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Cloning cDNA and functional characterization of UDP-glucose pyrophosphorylase in *Dendrobium officinale*

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Abstract

Dendrobium officinale is a traditional Chinese medicinal herb that produces promising bioactive polysaccharides. However, the biosynthetic pathway of polysaccharides in this herb remains to be elucidated. The uridine diphosphate glucose pyrophosphorylase (UGPase) is a key enzyme for the production of uridine diphosphate glucose, which is a major glycosyl donor for synthesis of polysaccharides. This study identified a novel *UGPase* gene from *D. officinale* termed as *DoUGP*. Bioinformatics and subcellular-localization of the DoUGP protein indicate that it belongs to the UGPase-A type and was localized in cytoplasm. The *DoUGP* was revealed to be constitutively expressed in all organs, and the highest mRNA content was detected in stems, the organs with the highest polysaccharide content. Furthermore, sucrose feeding experiments in *D. officinale* demonstrate that sucrose addition could increase *DoUGP* transcription significantly and enhance polysaccharide accumulation accordingly. Together, we conclude that *DoUGP* probably plays an important role in polysaccharide biosynthesis of *D. officinale* and is a potential target for quality breeding of this orchid.

Additional key words: gene expression, polysaccharide synthesis, subcellular localization.

Introduction

Dendrobium officinale is an endangered traditional Chinese medicinal herb (Chen *et al.* 2011, Xia *et al.* 2012). Previous studies (Xing *et al.* 2013) have primarily focused on the extraction of polysaccharides, structure determination, and analysis of their bioactivity, whereas biosynthetic pathways remain to be elucidated. *D. officinale* polysaccharides are water-soluble and are composed of more than 60 % of glucose and a small proportion of other monosaccharides such as galactose, mannose, arabinose, *etc.* (Huang *et al.* 1997, Yang *et al.* 2004). Thus, presumably consistent with common sugar metabolic pathways in this herb, the core biosynthesis pathway of polysaccharides occurs in cytosol with uridine diphosphate glucose (UDPG), being an important direct or indirect donor of the glycosyl moiety (Delmer and Amor 1995). Uridine diphosphate glucose pyrophosphorylase (UGPase) catalyze reversible production of UDPG and pyrophosphate from glucose-1-phosphate (Glc-1-P) and uridine triphosphate (Kleczkowski 1994). Extensive studies have revealed that UGPase is a key enzyme in synthesis of structural or storage polysaccharides such as cellulose and starch (Johansson 2003, Kleczkowski *et al.* 2004). Furthermore, several studies have revealed that it is also involved in synthesis of bioactive polysaccharides in *Zizyphus vulgaris* (Tomoda *et al.* 1973), *Astragalus membranaceus* (Wu

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Abbreviations: Glc-1-P - glucose-1-phosphate; RACE - rapid amplification of DNA ends; qPCR - quantitative PCR; UAGPase - UDP-N-acetyl-gucosamine pyrophosphorylase; UDPG - uridine diphosphate glucose; UGPase - uridine diphosphate glucose pyrophosphorylase; USPase - UDP-sugar pyrophoshorylase.
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et al. 2002), *etc.* Activity of UGPase is positively correlated with polysaccharide content in hairy roots of *Astragalus membranaceus* (Wu *et al.* 2000). The UGPases are divided into two distinct subclasses, UGPase-A and UGPase-B, both of which show the same catalytic function but have low homology (Kleczkowski *et al.* 2010). The UGPase-A type, mainly located in cytosol, Golgi apparatus, and cell wall, is involved in sucrose synthesis and degradation and convert Glc-1-P to UDPG for synthesis of polysaccharides (Kleczkowski *et al.* 2010). UGPase-B type, with a distinct chloroplastic location, mainly participates in biosynthesis of the precursor of the polar head of sulfolipids by producing UDPG (Okazaki *et al.* 2009, Kleczkowski *et al.* 2010).

Despite many investigations focusing on its role in sugar metabolism in various plant species, studies on the UGPase gene (*UGP*) in *D. officinale* have been limited, and its role in *D. officinale* polysaccharide synthesis remains unclear. Accordingly, in order to understand the *D. officinale* polysaccharide biosynthesis better, the first step was to check UGPase-A which is involved in synthesis of the main polysaccharide precursor UDPG. Our specific approaches included cloning a novel *UGP* from *D. officinale* (designated as *DoUGP*) and subsequently characterizing its structure, cellular localization, phylogenetic position, and expression patterns in different organs, and finally determining the correlation between its expression and polysaccharide content concomitant to sucrose feeding.

Materials and methods

Plants and treatments: *Dendrobium officinale* Kimura & Migo plantlets were derived from plants grown in a greenhouse in Sichuan Province, China and whose provenance was a wild individual from Yunnan Province, China. The seeds were cultivated in a growth chamber (a 12-h photoperiod, an irradiance of 40 μmol m^{-2} s^{-1}, and a temperature of 25 °C) *in vitro* on half strength Murashige and Skoog (1962) medium supplemented with 2.0 mg dm^{-3} naphthaleneacetic acid, 0.5 mg dm^{-3} benzyladenine, 6 g dm^{-3} agar, 2 g dm^{-3} activated carbon, and 30 g dm^{-3} sucrose, with pH adjusted to 5.8. After two months, protocorms emerged from the seeds and later formed plantlets. After seven months, the plantlets were harvested for cloning and expression analysis. For sucrose feeding experiments, uniform plantlets of *D. officinale* at the age of 10 months were subcultured on a medium described above, supplemented with varying concentrations of sucrose (0, 30, and 50 g dm^{-3}). The plantlets were cultured in 10 bottles for each treatment, with 6 plantlets per bottle. The stems of each treatment were sampled after bimestrial cultivation for determination of water-soluble polysaccharide content and *DoUGP* expression. For subcellular localization experiments, plants of *Nicotiana benthamiana* Domin and *Arabidopsis thaliana* L. (ecotype Columbia-0, Col-0) were grown in a culture room for four weeks at approximately 25 °C, a 14-h photoperiod, and an irradiance of 80 μmol·m^{-2} s^{-1}.

Amplification, isolation, and cDNA cloning *DoUGP*: The total RNA was extracted from leaves of *D. officinale* using a plant RNA isolation mini kit (*Omega*, Beijing, China) according to the manufacturer's recommendation. The RNA was treated with *RNase-free DNase I* (*TaKaRa*, Tokyo, Japan) to remove genomic DNA contamination. First-strand cDNA synthesis was achieved by an *iScript*™ cDNA synthesis kit (*Bio-Rad*, Hercules, USA) and the resulting cDNA was stored at -20 °C. Degenerate primers (Table 1 Suppl.) were designed based on the multiple sequence alignment of the known *UGP* gene sequences of *Oryza sativa* (GenBank accession No. NM-001070414), *Bambusa oldhamii* (acc. No. AY178448.1), *Saccharum officinarum* (acc. No. NM-001070414), *Dendrocalamus sinicus* (acc. No. EU195533), *Brachypodium distachyon* (acc. No. XM-003578553), *Musa acuminate* (acc. No. AF203909.1), *Solanum tuberosum* (acc. No. AY082622.1), *Gossypium hirsutum* (acc. No. GU067484.1), and *Eucalyptus grandis* (acc. No. EU737107). The primers were synthesized by *Invitrogen Biotechnology* (Shanghai, China). The amplified cDNA fragments were cloned into the pMD19-T vector (*TaKaRa*) and transformed into the *Escherichia coli* strain DH5α. Recombinant colonies were selected for ampicillin resistance, identified by blue/white screening and verified by PCR. Positive clones were confirmed by sequencing (*Sangon Biotech*, Shanghai, China).

A rapid amplification of cDNA ends (RACE) technique was employed to isolate the full-length cDNA of *DoUGP*. Gene-specific primers (Table 1 Suppl.) were designed by the *Primer Premier 5.0* software (*Premier Biosoft International*, Palo Alto, CA, USA) based on sequence information obtained from a previously cloned fragment. The total RNA of leaves was used as template to amplify the *DoUGP* cDNA. Both 3' RACE and 5' RACE were carried out by a *SMARTer*™ RACE cDNA amplification kit (*Clontech*, Beijing, China). The full length cDNA of *DoUGP* was obtained by splicing and verified by cloning and sequencing.

Sequence and phylogenetic analyses: The full-length cDNA of *DoUGP* was translated *in-silico* by the *ORF Finder* tool (http://www.ncbi.nlm.gov/gorf/gorf.html) for open reading frame analysis. After homology analyses by *BLAST* (http://www.ncbi.nlm.nih.gov/blast), *RefSeq* protein sequences from some well-conserved and most

identified isoforms from different typical species with completely sequenced genomes on GenBank were chosen for phylogenetic analysis. These species consist of human and mono-/di-cotyledons including model plants as *Arabidopsis thaliana* and *Populus euphratica*, food crops as *Oryza sativa*, *Glycine max*, and *Solanum tuberosum*, fruit crops as *Vitis vinifera* and *Cucumis melo*, and an industrial crop *Gossypium raimondii*, most of which were analyzed for AtUGP3 classification by Okazaki *et al.* (2009). After aligning by the *ClustalW* program, these sequences were used to construct a phylogenetic tree in the *MEGA 6.05* program using the neighbor-joining method followed by phylogeny test options of 1 000 bootstrap replicates.

Subcellular localization: A C-terminal green fluorescent protein (GFP) fusion of *DoUGP* was assembled into the pCamv35SGFP vector at *Xba*I/*Sma*I sites using T4 DNA ligase (*TaKaRa*) with primers PxbaIf and PSmaIr (Table 1 Suppl.). The resulting vector pCamv35S:DoUGP:GFP was confirmed by sequencing and transformed into the *Agrobacterium tumefaciens* strain EHA105, which was subsequently used for infiltrating leaves of *N. benthamiana* as per the methods reported by Sparkes *et al.* (2006). The plasmid vector pCamv35S:DoUGP:GFP was introduced into protoplasts of *Arabidopsis* Col-0 according to the methods described earlier (Yoo *et al.* 2007). The subcellular localizations of the fused protein and chlorophylls were visualized using laser confocal fluorescence microscopy (*Leica TCS SP8*, Bensheim, Germany) at excitation wavelengths of 488 and 552 nm, respectively.

Expression analysis of *DoUGP*: The real time or reverse transcription (RT)-qPCR was performed on an *iCycler IQ* detection system (*Bio-Rad*) with an *IQ SYBR Green Supermix* kit (*Bio-Rad*). Samples were collected from roots, stems, and leaves, and each sample was analyzed in triplicate. Extraction of RNA and cDNA synthesis were performed as above mentioned. Primers used are listed in Table 1 Suppl. Each PCR reaction was performed in a final volume of 20 mm^3 containing 10 mm^3 of *SYBR Supermix* (2×), 2 mm^3 of cDNA, 6 mm^3 of sterilized double- distilled H_2O, and 1 mm^3 of each of the primers. The amplification conditions were: an initial denaturation at 95 °C for 3 min followed by 39 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 15 s. The *D. officinale Actin* gene (GenBank accession number: KC831582.1) was chosen as reference gene based on a previous study (Jin *et al.* 2013).

Determination of polysaccharide content: Roots, stems, and leaves of the *D. officinale* seedlings were thoroughly washed with distilled water, dried at 60 °C, and powdered with a pulverizer. The powder was extracted with petroleum ether at 70 °C for 2 h to remove lipids. The extract was filtered, the residues were air-dried, refluxed again with 80 % (v/v) ethanol at 90 °C for 2 h, extracted with double-distilled water at 100 °C for 2 h three times, and the resulting solution was finally filtered. The combined filtrate was precipitated by adding four volumes of 95 % (v/v) ethanol and incubating at 4 °C for 24 h. After filtration and centrifugation, the precipitate was dissolved in double-distilled water and deproteinized five times with the *Sevag* reagent. It was subsequently dialyzed against deionized water for 72 h and lyophilized to finally yield the desired crude water-soluble polysaccharides (Luo *et al.* 2011). Polysaccharide content was determined by the phenol-sulphuric acid method (Dubois *et al.* 1956) according to the procedure outlined by Xiao *et al.* (2004).

Statistical analysis: Data were analyzed using the *Microsoft Excel* and *SPSS 16.0* software. The relative expression level of each transcript was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). All results are reported as means of three independent experiments, each of which was done in triplicate. One-way *ANOVA* was used for significant difference analysis followed by the least significant difference (LSD) post hoc test (α = 0.05 or 0.01). Pearson correlation coefficients were used to define relationships between *DoUGP* expression and polysaccharide content.

Results

Dendrobium officinale UGP deposited as KF711982 in the GenBank database contained a 1 419 bp open reading frame encoding 472 amino acid residues. All UGPases, UDP-sugar pyrophosphorylases (USPases, EC2.7.7.64), and UDP-N-acetyl-glucosamine pyrophosphorylases (UAGPases, EC2.7.7.23) belong to the nucleotidyl transferase superfamily (EC2.7.7). These enzymes contain a pyrophosphorylase consensus motif and a nucleotide binding motif involved in activating hexose, thereby resulting in formation of a nucleotide hexose, and all of the three can produce UDPG (Kleczkowski and Decker 2015). Based on the amino acid sequences of the three pyrophosphorylases obtained from different model species, a phylogenetic tree was constructed for classification and functional prediction of DoUGP (Fig. 1). The phylogenetic analyses reveal that USPases, UGPases (either A or B type), and UAGPases were categorized into distinct groups. The DoUGP protein belongs to the UGPase-A type, which is distinct from the previously characterized subfamilies constituting USPases, UAGPases, as well as another type of UGPase, UGPase-B. Furthermore, DoUGP had the closest

evolutionary relationship with a monocotyledon of the same kind, *O. sativa* UGP (confidence coefficient = 94 %).

To confirm cytoplasmic targeting the DoUGP protein, DoUGP-GFP transient expression was performed in *N. benthamiana* leaves and *A. thaliana* (Col-0) protoplasts, respectively. In cells of both the plants, fluorescence derived from GFP was targeted to cytoplasm and was not associated with chloroplasts as indicated by red autofluorescence from their chlorophyll molecules (Fig. 2). Our results demonstrate that DoUGP was localized in cytoplasm as expected. Its cytosolic localization further comfirmed that it was indeed a UGPase-A protein.

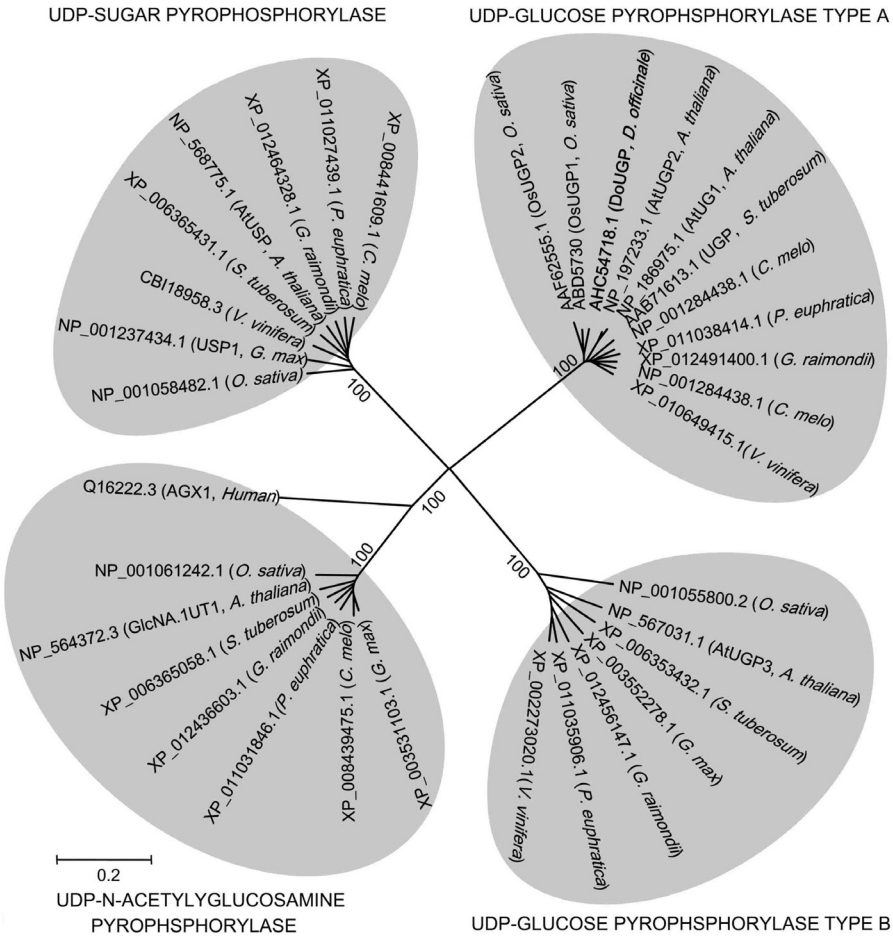

Fig. 1**.** An unrooted phylogenetic tree of UDP-hexose pyrophosphorylases. The phylogenetic tree was constructed by the neighbor-joining method using *MEGA 6.05*. The numbers on the branches represent bootstrap support for 1 000 replicates. The sequence number of every protein from human and plant species is shown, followed by the name of the reported protein, and the name of respective species in parentheses.

The expression patterns of *DoUGP* in roots, stems, and leaves from *D. officinale* were examined and polysaccharide content in the corresponding organs was measured. The results reveal that *DoUGP* was differentially expressed in the three organs, with the highest expression in stems, followed by leaves, and the lowest in roots (Fig. 3*A*). Polysaccharide content in stems was more than 3-fold higher than in roots and leaves, and roots and leaves had similar content (Fig. 3*B*). Therefore, the results suggest that the organ with a high expression of *DoUGP* also showed the highest polysaccharide content.

Previous studies have found that sucrose could be used as a specific elicitor for induction of upregulated expression of *UGP via* a hexokinase-independent pathway (Ciereszko *et al.* 2001) and promote synthesis of polysaccharides (Spychalla *et al.* 1994, Yang and Sun 2014). To further clarify functional correlation between *DoUGP* and polysaccharide biosyntheses, the tissue-cultured plantlets of *D. officinale* were fed with sucrose at different concentrations and the expression of *DoUGP* and polysaccharide content in stems were measured. The results indicate that *DoUGP* was strongly upregulated by increasing sucrose concentrations and reached the highest expression at 50 g dm^{-3} sucrose, which was about two times higher than without sucrose feeding (Fig. 3*C*). Polysaccharide content at 30 g dm^{-3} sucrose significantly increased ($P < 0.05$) and polysaccharide content at

50 g dm^{-3} sucrose remarkably increased ($P < 0.01$), as compared with that at 0 g dm^{-3} sucrose (Fig. 3*D*). The Pearson correlation analysis showed significant positive correlations between the expression of *DoUGP* and polysaccharide content ($R^2 = 0.904$).

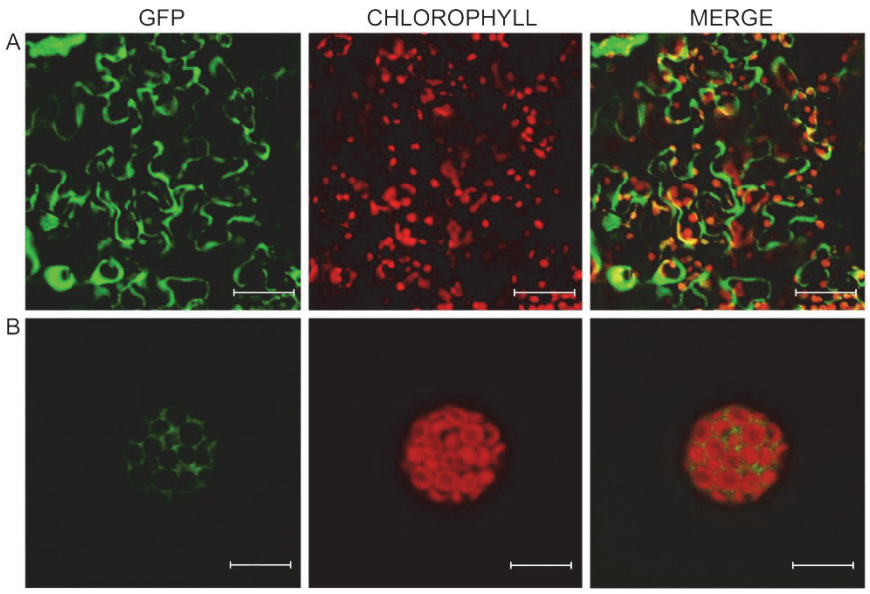

Fig. 2 The subcellular localization of DoUGP-GFP fusion protein transiently expressed in *Nicotiana benthamiana* leaves (*A*) and *Arabidopsis thaliana* Col-0 protoplasts (*B*). The first column shows a green fluorescence image derived from green fluorescent protein, the second shows red autofluorescence from chlorophyll, and the third shows an overlay of the two channels. The *bars* = 50 μm (*A*) and 15 μm (*B*).

Discussion

In order to elucidate the core biosynthesis pathway of *D. officinale* polysaccharides, we isolated a *UGP* in *D. officinale* by RACE because of its important function in saccharide metabolism. Functional motif analyses (data not shown) indicate that DoUGP is similar to UGPase from other species with several conservative catalysis/ substrate binding sites (Katsube *et al.* 1991, Martz *et al.* 2002, Wang *et al.* 2011). A 3D structure model of DoUGP is a typical bowl-shaped monomer, contains three large domains: an N-terminal domain, a centrally located catalytic domain including a nucleotide binding loop, and a C-terminal domain that includes an insertion loop (data not shown). It shows that the structure of DoUGP has a high degree of conservation (Geisler *et al.* 2004, McCoy *et al.* 2007) as previously described in other species (Chen *et al.* 2007, Meng *et al.* 2008). Additionally, phylogenetic analyses also indicate that DoUGP serves as a typical UGPase-A type, contrasting to other pyrophosphorylases, which is consistent with conclusions from previous studies (Geisler *et al.* 2004, Litterer *et al.* 2006a,b, Okazaki *et al.* 2009, Kleczkowski *et al.* 2010, 2011). Furthermore, the cytosolic location of DoUGP is consistent with its functional annotation. Thus, our results demonstrate that DoUGP belongs to the UGPase-A type, which prefers to react with Glc-1-P to produce UDPG providing an activated glycosyl donor for polysaccharide biosynthesis.

It is widely acknowledged that *UGP* is highly expressed both in source organs and in sink organs, resulting in a high enzymatic activity, protein content, and mRNA abundance in several plant species, *e.g.*, barley (Eimert *et al.* 1996), rice endosperm (Abe *et al.* 2002, Chen *et al.* 2007), *Arabidopsis* (Ciereszko *et al.* 2001), and aspen (Meng *et al.* 2007). Stems, as an important sink organ of *D. officinale*, have the highest content of polysaccharides and are the preferred organs of medicinal use. Our results show that *DoUGP* expression was highest in stems and positively correlated with the highest polysaccharide content. A similar result has also been reported in *Astragalus membranaceus* (Wu *et al.* 2000). Expression of genes varies among species and organs within a species, being usually consistent with its function (Pua *et al.* 2000). Therefore, *DoUGP* may play an important role in biosynthesis of *D. officinale* polysaccharides.

It has been characterized that the UGPase reaction is channeled towards formation of Glc-1-P or UGPG, involved either in synthesis or in degradation of sucrose, respectively (Kleczkowski *et al.* 2004). Catalytic activity of UGPase appears to be triggered by binding of UTP or

UDPG prior to binding of Glc-1-P or pyrophosphate (Tsuboi *et al.* 1969). Therefore, with a sufficient sucrose supply, UDPG is likely to be produced from sucrose by sucrose synthase, which subsequently forms Glc-1-P by UGPase, and then, Glc-1-P is finally utilized in synthesis of polysaccharides by glycosyltransferases (Kleczkowski and Decker 2015). Previous studies revealed that polysaccharide production both from protocorms of *D. officinale* and from protocorm-like bodies of *D. huoshanense* increases in suspension cultures by feeding sucrose (Zha *et al.* 2007, He *et al.* 2007b). Likewise, in our study, when fed with sucrose, *DoUGP* transcription in stems of *D. officinale* was upregulated and positively correlated with *D. officinale* polysaccharide content. Yang *et al.* (2012) found that sucrose synthase and invertases are the most important enzymes in biosynthesis of polysaccharides in *D. officinale*, and also that degradation of sucrose to monosaccharides is a starting point in this pathway. Based on the above, *DoUGP*, at the center position in the sucrose metabolic pathway, is probably involved in polysaccharide biosynthesis of *D. officinale*.

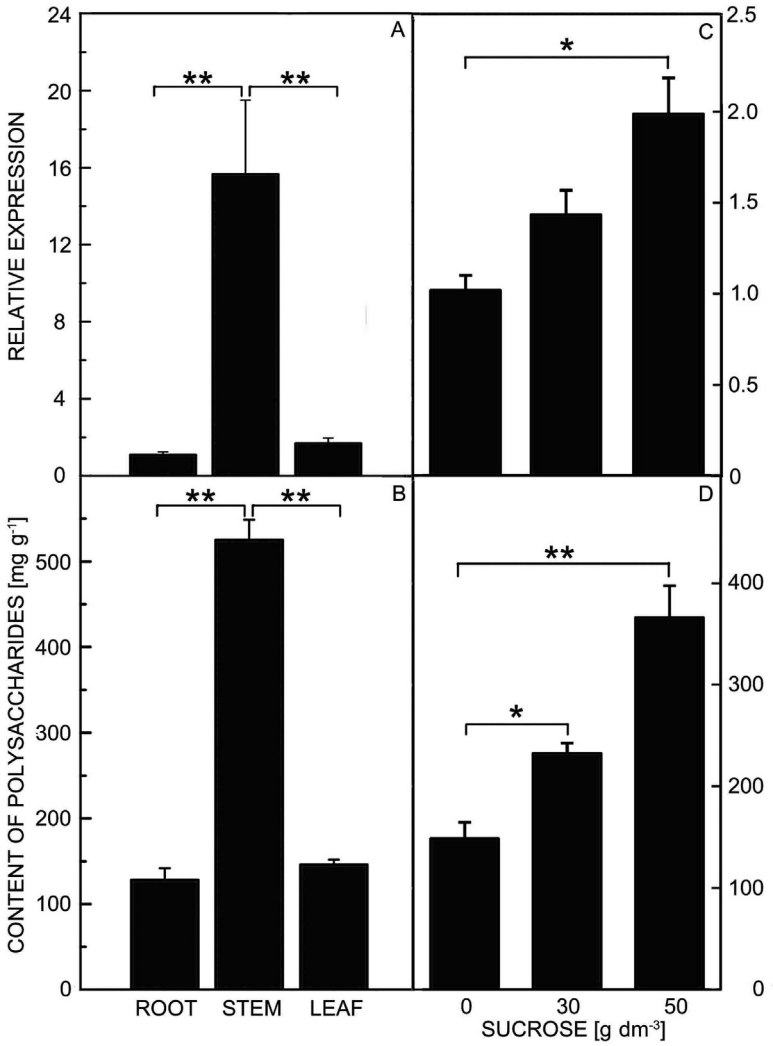

Fig. 3. The relative expression of *DoUGP* and the content of *D. officinale* polysaccharides in different organs (*A*, *B*) or in stems under sucrose feeding at different concentrations (*C*, *D*). The expression of *DoUGP* was normalized to that of *Actin*. The expression of *DoUGP* in roots was set to 1 and also in the control without sucrose treatment. Means ± SDs, $n = 9$. *, ** significant differences at $P < 0.05$ and $P < 0.01$, respectively.

Additionally, 30 g dm^{-3} sucrose is usually used for tissue culture plantlet production. However, in our studies, compared with the 30 g dm^{-3} sucrose treatment, the 50 g dm^{-3} sucrose treatment not only promoted accumulation of *D. officinale* polysaccharides, but also enhanced stem diameter enlargement with none negative growth effects (data not shown). Therefore, we suggest that sucrose content needs to be appropriately increased for high quality *in vitro* seedlings of *D. officinale*.

In conclusion, a novel *DoUGP* in *D. officinale* was

cloned, located in cytoplasm, and corresponding UGP showed the functional characteristic of the UGP-A type. It predicts to be of a vital importance in biosynthesis of polysaccharides. Polysaccharide composition represents an important parameter of *D. officinale* quality. Our work may extend our knowledge towards understanding the poorly-studied polysaccharide biosynthesis process in *D. officinale* and facilitate breeding of the *Dendrobium* for improved economic properties.

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