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Prokaryotic Expression of the UGPASE Gene of *Dendrobium Officinale* and Its Functional Analysis

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ABSTRACT

As is proven, the UGPase gene as a sugar metaboliser in *Dendrobium officinale* weigh heavily, yet little is known about the substance's structural and functional roles. To solve this problem, the biological properties of UGPase were investigated in this paper, and the gene encoding UGPase (UDP - glucose pyrophosphorylase) from *Dendrobium officinale* was successfully transformed into *Escherichia coli* BL21 (DE3) to construct an overexpression recombinant strain. UGPase is a stable hydrophilic protein nucleotidyltransferase. The molecular formula is C₂₃₅₁H₃₇₄₇N₆₀₃O₇₀₈S₈, the theoretical molecular weight is 52.04 kD, the theoretical isoelectric point is 5.08 and the instability index is 27.95. Irregular convolutions as well as α -helices are the main secondary structures of UGPases, and they constitute the main backbone of UGPases. The fusion protein was obtained in vitro and confirmed by Western Blot.

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The enzymatic activity of the recombinant protein UGPase was determined using different substrates and the recombinant protein UGPase was enzymatically active. Compared with the control group, recombinant pET-32a-UGPase was more tolerant to low temperature at -20 °C, high temperature at 50 °C, in 1200 mmol/L sorbitol. This study aims to predict the protein structure, express the protein in a prokaryotic expression system and to detect the resistance of UGPase in *Dendrobium officinale*, which provides an insight into the biological properties of UGPase in *Dendrobium officinale*.

Keywords *Dendrobium officinale*, UGPase, Prokaryotic expression, Functional analysis

Introduction

Encoded by the UGP gene, UGPase (UDP-glucose pyrophosphorylase) is a family of enzymes found in a variety of organisms. It is an important rate-limiting enzyme involved in the entire life process of plants as far as is known¹. UGPase catalyses a reversible reaction, converting Uridine triphosphate (UTP) and glucose-1-P (Glc-1-P) into UDP-glucose (UDPG) and Pyrophosphoric acid (PPi). It is an important precursor to hundreds of glycosyltransferases in all organisms. It is also a substrate for the biosynthesis of a large number of oligo and polysaccharides, which plays an important role in plant growth and development².

Dendrobium officinale Kimura et Migo (*D. officinale*), a member of the genus *Dendrobium* in the family *Orchidaceae* is widely used as medicine and food³. Modern studies have shown that polysaccharides are the main active constituents in *D. officinale*, with a wide range of biological activities⁴. Consequently, as a key enzyme in polysaccharide synthesis, plays an important role in the measurement of the medicinal value of *D. officinale*. However, research on the UGPase gene in *D. officinale* remains limited, despite its widespread distribution across various plant species. Currently, the gene has been isolated in poplar⁵, rice⁶, jute¹ and other plants, suggesting its significant function in the biosynthetic pathways related to sugar metabolism. In addition to providing energy for living organisms, sugars can also exist as components of cellular structures.

UGPase plays a key role in sugar metabolism, and in addition, it also has an effect on cellular redox balance and pollen development^{7,8}. There is an overlap with the function of the glutathione S-transferase gene⁹. Then the hypothesis comes: is it possible that UGPase can directly participate in intracellular oxidative reactions and have an effect on cellular metabolism beyond polysaccharide synthesis? UGPase is able to influence cell wall development by affecting cellulose and hemicellulose content^{1,10}.

¹¹. Cellulose and hemicellulose are the main components that make up plant cell walls¹². The plant cell wall is the first barrier of the plant against various stresses¹³. So, can overexpression of UGPase increase the resistance of *D. officinale* to abiotic stresses?

In our laboratory, one UGPase protein gene was cloned from *D. officinale* in the early stage, containing a complete coding frame of 1,419 bp and encoding 472 amino acids. It was confirmed that UGPase in the photoadaptability of *D. officinale* is related to the unique photosynthetic metabolic pathway it contains¹⁴, suggesting that the expression of this gene has an effect on the growth of *D. officinale*. In order to further understand the function of the *D. officinale* UGPase gene, the researchers constructed the *D. officinale* UGPase protein, conducted bioinformatic analyses, and evaluated its tolerance to adverse stresses through the *Escherichia coli* (*E. coli*) system. The results showed that UGPase in *D. officinale* had a positive effect on stress tolerance. This study provides ideas for the quality improvement of *D. officinale*, and helps further understand the function of *D. officinale* UGPase gene.

Materials and Methods

Test Materials and Treatments

The material used in this experiment was two-year-old *D. officinale*, provided by *D. officinale* plantation base in Nongfeng Mountain, Guangxi, China. The base adopts the planting mode of standardised planting greenhouses and off-ground seedbeds, using pine bark fermented by organic biofungus as the planting substrate, with organic planting standards for planting management. The plant was identified by Professor Teng Jianbei of Guangxi University of Chinese Medicine as *Dendrobium officinal* Kimura et Migo, categorised into the genus *Dendrobium* (*Dendrobium Sw.*), family *Orchidaceae*. The prokaryotic expression vector pET28a and its *Escherichia coli* DH5 α and Rosetta (DE3) are kept in this research laboratory, where the *D. officinale* UGPase gene was cloned and is preserved either.

Bioinformatics Analysis

The protein structural domains were predicted using NCBI-CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The physicochemical properties of the protein were predicted with ExPASy ProtParam (<https://web.expasy.org/protparam/>). The secondary structure and hydrophilicity of the protein were assessed using SOPMA SPLIT 4.0 Server (<http://split.djpept.com/split/4/>). The tertiary structure of the protein was analysed with SWISS-MODEL

(<https://swissmodel.expasy.org/>). Transmembrane regions and signal peptides were predicted using the TMHMM Program (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>) and SignalP 4.1 Server (<https://services.healthtech.dtu.dk/services/SignalP-4.1/>). Protein phosphorylation sites were predicted using NetPhos 3.1 (<https://services.healthtech.dtu.dk/services/NetPhos-3.1/>). The subcellular localization of UGPase was predictively analysed with WoLF PSORT (<https://wolfsort.hgc.jp/>).

Cloning of UGPase Gene and Construction of Expression Vector

The study analyse the sequence of the *D. officinale* UGPase gene, which has been cloned in this research laboratory. The open reading frame of this gene is 1,416 bp, and 472 amino acids are encoded. Primers were designed at both ends of the coding region of the UGPase gene with the corresponding enzymatic sites added.

The first strand of cDNA was synthesised from the *D. officinale* RNA according to the instructions of the PrimeScript™ II 1st Strand cDNA Synthesis Kit, and then reverse transcribed to be the template for PCR amplification. After amplification by PrimeSTAR™ HS DNA Polymerase and recovery by gel electrophoresis, the product and expression vector pET28a (+) were purified, recovered and ligated following double digestion by *NheI* and *NotI*, and transformed into *E. coli* DH5 α sensory state for single colony PCR. Positive recombinants were sent to the company for sequencing. The extracted plasmid from the correctly sequenced positive recombinants (named ET28a-UGPase) was transformed into the expression strain *E. coli* Rosetta (DE3) sensory state, and the positive recombinants were screened for subsequent induced expression.

Induced Expression and Detection of Recombinant Plasmids in *E. coli*

Protein Expression

Under thermal stimulation, the recombinant plasmid pET28a-UGPase was transformed into *E. coli* Rosetta (DE3) receptor cells using thermal stimulation. 42 °C-heat-excited *E. coli* is spread on plates with 30 µg/mL kanamycin and 34 µg/mL chloramphenicol. After overnight incubation at 37 °C, the monoclonal colonies were picked and incubated at 37 °C in liquid medium containing 30 µg/mL kanamycin and 34 µg/mL chloramphenicol. When the OD₆₀₀ value reached 0.6, 0.5 mM inducer IPTG was added and the culture was continued overnight at 16 °C and 20 °C respectively. The specimens without added inducer were marked as negative control. At the end of induction, the researchers

centrifuged the samples at 4,000 rpm for 10 min, discarded the supernatant and collected the organisms. Then Buffer A was added to the collected organisms which were fully solubilised using an ultrasonic crusher later. The supernatant and precipitate were collected by centrifugation, with the latter solubilised using Buffer B. Separate samples of supernatant and precipitate proteins were prepared for the gelatinisation assay. The bacteria were cultured in medium containing 30 µg/mL kanamycin and 34 µg/mL chloramphenicol. When the OD₆₀₀ value reached 0.6, 0.5 mM inducer IPTG was added, and the culture was induced overnight at 20 °C for bulk expression. At last, the cyto bacteria were collected by centrifugation.

Protein Purification

The above collected cyto bacteria were lysed in buffer C, broken by sonication and centrifuged to collect the supernatant crude proteins. The researchers took 5 mL of Ni-NTA and washed the equilibrated column with 10 times the volume of Binding buffer in the column bed. The crude protein was incubated with the equilibrated column packing for 1 h and the effluent was collected. The equilibrium column was washed with Binding buffer again, and then with Washing buffer. The effluent was eluted with Elution buffer and collected.

The crude protein and effluent fractions were processed separately, and samples were prepared for SDS-PAGE purification and detection. The purified fractions were dialysed into the pH 8.0 protein preservation buffer with 50 mM Tris and 300 mM NaCl. Subsequently, the samples were concentrated with PEG20000, filtered through 0.45 µm membrane, dispensed into 1 mL tubes, and stored at -80 °C. Expression of target proteins was detected by SDS-PAGE electrophoresis. The above prokaryotic expression of target proteins was further verified by Western Blot. SDS-PAGE assay: protein samples were processed and prepared. Using 12% resolving gel and 5% stacking gel for electrophoresis to detect molecular weight. Western Blot Validation: protein samples were processed and prepared. The validation utilises a gel electrophoresis setup with a 5% stacking gel and a 12% resolving gel. The primary antibody used is a mouse-derived antibody specific for the His-tag, while the secondary antibody is a goat-derived antibody against mouse IgG. Tetramethylbenzidine (TMB) is employed as the chromogenic substrate for visualisation, and labeled antibodies were used for validation.

In Vitro Enzymatic Activity of UGPase

Kit Method

According to the protocol of the plant UDPase assay kit (Shanghai Baililai Biology Technology Co., Ltd.), the researchers conducted enzymatic activity assay of purified recombinant proteins containing the UGPase gene. Protein content was quantified according to an enzyme-linked immunoassay (ELISA) assay kit (Shanghai Baililai Biology Technology Co., Ltd.). The target protein inactivated by boiling was used as a control, with three parallels per treatment. UGPase activity was determined by reaction spectrophotometry at 340 nm in the reaction mixture (from the UGPase assay kit) using the Epoch2 microplate spectrophotometer enzyme marker. One unit of UGPase activity was defined as the amount of enzyme required to reduce 1 mmol NADP⁺ per minute.

UGPase Activity toward Other Substrates

Referring to Edwards¹⁵ and Habig¹⁶ approach, of measuring the ability of UGPase protein to catalyze 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), 4-Nitrobenzyl chloride (NBC), 7-Chloro-4-nitrobenzo-2-oxa-1,3-di-azole (NBD-Cl), L-threo-2,3-Hexodiolosonic acid (DHA), 2-Hydroxyethyl disulfide (HED), the increase in product per unit volume per unit time was measured. The target protein inactivated by boiling was used as a control, with three parallels per treatment. All assays were carried out on a UV2600A UV-visible spectrophotometer.

An appropriate amount of substrate assay buffer, an appropriate amount of the first substrate GSH, an appropriate amount of the second substrate (CDNB or DCNB or NBC or NBD-Cl or DHA or HED), and an appropriate amount of purified recombinant proteins or inactivated proteins after boiling were added to the cuvettes of the control group and the experimental group, respectively. Specific substrates and dosages are shown in Table 1.

After mixing evenly, the researchers used a spectrophotometer to detect the increase in absorbance of the reaction product at room temperature, and read every 30 seconds for 3-5 minutes until the substrate was completely reacted. The absorbance change value for 1 minute came from the following formula. Specific activity [$\mu\text{mol}/(\text{min}/\text{mg})$] = $(\Delta \text{ODABS} * V) / (\epsilon * T * L * E)$

ΔODABS is the change in absorbance per unit time, V is the volume of enzymatic reaction (μL), ϵ is the extinction coefficient of the product (9.6 L/mmol-cm), T is the reaction time (min), L is the optical range (1 cm), and E is the amount of enzyme added (g).

Table 1 Enzyme Activity Substrate Table

First substrate and dosage	Substrate buffer and dosage	secondary substrate	Amount of protein	Reaction product absorption wavelength
50μl 60mM CDNB	2.85ml 0.05 M K ₃ PO ₄ , pH=6.5	50μl 60mM GSH	10μl	340nm
50μl 60mM DCNB	2.85ml 0.05 M K ₃ PO ₄ , pH=7.5	50μl 60mM GSH	10μl	345nm
50μl 60mM NBC	2.85ml 0.05 M K ₃ PO ₄ , pH=6.5	50μl 60mM GSH	10μl	310nm
50μl 10mM NBD-Cl	2.85ml 0.1M sodium acetate, pH=5	50μl 60mM GSH	10μl	419nm
25μl 20mM DHA	900μl 0.1M K ₃ PO ₄ , pH=2.5	50μl 100mM GSH	10μl	265nm
100μl 7mM HED	500μl 0.2M Tris-Cl 100μl 2.5mM NADPH in 0.1%NaHCO ₃ 中	100μl 10mM GSH	100μl	340nm

168

169 **Resistance Analysis of Recombinant *E. coli* pET28a-UGPase**170 **Determination of Growth Kinetics of Transformed *E. coli* under Adverse Stresses.**

171 Referring to the experimental methods of He Xuegao¹⁷ and Liu Linya¹⁸, the expression strain *E. coli*

172 Rosetta (DE3), which was transformed with the recombinant plasmid of pET28a-UGPase, was

173 inoculated into 2 mL of LB culture medium containing kanamycin (50 μg/mL), and incubated at 37 °C

174 with shaking overnight. On the next day, 0.2 mL of the culture was transferred into 20 mL of new

175 kanamycin-resistant LB liquid medium and incubated at 37 °C for 4 h, until the OD₆₀₀ of the culture was

176 increased to 0.8, and then the researchers added IPTG whose final concentration was 1 mmol/L and

177 induced for 4 h at 25 °C. The OD₆₀₀ of *E. coli* transformed with pET28a-UGPase and pET28a blank

178 plasmid at the end of IPTG induction was conformably adjusted to 0.8. 1 mL of recombinant and non-

179 recombinant bacterial fluids were taken into EP tubes and subjected to low temperature (-20 °C, 24 h)

180 and high temperature (50 °C, 40 min) treatments, respectively. The treated bacterial solution was added

181 into LB medium at a volume ratio of 1:100, and incubated at 37 °C, 200 r/min for 24 h, and the OD₆₀₀

182 value was determined by sampling every 2 h, which was used to draw the growth curve. The pH of LB

183 medium was adjusted to 5.0 and 9.0 to simulate acid/alkaline stress. A final concentration of 500 mmol/L

184 NaCl, 1,200 mmol/L sorbitol was added to LB medium as high-salt and hyperosmotic treatments. In the

185 treated medium, recombinant and non-recombinant bacteria were added at a volume ratio of 1:100 and
186 incubated at 37 °C and 200 r/min for 24 h. The OD₆₀₀ values were determined by sampling every 2 h and
187 the growth curves were plotted. The experiment was carried out on LB medium without any stressor as
188 a blank control group and three replicates were set up for each treatment.

190 **Effect of UGPase-transformed *E. coli* on the Stress Resistance of *E. coli***

191 For *E. coli* culture, IPTG induction and stress treatment, shares the same method as for the growth
192 kinetics assay. At the end of IPTG induction, the researchers transformed the *E. coli* with pET28a-
193 UGPase recombinant plasmid and pET28a (+) blank plasmid, and conformably adjusted the OD₆₀₀ of its
194 culture to 1. The culture was then diluted with sterilised water into 6 gradients of 10⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵.
195 Operator took 50 µL of each dilution gradient of the bacterial solution, respectively spread onto
196 LB solid medium containing NaCl (final concentration of 500 mmol/L) and sorbitol (of 1,200 mmol/L),
197 and incubated at 37 °C for 12 h, with subsequent photographic observation of bacterial growth. At the
198 same time, 1 mL of undiluted recombinant and non-recombinant bacterial culture was divided into EP
199 tubes and treated with low temperature (-20 °C, 24 h) and high temperature (50 °C, 40 min) respectively,
200 diluted according to the above method, and then placed in 37 °C to culture for 12 h, with subsequent
201 photographic observation of bacterial growth. It was verified that *E. coli* transformed with the pET28a-
202 UGPase recombinant plasmid exhibited resistance. The experiment was carried out on LB medium
203 without any stressor as a blank control group with three replicates for each treatment.

205 **Results and Analyses**

206 **Bioinformatics Analysis**

207 Protein structure analysis showed that UGPase proteins belong to the PLN02474 family (Fig.1a). The
208 molecular formula of UGPase protein is C₂₃₅₁H₃₇₄₇N₆₀₃O₇₀₈S₈. Its theoretical molecular weight is 52.04
209 kD. Its theoretical isoelectric point 5.08. It is acidic protein. The instability index is 27.95, the number of
210 negatively charged residues is 65 and the number of positively charged residues is 50, with an extinction
211 coefficient of 37,025, a fat coefficient of 103.4, and an average hydrophobicity of -0.084. According to
212 the rule that the lower the amino acid score the more hydrophilic and the higher the score the more
213 hydrophobic, amino acids between +0.5 and -0.5 are mainly amphoteric amino acids, so the protein is
214 hydrophilic and stable(Fig.1b).

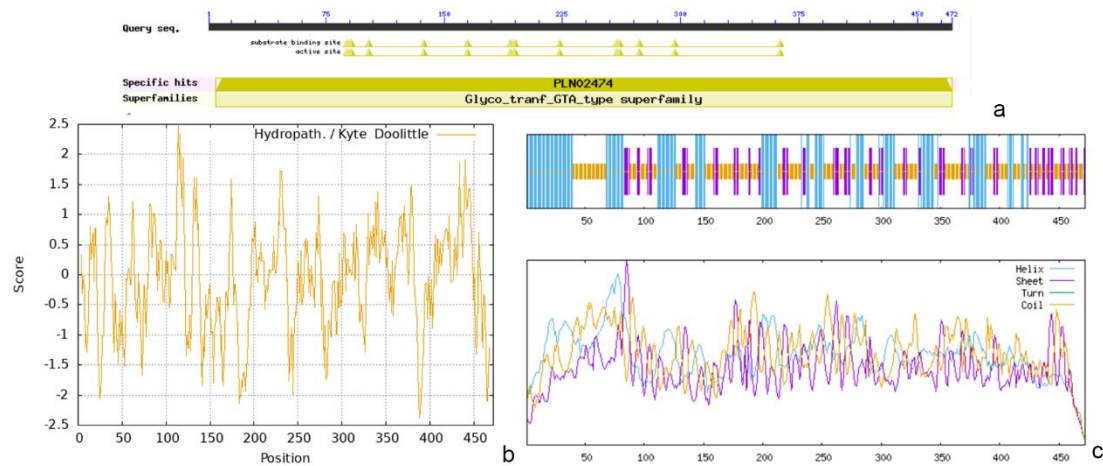


Fig.1 Domain analysis of UGPase protein (a), prediction of hydrophobicity (b) and secondary structure (c)

The secondary structure of UGPase protein is characterised by irregular coils, which accounted for 45.97% of the total, followed by α -helices with 34.11%, and extensions with 19.92% (Fig.1c). In order to further resolve the functions played by UGPase genes in *D. officinale*, the possible formation of higher structures is predicted by Phyre. In addition to the core domain, the typical UGPase protein had a β -folding carboxyl-terminal domain to form a barrel structure (L-loop), an amino-terminal domain with an NB-loop, and a glycan-binding domain, which was characteristic of UGPase proteins. The prediction results showed that UGPase has the above typical characteristics, and it was speculated that UGPase plays a typical function in *D. officinale* (Fig.2a).

TMHMM prediction showed that the UGPase protein does not have a transmembrane region (Fig.2b) and has a 100% probability of being located outside the membrane. Thus, it was presumed to be a possible secreted protein with no signal peptide (Fig.2c). Furthermore, the WoLF PSORT website was used to predict the localisation of UGPase proteins in cells, which showed that UGPase proteins may be localised in the cytoplasm. The UGPase peptide chain contains 39 phosphorylation sites, including 21 serine (Ser) sites, 14 threonine (Thr) sites and 3 tyrosine (Tyr) sites (Fig.2d).

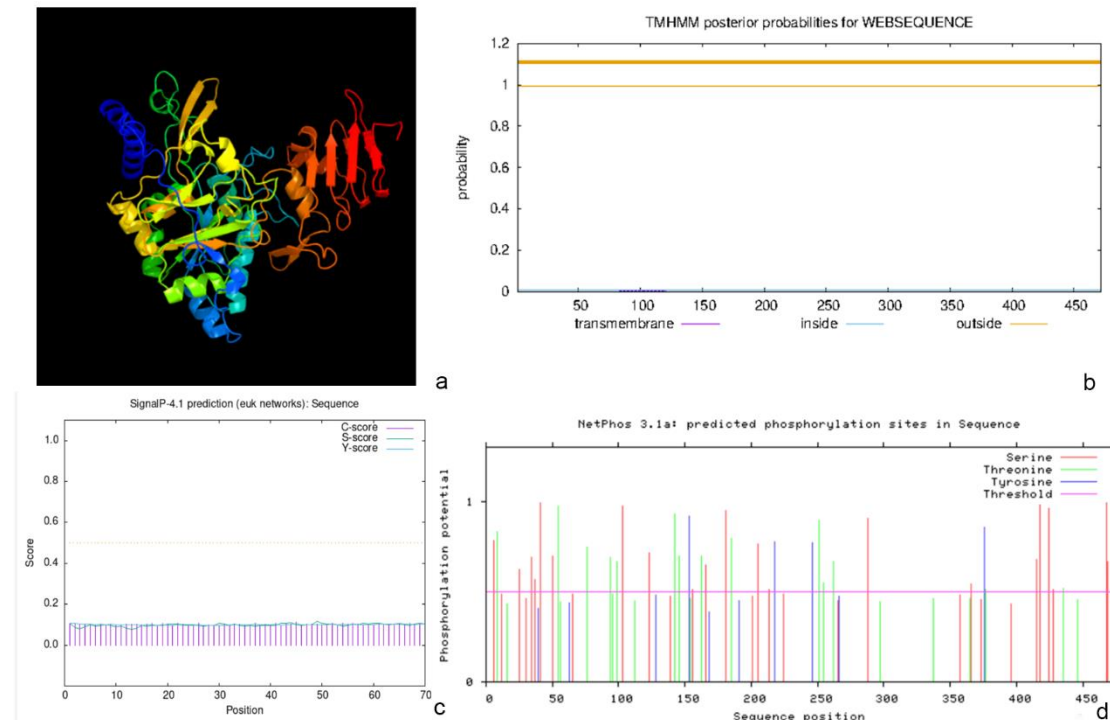


Fig.2 Prediction analysis of tertiary structure (a), transmembrane helix (b), signal peptide of UGPase protein (c) and phosphorylation sites (d) of UGPase protein

Construction of UGPase Gene Prokaryotic Expression Vector

With *D. officinale* cDNA as a template, the cDNA coding region of UGPase gene was amplified by primers with added cleavage sites, and the target band of about 1,500 bp was assessed (Fig.3). The amplified fragments were recovered and purified, and were ligated into the pET28a (+) vector after double digestion by restriction endonucleases *NheI* and *NotI*. The recombinant vector was transformed into *E. coli* DH5 α , and then the positive clones were sent to the company for sequencing, which showed that the sequence of the inserted fragment was consistent with that of the cDNA coding region of the UGPase gene, and the sequence of the inserted fragment was consistent with that of the cDNA coding region of the UGPase gene. It indicates that the pET28a-UGPase prokaryotic expression vector has been successfully constructed.

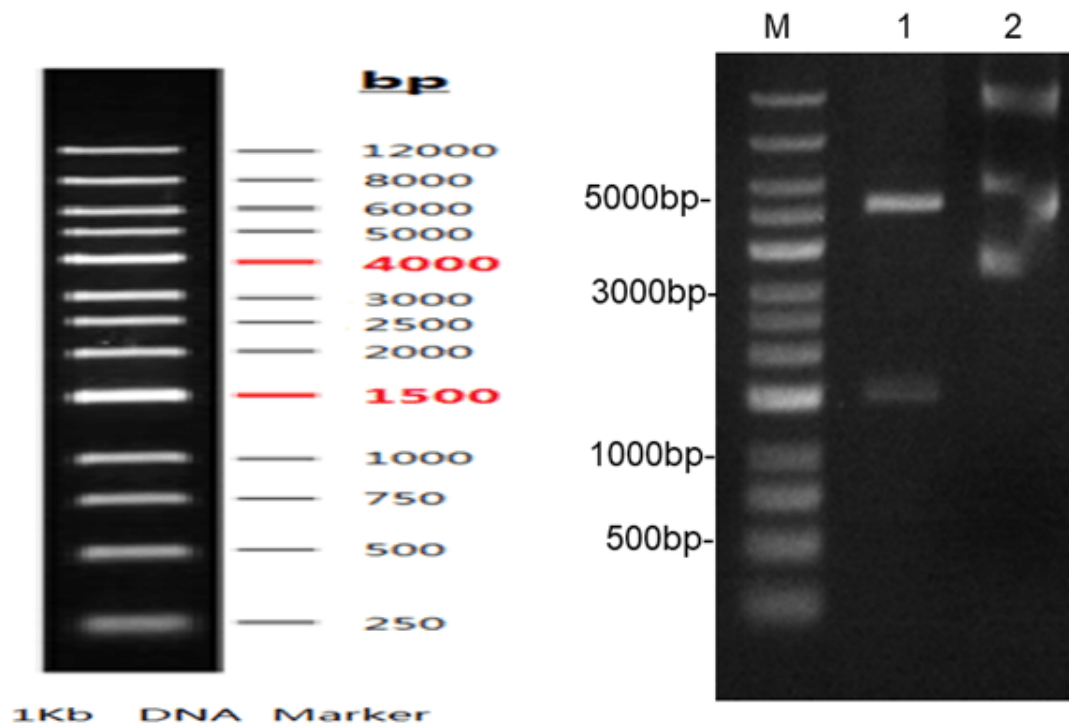


Fig.3 Restriction map of the recombinant plasmid (digested with *NdeI*-*XhoI*)

Lane 1: Plasmid Digested with *NdeI*-*XhoI*

Lane 2: Plasmid

Lane M: DNA Marker

Induction of Expression and Detection of Recombinant Plasmid pET28a-UGPase

The constructed pET28a-UGPase prokaryotic expression vector was transformed into *E. coli* expression strain *E. coli* Rosetta (DE3). The recombinant plasmid was identified by colony PCR amplification to show that the recombinant plasmid had been transformed into *E. coli* Rosetta (DE3). After induced expression of the expression strain transformed with PET28a-UGPase recombinant plasmid with 1 mmol/L IPTG at 25 °C, it was ultrasonically broken for SDS-PAGE. The results showed that induction with 1 mmol/L IPTG for 4 h at 25 °C induced the target proteins with molecular weights consistent with those of UGPase proteins, and the recombinant proteins existed in the form of both soluble proteins and insoluble inclusion bodies (Fig.4). The fusion proteins were then purified by chromatography using a nickel column. Based on the SDS-PAGE staining results (Fig.5), it was found that the fusion proteins were predominantly enriched in the supernatant before purification. After purifying and eluting the supernatant, the target bands appeared above 60 kD.



Fig.4 SDS-PAGE analysis of the fusion protein expression test.

M: Protein Marker; 1: Total protein before induction;

2: Soluble supernatant after 20°C induction;

3: Insoluble fraction after 20°C induction;

4: Soluble supernatant after 37°C induction;

5: Insoluble fraction after 37°C induction.

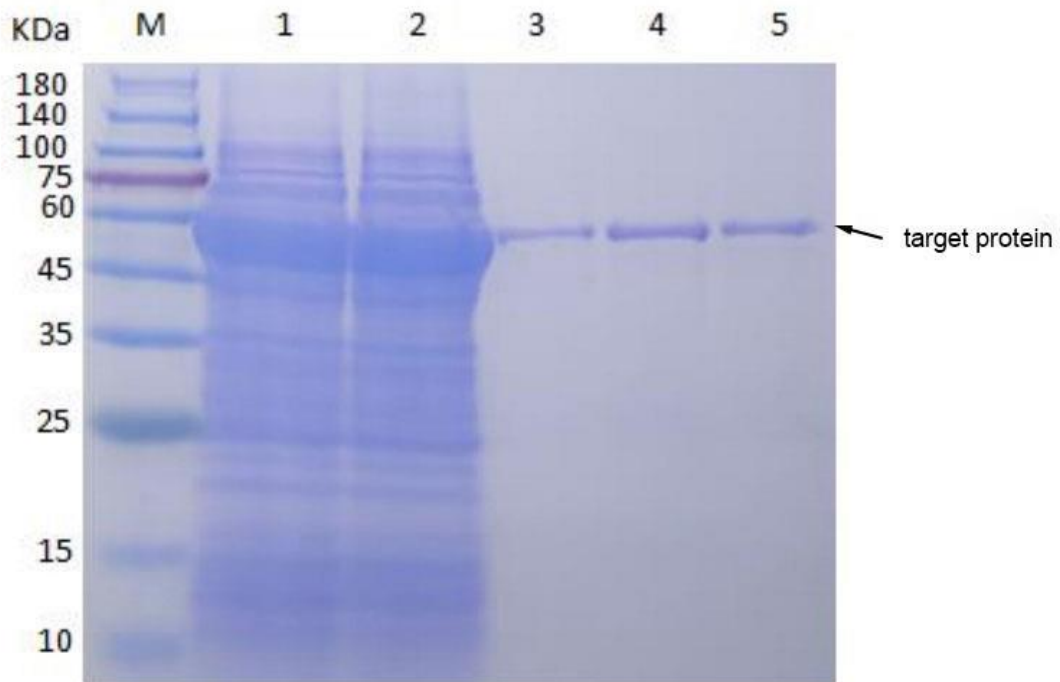


Fig.5 SDS-PAGE analysis of fusion protein purified by nickel agarose affinity chromatography

M: Protein marker; 1: first sample; 2: disgorge; 3: 20 mM Imidazole elution component; 4: 50 mM Imidazole elution component; 5: 500 mM Imidazole elution component

After purification of the fusion protein, SDS-PAGE analysis showed obvious bands near the theoretical molecular weight(Fig.6). The performance can help judging initially that the fusion protein has been successfully purified.

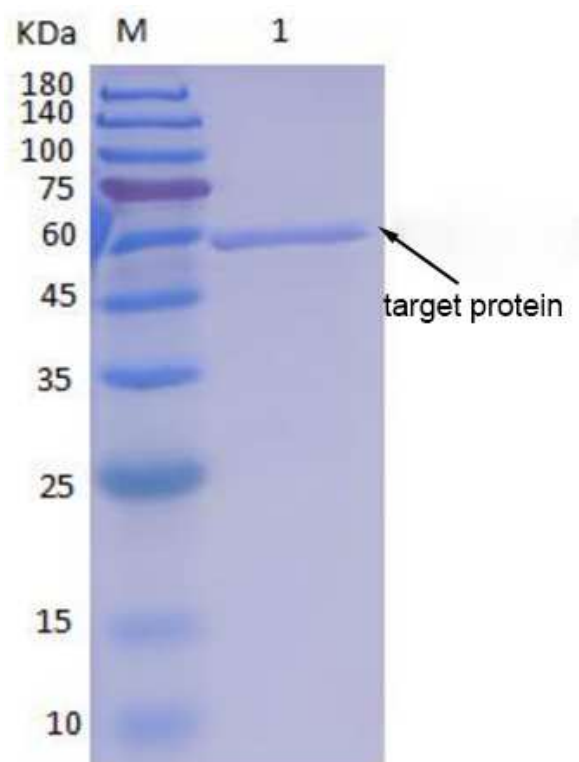


Fig.6 SDS-PAGE analysis of the final purified protein.

M: Protein marker; 1: Fusion target proteins

The purified proteins were further verified by Western Blot. It was verified with labeled antibodies and visualised using the TMB Colour Kit. As shown in Fig.7. The fusion proteins also showed target bands near the corresponding positions, indicating the accuracy of target protein expression. 3 mL of the target protein was successfully purified at a concentration of 0.35 mg/mL, totalling 1.05 mg.

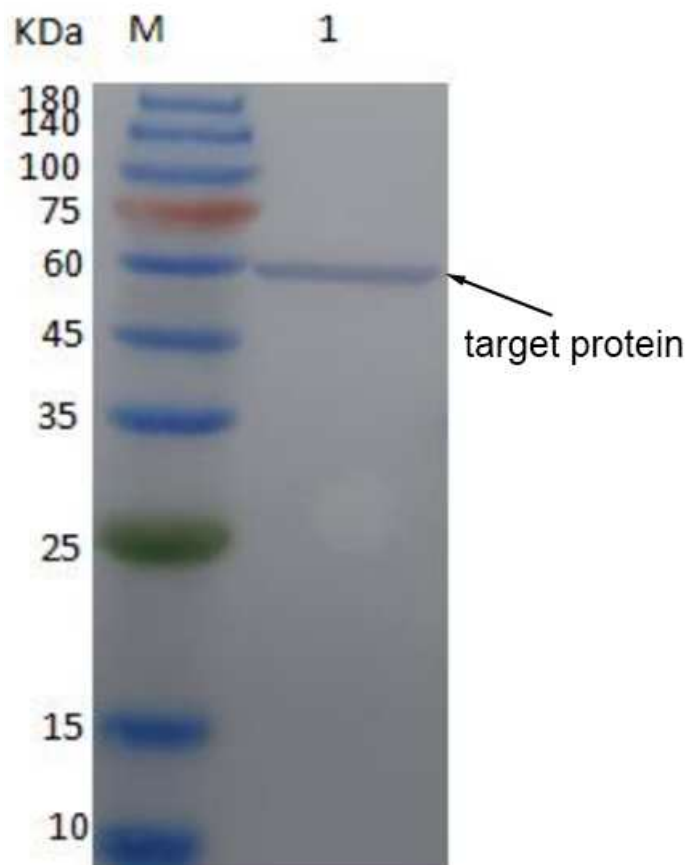


Fig.7 Western Blot analysis of the final purified protein.

M: Protein marker; 1: Fusion target proteins

In Vitro Enzymatic Activity of UGPase

Kit Method

The standards were prepared and assayed according to the method of enzyme-linked immunoassay (ELISA) test kit of Shanghai Baililai Biology Technology Co., Ltd. The extracted UGPase protein and the OD value of the boiled protein were brought into the equation to calculate the concentration of the sample, and then multiplied by the number of times of dilution (5 times), leading to the actual enzyme activity of the extracted protein. The specific enzyme activity was calculated according to the following formula.

$$\text{Enzyme specific activity} = \text{enzyme activity (U)} / \text{enzyme protein concentration (mg/mL)}$$

Table 2 Determination of protease activity by kit method

samples	absorbance	enzyme activity (U/L)	Specific enzyme activity (U/mg)
proteins	0.782	97.768	279.33
	0.766	95.295	272.27
	0.777	96.994	277.13
	0.347	33	94.28
boiled protein	0.277	23.06	65.88
	0.291	25.04	71.54

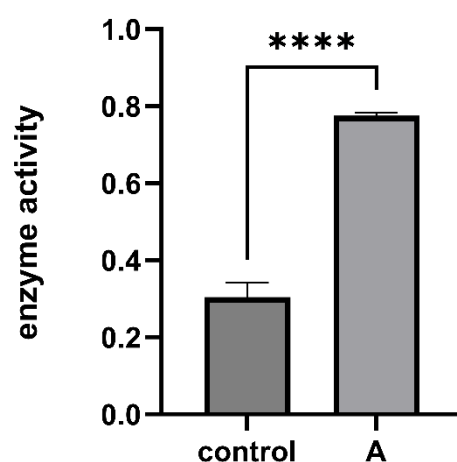


Fig. 8 Comparison of protease activity of purified proteins and after boiling

As shown in Table 2, Figure 8, enzymatic activity was present in the purified UGPase proteins and there was a significant difference between the enzymatic activity of the purified proteins and that of the boiled proteins. The mean value of purified protease activity was 96.69 U/L and the mean value of specific activity was 276.24 U/mg.

UGPase Activity toward Other Substrates

To further analyze whether UGPase has a function other than its involvement in polysaccharide synthesis, the antioxidant capacity of UGPase was probed with a number of different substrates. The activities of UGPase protein on six substrates, including CDNB, DCNB, NBC, NBD-Cl, DHA and HED, were tested and the results are shown in Table 3.

318

Table 3 Viability of purified proteins against different substrates

Substrate	Δ ODABS	Specific enzyme activity $\mu\text{mol}(\text{min} \cdot \text{mg})$
CDNB	0.003	0
DCNB	0.003	0
NBC	0.012	0
NBD-Cl	0.015	0
DHA	0.01	0
HED	0.012	0

319

As can be seen from the table, the UGPase protein showed no detectable activity against any of the six substrates.

320

321

322 **Growth Kinetics of UGPase-transformed *E. coli* under Adverse Stresses**

323 The researchers analysed the resistance of recombinant bacteria pET28a-UGPase and non-recombinant
 324 bacteria pET28a, which were induced by IPTG for 3 h, under various stress conditions including low
 325 temperature at -20°C, high temperature at 50°C, pH 5.0, pH 9.0, 500 mmol/L NaCl, and 1,200 mmol/L
 326 sorbitol, using recombinant bacteria and non-recombinant bacterial suspensions without stress treatment
 327 as controls. The results of growth kinetic analysis of the transformed cells (Fig.9) showed that under
 328 normal conditions (Fig.8a), the growth curves of pET28a-UGPase and pET28a transformed cells were
 329 basically the same, and there was no significant difference between them. Both strains entered the
 330 logarithmic growth phase after a latency period of approximately 3 h, reaching a plateau after 12 h,
 331 indicating that UGPase expression in *E. coli* does not adversely affect *E. coli* growth. After treatment
 332 with 1,200 mmol/L sorbitol (Fig.9b), recombinant bacterium pET28a-UGPase showed better growth
 333 compared to the non-recombinant strain. Conversely, in medium containing 500 mmol/L NaCl (Fig.9c),
 334 the non-recombinant strain exhibited superior growth. When subjected to pH=9 (Fig.9d) and pH=5
 335 (Fig.8e), both strains had similar growth rates for the first 6 h, after which pET28a-UGPase outperformed
 336 the non-recombinant strain. The growth status of pET28a-UGPase cells was also slightly better than that
 337 of pET28a after low temperature (Fig.9f) at -20 °C and high temperature (Fig.9g) at 50 °C. These results
 338 suggest that overexpression of UGPase can improve the resistance of *E. coli* to all adversities to a certain
 339 extent.

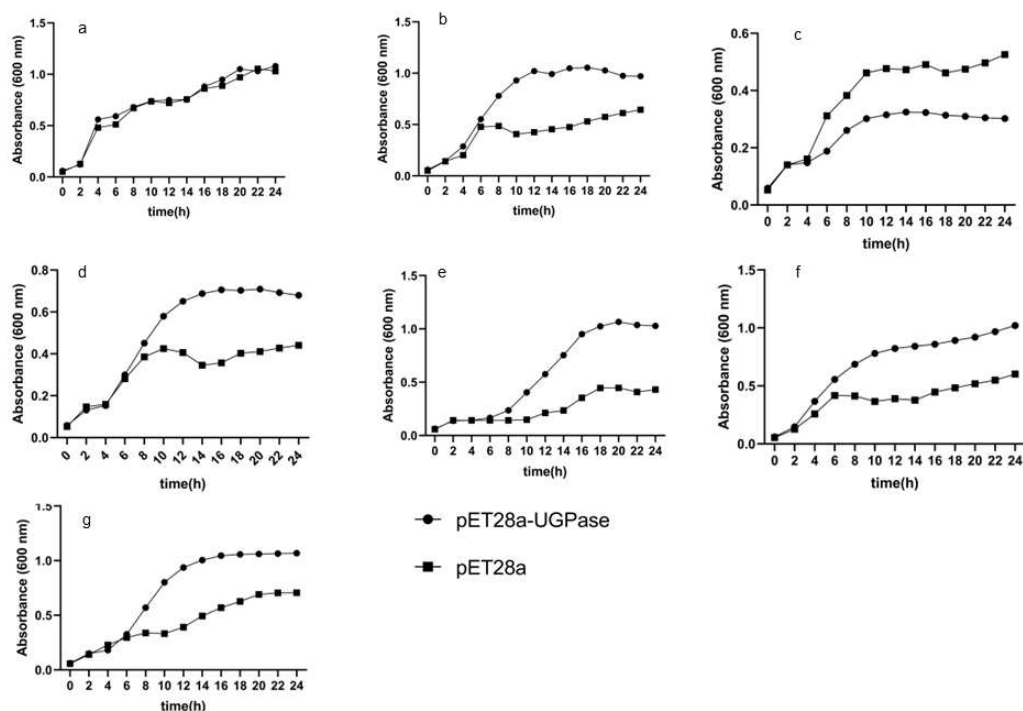


Fig.9 Detection of OD600 value of recombinant strain and control strain in different stresses

Note: a: Control; b: 1,200 mmol/L sorbitol; c: 500 mmol/L NaCl; d: pH=9; e: pH=5; f: -20 °C for 24 h; g: 50 °C for 40 min

Effect of UGPase-transformed *E. coli* on the Stress Resistance of *E. coli*

To investigate the effect of overexpression of UGPase protein on the growth of *E. coli* recombinants under different environmental stresses, the researchers diluted and spread recombinant bacterial pET28a-UGPase and non-recombinant bacterial pET28a cultures on different plates. Fig.10 shows that in overnight cultures, pET28a-UGPase and pET28a grew similarly on Luria-Bertani (LB) medium. In normal medium simulating high and low temperature stress as well as in medium with 500 mol/l sorbitol added for simulating high osmotic pressure, pET28a-UGPase grew better and had more colonies compared to pET28a.

Fig.10a shows the growth of recombinant pET28a-UGPase and non-recombinant pET28a in LB medium under non-stressed conditions. The growth status of pET28a was similar to that of pET28a-UGPase. Compared with pET28a, pET28a-UGPase formed smaller colonies on the plate, and the number of colonies formed by both was basically the same.

After high-temperature (Fig.10b 50°C, 40 min) and low-temperature (Fig.10c -20°C, 24 h)

treatments, the cultures were coated with gradient dilutions and incubated on LB medium. Compared with pET28a, pET28a-UGPase showed significantly better survival and higher persistence. When cultures were diluted up to thousandfold, pET28a formed fewer colonies on the plates, while pET28a-UGPase colonies were densely packed. It was shown that the expression of UGPase gene enhanced the tolerance of *E. coli* to high and low temperature stress.

The response of UGPase proteins to high osmotic pressure (Fig.10d) was examined by adding 1,200 mmol/L sorbitol to LB medium. In sorbitol-induced high osmotic stress, pET28a-UGPase grew significantly better compared with pET28a. In plates with different dilutions, the growth situation of pET28a was similar to that of blank medium, whereas pET28a-UGPase growth was somewhat restricted. It suggests that UGPase gene expression provides tolerance to hyperosmotic stress in *E. coli*.

In high salt medium containing 500 mol/L NaCl (Fig.10e), pET28a grew better than pET28a-UGPase. The pET28a-UGPase colonies decreased significantly with increasing dilution, and at 10⁻⁶-fold dilution, pET28a-UGPase hardly grew. Meanwhile at different dilutions, pET28a had significantly more colonies than pET28a-UGPase.

The researchers quantitatively analysed the growth of colonies in different treatments was quantitatively analyzed using plate counting method, and selected colonies within countable range were selected for statistical analysis. The number of colonies in different treatment groups is shown in Fig. 11. In the environment of high and low temperature and high osmotic stress simulated by sorbitol, the number of colonies of pET28a-UGPase was significantly more than that of pET28a. It was shown that the expression of UGPase gene could enhance the tolerance of *E. coli* to high and low temperature and hyperosmotic stress.

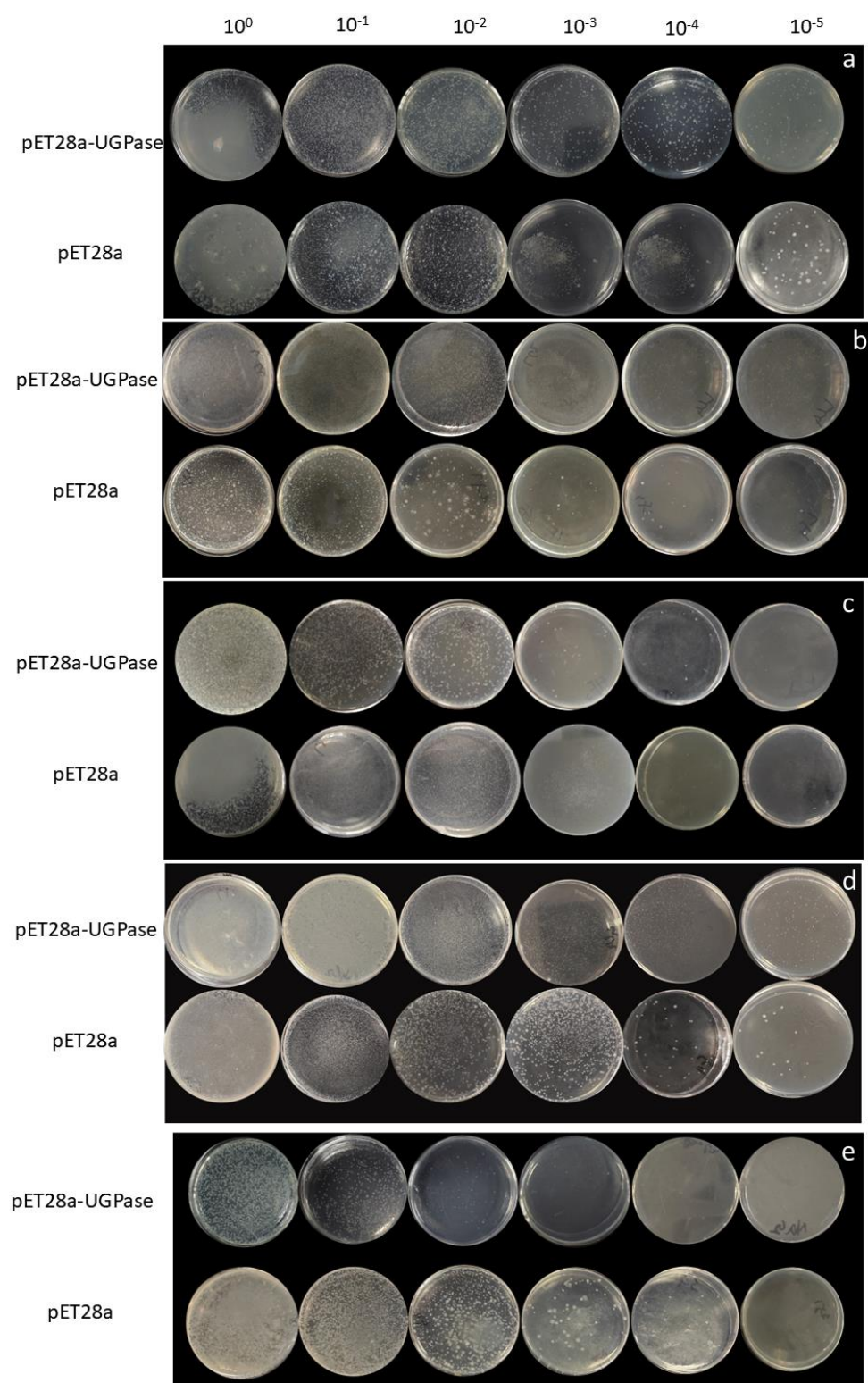


Fig.10 Functional characterization of UGPase in *E. coli* Transetta

Note:a:Control; b: -20 °C for 24 h stress; c: 50 °C for 40 min stress;

d: 1200 mmol/L sorbitol stress; e: 500 mmol/L NaCl stress

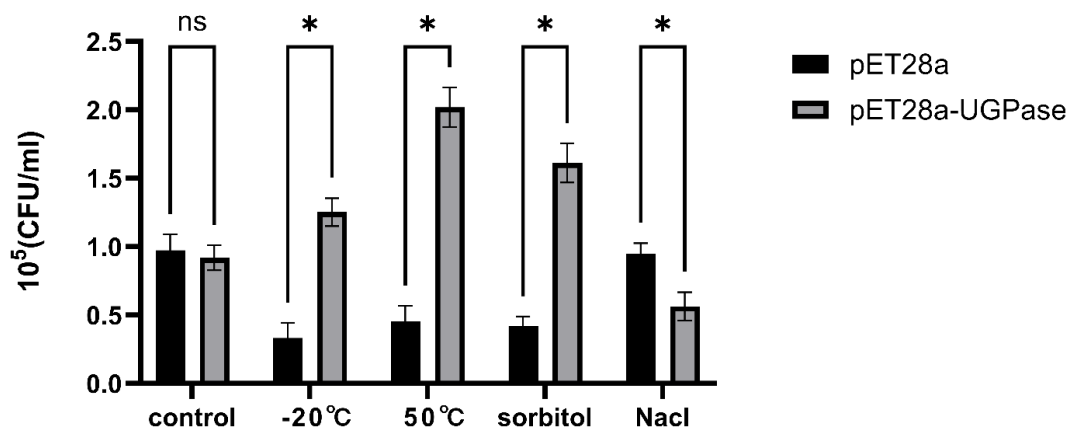


Fig. 11 Comparison of the number of colonies in different environments

Discussions

Dendrobium officinale holds significant medicinal value, with polysaccharides recognised as one of its most active components. UGPase is a key link in the glucose metabolism pathway, so the study of UGPase in *D. officinale* is of great significance. The effect of light quality on the UGPase of *D. officinale* has been previously understood by the researchers' group¹⁴.

UGPase uses UTP and glucose-1-P as substrates in the forward direction and UGPase and pyrophosphate (PPi) in the reverse direction. Thus, UGPases are located at key junctions in plant metabolism and are indirectly involved in the synthesis of complex carbohydrates as well as in sugar and energy metabolism¹⁹. The UDP-catalysed production of UDP-glucose is vital for the sugar metabolism cycle in plants, facilitating the formation of the primary cell wall during early development and promoting polysaccharide accumulation throughout growth stages. For these reasons, the aim of the present study was to understand the nature of the protein and to gain a preliminary understanding of its resistance. Notably, the overall morphology of many UGPase proteins resembles a bowl, featuring a concave interior that can be divided into three major structural domains: N-terminal structural domains, central structural domains with nucleotide-binding loops and C-terminal structural domains with insertion loops. These structural domains constitute the catalytically active region of the UGPase, which is the UGPase functional domain^{20, 21}. Whereas the N-terminal and C-terminal domains of UGPase enzymes from different plants have enzyme-specific folds, they may have different regulatory functions in different pyrophosphorylases²².

The UGPase proteins are UGPase-A and UGPase-B, both localised in the cytoplasm²³. However,

later studies have demonstrated that the localisation of the UGPase is not the same in different plants. For example, the rice mutant UGPase protein is localised to starch grains²⁴ and the Xing'an Larix UGPase proteins may be found in chloroplasts¹¹. The difference in their localisation is probably due to the different structures formed at the N-terminal or C-terminal of the protein sequence. The functional diversity of different enzymes and isoenzymes involved in UGPase synthesis in plants is influenced by subcellular localisation²⁵, which coincides with the place where it functions. The cytoplasmic membrane is crucial for maintaining the cellular homeostasis and regulating cellular activities, suggesting a potential link between UGPase localisation and its role in stress resistance.

In plants, UGPase proteins are directly involved in carbohydrate metabolism and polysaccharide synthesis, and their metabolites indirectly affect redox and growth and developmental status in vivo²⁶. For example, uridine diphosphate glucose (UDP-Glc), generated by the UGPase reaction, is a key sugar molecule involved in more than 270 reactions in plants and is a direct precursor for the synthesis of polysaccharides, glycoproteins, glycolipids, and cell wall components^{27, 28}. It can also act as signaling molecules with potential roles in mediating ROS signaling and programmed cell death (PCD)²⁹. Proteins exhibit different enzymatic activities in vivo and in vitro³⁰. On the one hand, because of the different environments, enzyme activity in vitro may be affected by a variety of factors such as ionic composition, pH, etc., which may be regulated by intracellular homeostatic mechanisms in vivo^{31, 32}. On the other hand, during purification, protein activity is affected due to prolonged exposure of proteins to extreme conditions or changes in protein structural conformation³³. Therefore, the UGPase enzyme activity assayed in vitro does not represent the true activity it exerts in vivo.

The *E. coli* expression system (prokaryotic expression system) is renowned for its ability to produce high levels of exogenous proteins, thanks to its well-characterised genetic background and biochemical properties, along with its operational simplicity. It is therefore commonly used for functional and mechanistic studies of plant resistance-related genes³⁴⁻³⁸. The results of Adriana et al.³⁹ showed that expression of plant LEA proteins directly contributes to increase stress tolerance in bacterial host cells. Additionally, the ApCystatin gene from *Bacopa monnieri* was overexpressed in recombinant *E. coli* Transetta (DE3) to improve the tolerance of *E. coli* to osmotic, drought, and low-temperature stresses⁴⁰. The colonies formed by recombinant bacteria, however, are likely to be affected by transformation efficiency and plasmid stability⁴¹. High salt environments may also affect protein stability and correct folding, and recombinant proteins may form non-functional aggregates or inclusion bodies, affecting

their expression and activity⁴². In this experiment, the researchers successfully constructed prokaryotic expression vectors, seeing that the target genes were effectively expressed under IPTG-induced conditions, and the recombinant strains were significantly more resistant than the control bacteria. Moreover, recombinant bacteria were more tolerant than non-recombiners in simulating high and low temperatures, acidity and high osmotic stress.

UGPase expression is co-regulated by phosphorus content, glucose or sucrose content, as well as light conditions and low-temperature signalling, and these conditions may have a superimposed effect on UGPase expression^{14, 43, 44}. Previous studies have indicated that UGPase activity is regulated by temperature changes in some organisms with abiotic stress and phloem organisation-associated promoter progenitors⁴³. Therefore, it is reasonable to hypothesise that the significant increase in survival of overexpressed strains under abiotic stress is due to the increase in polysaccharide production resulting from overexpression of UGPase. This increase likely contributes to the protection of the strains and, by extension, enhances the plant's tolerance to abiotic stress. In the *D. officinale* over-expressing UGP, the transgenic plants showed certain resistance to mannitol-mimicked osmotic stress and low-temperature conditions⁴⁵, demonstrating that UGP in the phytosome also enhances the plant's tolerance to abiotic stresses.

Conclusion

In this study, the researchers successfully cloned the UGPase gene from *D. officinale* using RT-PCR, resulting in a UGPase protein with a complete coding sequence of 1,419 bp that encodes 472 amino acids. To further elucidate the function of *D. officinale* UGPase proteins, the purified proteins were assayed for enzymatic activity, the researchers conducted bioinformatics analyses and verified the expression of recombinant proteins alongside their resistance to abiotic stress.

This research lays a theoretical foundation for understanding the structure of *D. officinale* UGPases and contributes to expanding the existing database on these proteins.

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Author contributions

The study design was contributed by LC, JSG, HT, and HZ; funding was obtained by HZ; the experiments were performed by SH, LC and RL; data analysis was conducted by SH, LC, HW and ZD; the manuscript was written by SH and LC. The article was contributed to by all authors and the submitted version was approved by them.

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Data availability

All raw data used in this study can be found in the following repositories. UGPase gene sequence of *D.*

590 *officinale* was deposited into in the NCBI repository (National Center for Biotechnology Information)
591 under accession number: GenBank:KF711982.1 (Dendrobium officinale isolate T03-(1)
592 pyrophosphorylase (UGP) mRNA, co - Nucleotide - NCBI)

593 **Declarations**

594 **Ethical approval**

595 This article does not contain any studies with human participants or animals performed by any of the
596 authors.

597 **Ethics approval and consent to participate**

598 The field and laboratory studies were carried out in accordance with all applicable institutional, national,
599 and international laws.

600

601 **Conflict of interest**

602 The authors declare no competing interests.

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