significantly, but at 0.5%, the PlUGT1 protein was maximally induced. Therefore, methanol concentration 0.5% was used in the later experiment.

At concentration 0.5%, the optimal induction time was tested and the result is shown in Figure 5b. The maximum

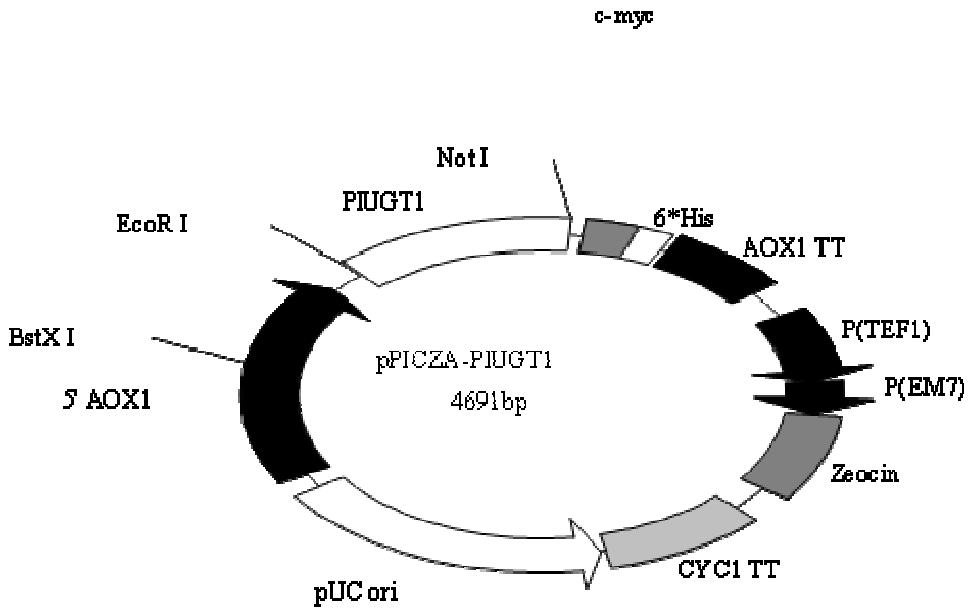


**Figure 3.** The recombinant *P. pastoris* expression plasmid pPICZA-PIUGT1. The 5' AOX1 promoter is a methanol-inducible promoter, PIUGT1 represents the coding regions of the *P. lobata* UDP glycosyltransferase gene. *Bst*X I is the restriction site used to linearize the plasmid before electroporating into *P. pastoris*.

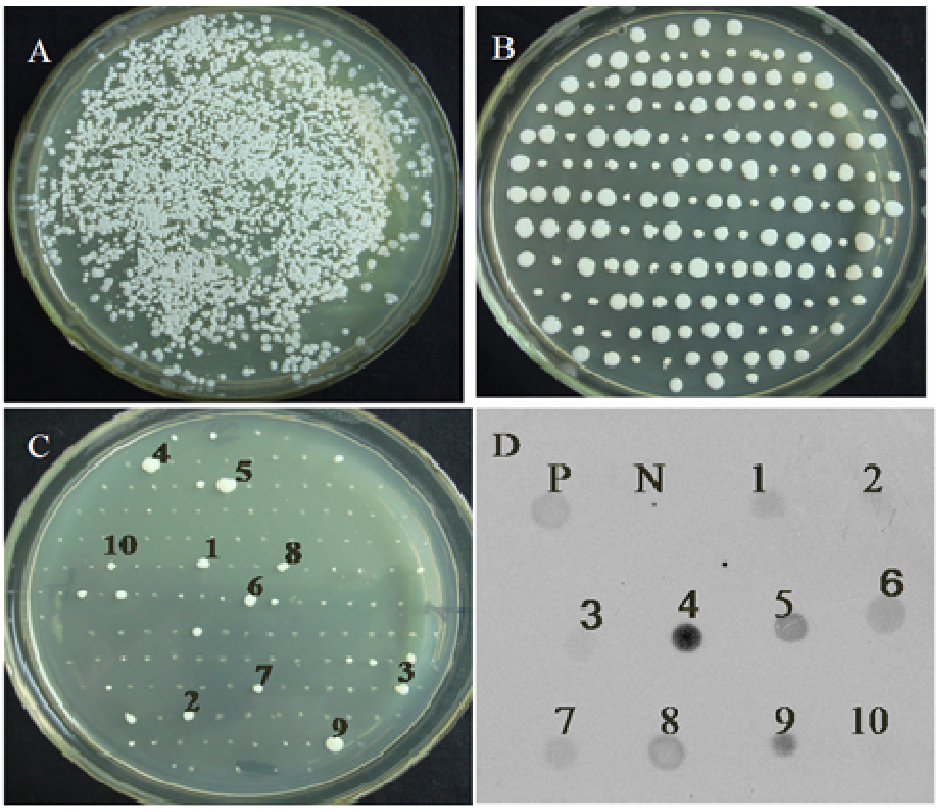


**Figure 4.** Screening for multicopy recombinants with (A), 100 µg/ml (B), 1000 µg/ml; (C), 4000 µg/ml zecoin; (D) southern dot blotting analysis of recombinants' genomic DNA; Dot P, plasmid pPICZA-PIUGT1; dot N, GS115/pPICZA genomic DNA; dots 1-10, genomic DNA of recombinants HC1-HC10.

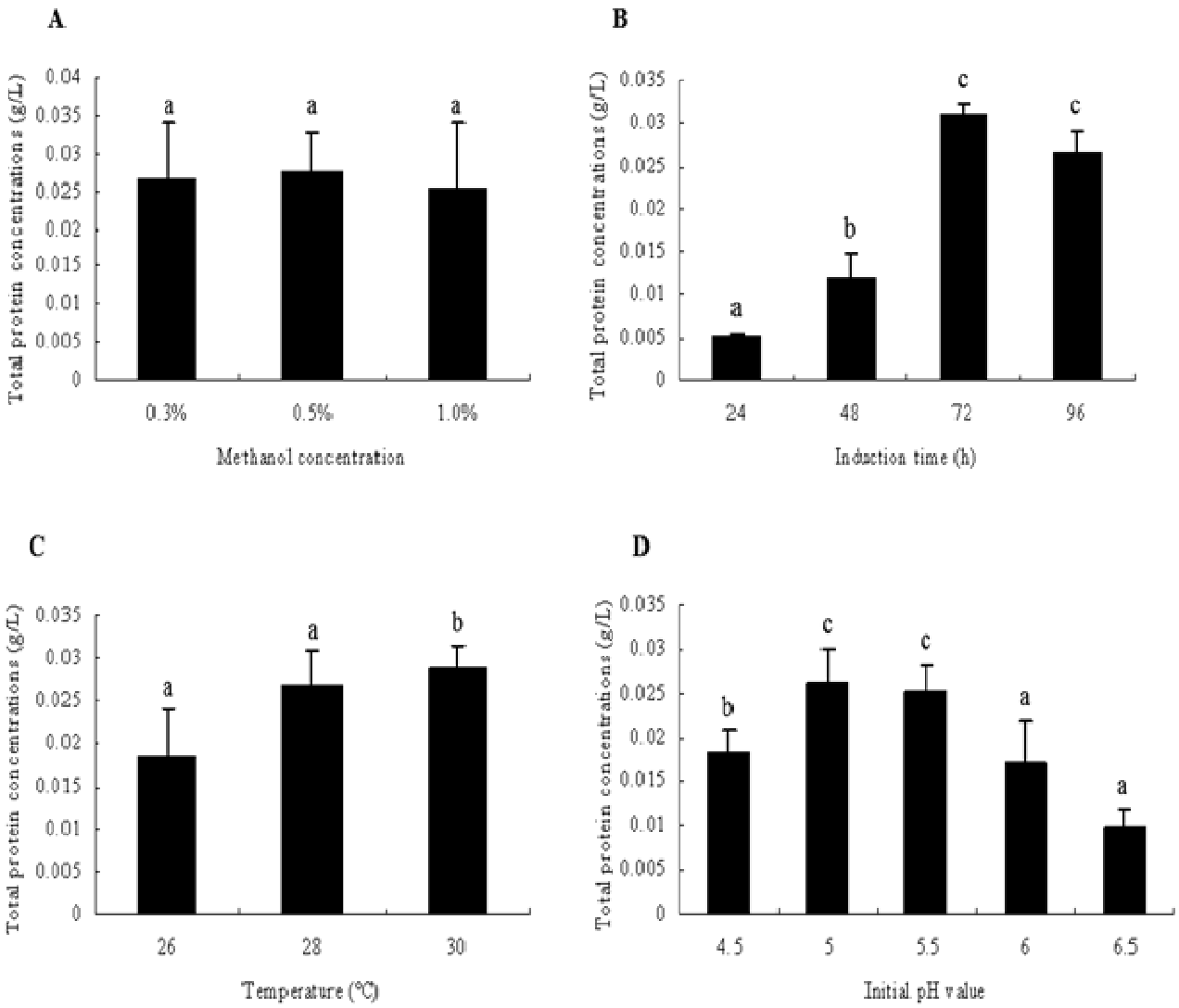


**Figure 5.** The optimal expression conditions for PIUGT1 production in the *P. pastoris* expression system. The optimal condition of PIUGT1 expression was examined in (A), different methanol concentrations (B), time course; (C), cultivation temperature; (D), initial pH values. These data are shown as mean ± S.D. and the different letters above the bars indicated the significant differences at $p < 0.05$.

expression level of PIUGT1 occurred after induction for 72 h. During 72 to 96 h, the expression level decreased. Therefore, the induction time of 72 h was used in the later experiment. *P. pastoris* cells were induced with 0.5% methanol for 72 h, the optimal cultivation temperature was investigated and the result shows that, the yield of PIUGT1 protein at 30℃ reached 0.029 g/l, significantly higher than that at 26℃ (Figure 5c). The optimal pH value was also determined when cells grew at 30℃ and were induced at 0.5% methanol for 72 h. As Figure 5d shows, from the initial pH value of 4.5 to 6.5, the expression level was increasing and then decreasing. At the initial pH of 5.0, the yield reached up to 0.026 g/l.

The HC3, HC4, HC5 and HC6 clones were finally selected for further studies of the effect of copy number on the concentration of expression protein. As the copy number of expression cassettes integrated into GS115 genomic DNA increased, the concentration of expression protein PIUGT1 was concomitantly higher (Figure 6), demonstrating that the copy number of PIUGT1 gene in the genome of *P. pastoris* is positively correlated with the intracellular expression levels of PIUGT1 fusion protein.

## Analysis of recombinant PIUGT1 proteins

The HC4 clone was chosen for characterization of recombinant PIUGT1. Protein expression was initiated under optimal condition. After induction, SDS-PAGE was performed to investigate protein production and purity using

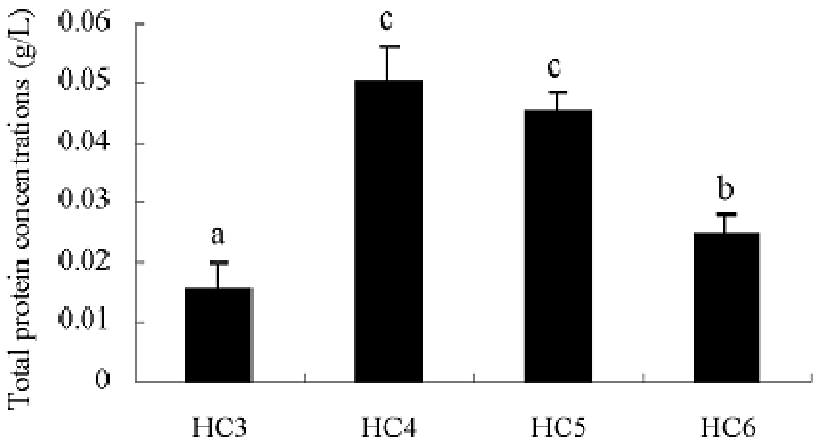


**Figure 6.** Effect of copy number on the concentration of PlUGT1 proteins expressed in *P. pastoris*. Recombinants HC3 to HC6 represented the copy number of PlUGT1 integrated into the genomic DNA of *P. pastoris* was different. These data are shown as mean ± S.D. and the different letters above the bars indicate significant differences at $p < 0.05$.

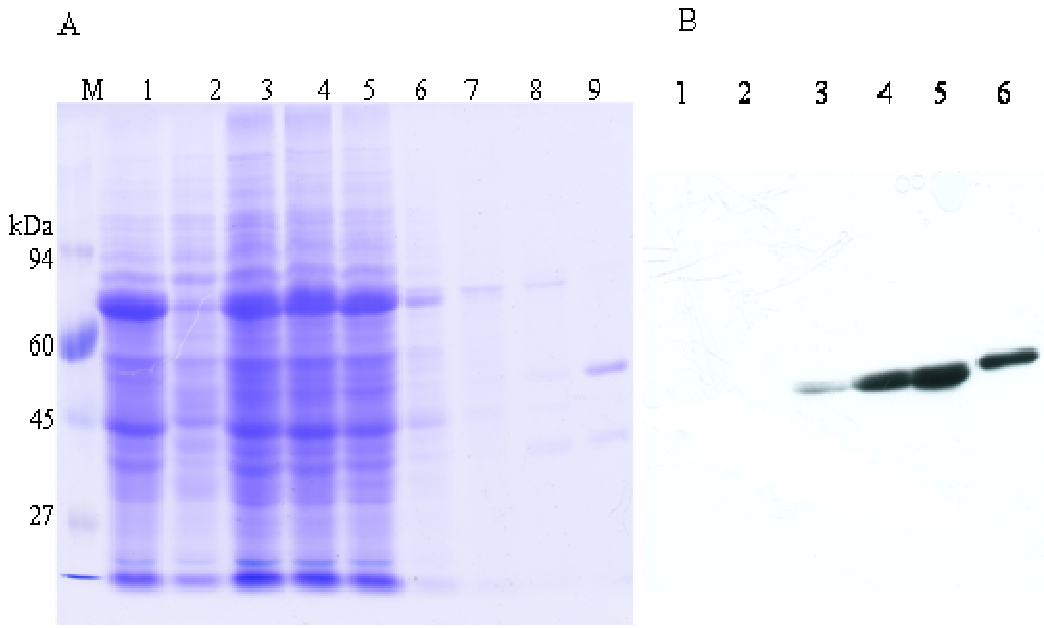


**Figure 7.** SDS-PAGE and western blot analysis of the PlUGT1 proteins expressed in *P. pastoris*. M, molecular mass marker; (A) Coomassie blue-stained SDS-PAGE showing total cell extracts from different recombinants. Lane 1, cell lysates of GS115/pPICZA after induction for 72 h (as negative control); lanes 2 to 5, cell lysates of GS115/pPICZA-PlUGT1 after induction for 0, 24, 48 and 72 h, respectively; lanes 6 to 9; purified protein eluted with 0.05 M sodium phosphate buffer containing 5, 10, 20 and 400 mM histidine, respectively. (B) western blot of a matching gel to shoe binding of PlUGT1 protein with Anti-Myc antibody. Lanes 1 to 5, the same as Panle (A); lane 6, purified protein eluted with 0.05 M sodium phosphate buffer containing 400 mM histidine

slab gels containing 8% (w/v) polyacrylamide. Compared with the negative control, an extra band of approximately 55 kDa was found in cells of *P. pastoris* with pPICZA-PlUGT1 (Figure 8A, Lane 3 to 5) and a single band can be seen after purification (Figure 7A, Lane 9). Furthermore, mouse monoclonal antibody against *c-myc* epitope also recognized approximately 55 kDa (Figure 7B). In addition, the signal showed a gradual increase from lane 3 to 5, which indicated that, the protein expression reached it highest level after methanol induction for 72 h and this

was consistent with the result shown in Figure 5b.

## DISCUSSION

Despite numerous studies on the occurrence, chemical structure and varying pharmaceutical activity of puerarin, its biosynthesis pathway is still poorly understood. Glucosylation of isoliquiritigenin is thought to be the final step in puerarin biosynthesis (Inoue and Fujita, 1974, 1977; Chen et al., 2010). However, no cDNA encoding glucosyltransferase responsible for puerarin synthesis has been isolated. In all plants for which genomic information is available (*Arabidopsis*, rice, poplar and *M. truncatula*), UDP-dependent glycosyltransferases seem to be encoded by comparatively large multigene families. Even though many UGTs have been cloned and heterologously expressed in the past few years, the catalytic activity of only a handful has been fully demonstrated *in vivo*. The UGT super family in higher plants is thought to encode enzymes that glycosylate a broad array of aglycones, including plant hormones, all major classes of plant secondary metabolites and xenobiotics such as herbicides (Vogt and Jones, 2000). The level of similarity between these UGT amino acid sequences varies from over 95% to lower than 30% identity. Considering the highly conserved domain involved in binding the nucleotide sugar substrate of plant UGTs (Gachon et al., 2005), several plant UGTs was chosen to design nested primers (Lim et al., 2002; Ma et al., 2007) and PlUGT1 encoding a putative glycosyltransferase, was first isolated from *P. lobata*. At the same time, we found that there was no intron in the genomic DNA sequence corresponding to PlUGT1. There have been similar results in previous studies (Kita et al., 2000). Ross et al. (2001) showed that, genes encoding *A. thaliana* UGTs contain up to two introns, but over half contain no introns.

Various factors affect the level of expression when *P. pastoris* integration vectors are used, such as inductive duration time, cultivation temperature, initial pH value, gene dose, etc. We paid particular attention to the importance of optimizing the physicochemical environment for efficient and maximal recombinant protein production in the *P. pastoris* expression system. The inductive duration time is crucially important to the production of proteins when a foreign gene is expressed in the yeast *P. pastoris*. So the appropriate inductive duration time for the expression of PlUGT1 protein was confirmed to be 72 h after induction. Lower cultivation temperature can also influence yields of recombinant protein, possibly due to poor stability of the recombinant protein at higher temperatures, release of more proteases from dead cells and folding problems at higher temperatures (Hong et al., 2002). Li et al. (2001) have shown that, lowering the process temperature (from 30 to 23°C), increased the yield of herring antifreeze proteins from 5.3 to 18.0 mg/l. However, we found that, lowering cultivation temperature from 30 to 26°C did not elevate but decrease the protein expression level. *P. pastoris* is capable of growing across a relatively broad pH range (3.0 and 7.0). This range does not affect the growth significantly, but has a marked impact on the protein expression level. Different pH values were found to be optimal from the point of view of a recombinant proteins stability; pH 6.0 was optimal in production of recombinant mouse epidermal factor and human serum albumin (Clare et al., 1991a; Kobayashi et al., 2000) and pH 3.0 was optimal in production of insulin-like growth factor-I and cytokine growth-blocking peptide (50 mg/l) (Brierley et al., 1994; Koganesawa et al., 2002). Thus, it can be deduced that pH 5.0 was optimal for the production of PlUGT1 in *P. pastoris*. The combination of one or all of these cultivation-level strategies would prove an effective means for obtaining maximal amounts of recombinant proteins from *P. pastoris*.

Gene copy number has been identified as a rate-limiting step in the production of recombinant proteins from *P. pastoris* (Clare et al., 1991a). Although, most *P. pastoris* integrative transformants contain only single copy insertions, a small proportion (1 to 10%) contain multiple integrated copies (Clare et al., 1991a, b; Romanos et al., 1991). Increasing the number of copies of the expression cassette generally has the effect of increasing the amount of protein expressed (Clare et al., 1991a; Romanos, 1995; Vassileva, 2001), which was the same as the result showed (Figure 6). Hohenblum et al. (2004) did not note any increase in the expression of a recombinant human trypsinogen when the gene dosage was increased from one to three copies under the control of the GAP promoter. However, an increase in the expression level of this protein was observed using the AOX1 promoter when the gene copy number was increased to two, but levels of expression fell upon further copy number increases. A number of researchers have had significant success with increasing gene dosage utilizing both the AOX1 and GAP promoters (Clare et al., 1991a; McGrew et al., 1997; Vassileva et al., 2001), therefore, before considering increasing the gene copy number as an optimization strategy for recombinant protein production from *P. pastoris*, the identity of the promoter must be considered in advance.

This study should be valuable for further research on the catalytic function of PlUGT1 involved in the biosynthesis of puerarin, which is ongoing in our laboratory. The stated data will provide a useful foundation for starting to help elucidate the intricate molecular pathway of puerarin biosynthesis and a potential strategy for the production of puerarin in industry utilized the *P. pastoris* expression system to produce PlUGT1.

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## Abbreviations

**UGT,** UDP-dependent glycosyltransferase; **PSPG,** plant secondary product glycosyltransferase; **HC,** high copies; **ECL,** electrochemiluminescence; **SDS-PAGE,** SDS polyacrylamide gel electrophoresis; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcription polymerase chain reaction (RT-PCR); **RACE,** rapid amplification of cDNA ends.

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