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Characterization of the alkaline/neutral invertase gene in *Dendrobium officinale* and its relationship with polysaccharide accumulation

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ABSTRACT. *Dendrobium officinale* is one of the most well-known traditional Chinese medicines, and polysaccharide is its main active ingredient. Many studies have investigated the synthesis and accumulation mechanisms of polysaccharide, but until recently, little was known about the molecular mechanism of how polysaccharide is synthesized because no related genes have been cloned. In this study, we cloned an alkaline/neutral invertase gene from *D. officinale* (*DoNI*) by the rapid amplification of cDNA ends (RACE) method. *DoNI* was 2231 bp long and contained an open reading frame that predicted a 62.8-kDa polypeptide with 554-amino acid residues. An alkaline/neutral invertase conserved domain was predicted from this deduced amino acid sequence, and DoNI had a similar deduced amino acid sequence

to *Setaria italica* and *Oryza brachyantha*. We also found that *DoNI* expression in different tissues was closely related to DoNI activity, and more importantly, polysaccharide level. Our results indicate that *DoNI* is associated with polysaccharide accumulation in *D. officinale*.

Key words: *Dendrobium officinale*; Polysaccharide; Alkaline/neutral invertase

INTRODUCTION

Dendrobium officinale Kimura et Migo stems are rich in active compounds, particularly polysaccharides. Water-soluble polysaccharides from *D. officinale* are well known in China because they enhancing immune functions of body (Liu et al., 2011). Polysaccharides are usually synthesized from glucose, mannose, and fructose, which are all hydrolyzed monosaccharides or are derived from sucrose (Hua et al., 2004; He et al., 2016). There is a close relationship between sucrose metabolism and polysaccharide synthesis.

Sucrose is hydrolyzed to glucose or fructose for use in plant growth. Enzymes such as sucrose invertase are involved in this process (Tymowska-Lalanne and Kreis, 1998), and alkaline/neutral invertase (NI) is involved in sucrose hydrolysis in the cytoplasm (Egger and Hampp, 1993; Du et al., 2010). NI regulates sucrose metabolism in many plants, including *Saccharum officinarum*, *Beta vulgaris*, and *Citrus reticulata* (Hubbard et al., 1991), and plays an important role in monosaccharide-dependent biosynthesis (Nonis et al., 2007). In order to investigate the role of NI in polysaccharide-synthesis regulation, several *NI* cDNAs have been cloned from *Solanum lycopersicum*, *Hevea brasiliensis*, *Saccharum officinarum*, and *Oryza sativa* (Gallagher and Pollock, 1998; Murayama and Handa, 2007; Bocock et al., 2008; Liu et al., 2015), and differential *NI* expression has been reported in different plant tissues, e.g., in tomato, higher mRNA levels have been detected in the fruit and leaves than in the flowers and stems. In contrast, in *Hevea brasiliensis*, the highest mRNA levels were detected in the bark and leaves.

In this study, we cloned *NI* cDNA from *D. officinale* (*DoNI*) by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). We investigated *NI* gene expression and enzyme activity, and polysaccharide level, in different *D. officinale* tissues, in order to elucidate its role in polysaccharide synthesis.

MATERIAL AND METHODS

Plant material and RNA extraction

Total RNA was extracted from *D. officinale* by the CTAB method, verified by ethidium bromide staining on a 1% agarose gel, and scanned using an AlphaImager® HP System (Alpha Innotech, USA). RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Approximately 2 mg total RNA was used as a template for first-strand cDNA synthesis with a Prime Script™ 1st cDNA Synthesis Kit (TaKaRa, Japan), following the manufacturer instructions.

Sequencing of a partial cDNA sequence

To obtain a partial cDNA sequence, primers (Table 1) were designed based on *NI*

sequences that were conserved in other plant species. Synthesized cDNA (described above) was used as a template, and a PCR was conducted in the following manner: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 1 min, and then 72°C for 7 min. A generated DNA fragment of the expected size was used to construct a recombinant plasmid with a pMD-19 T vector (TaKaRa, Japan), transferred to *Escherichia coli* DH5α, and sequenced by Sangon Biotech Co. Ltd. (Shanghai, China).

Table 1. Primers used for the sequencing of *Dendrobium officinale* alkaline/neutral invertase *(DoNI*).

Primer	Primer sequence	Used for	GenBank accession No.
RACE-NI-F	3'-GCCTACGCGTGGCGGCTACTTCATT-5'	RACE	KT288204
RACE-NI-R	3'-GGTCTACCCGCTTTTCCCAGCCTTG-5'	RACE	KT288204
q-NI-F	3'-GCCATTTTATCATCACTTGCTACTC-5'	qPCR	KP742351
q-NI-R	3'-ACAATCCGCCATTCATGTCCCTCAA-5'	qPCR	KP742351
q-Ds18s-F	3'-GCTGTTTGATGAGCCTGCGTAGTAT-5'	qPCR	KC465231
q-Ds18s-R	3'-GTGTGGCTGATCATCCGAAAAGAC-5'	qPCR	KC465231
RT-NI-F	3'-AACTCTTTCGAGCCACATCCAATG-5'	RT-PCR	KP742351
RT-NI-R	3'-CCTGAATCTCAATCGGATACCCATA-5'	RT-PCR	KP742351
RT-Ds18s-F	3'-GTCTTTTCGGATGATCAGCCACAC-5'	RT-PCR	KC465231
RT-Ds18s-R	3'-GTAGACCAAGGGCGAACACTCAT-5'	RT-PCR	KC465231

RACE, rapid amplification of cDNA ends; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription PCR.

RACE

The full-length *NI* cDNA sequence was cloned by the RACE method. Primers (Table 1) were designed from partial *NI* sequences, as described above. Synthesized cDNA was used as a template, and 3'- and 5'-RACE was performed using a SMART™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer instructions. PCR fragments were subcloned into the pMD-19 T vector, transferred to *E. coli* DH5α, and sequenced by Sangon Biotech.

Analysis of the cDNA sequence

BLASTn and BLASTp online software was used to identify the nucleotide and deduced amino acid sequence of our cloned cDNA sequence. Multiple alignments between DoNI and its homologs were conducted using DNAMAN software, and nucleotide and deduced amino acid sequence profiles were analyzed by DNASTAR software. A phylogenetic tree was constructed using the neighbor-joining algorithm in the MEGA software.

Quantitative PCR (qPCR)

To normalize differences in the amount of total RNA that was reverse-transcribed and added to each reaction, 18S RNA from *D. officinale* (GenBank No. JX399880) was used as an endogenous control. All of the qPCR assays were performed using an ABI PRISM® 7500 Fast Real-Time PCR System (Applied Biosystems, USA) in 20-μL reactions. Each 20-μL reaction contained 2 μL template cDNA, 10 μL THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Japan), and 0.5 μM each forward and reverse primer. Three replicates were included, and qPCR Ct values were analyzed using the $2^{-\Delta\Delta Ct}$ method (Shi et al., 2013) and further tested by performing a one-way analysis of variance (ANOVA).

Enzyme activity assay

NI enzyme activity in *D. officinale* leaves, stems, and roots was determined according to the method described by Lowell et al. (1989). Three independent experiments were conducted.

Polysaccharide content

The polysaccharide content of *D. officinale* leaves, stems, and roots was determined according to the method described by The State Pharmacopoeia Commission of PR China (2010). Again, three independent experiments were conducted.

Data analysis

Significant differences between treatments were identified using ANOVA in SPSS 17.0, and significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

cDNA sequence analysis

Full-length *DoNI* cDNA was 2231 bp long and contained a 1665-bp open reading frame that encoded a 554-amino acid residue protein. A 319-bp 3'-untranslated region (UTR) and a 247-bp 5'-UTR were at the 3'- and 5'-ends, respectively, of the cDNA sequences (Figure 1). The deduced amino acid sequence was then searched in the National Center for Biotechnology Information database; it showed a high degree of identity with the *NI* sequences of other plants.

Multiple alignments

The EditSeq function in DNASTAR revealed that DoNI was 554 amino acid residues long. The amino acid sequence was then compared with those from *Elaeis guineensis*, *Phoenix dactylifera*, *S. italica*, *O. brachyantha*, and *Musa acuminata* by multiple alignments. The results showed that the DoNI amino acid sequence had a high similarity with those from the other species, and indicated that they have similar functions (Figure 2).

Evolutionary relationships

The NI proteins of all of the sequences we selected were classified into two major groups; the DoNI protein was clustered with *S. italica* and *O. brachyantha*, which are both monocotyledons (Figure 3). Our results also showed that all of the dicotyledons were clustered into another group. This suggests that our analysis was accurate, as the results were similar to those of previous studies (Huang and Ke, 2014; Pang et al., 2014).

DoNI expression and polysaccharide accumulation

Polysaccharide level and NI mRNA and enzyme activity were highest in *D.*

officinale stems (Figure 4A, B, C), indicating that DoNI is associated with polysaccharide accumulation. Since polysaccharide, which is the most remarkable material in *D. officinale*, often with a complex structure (Zha et al., 2012), our results will facilitate the researches about polysaccharide and *D. officinale*.

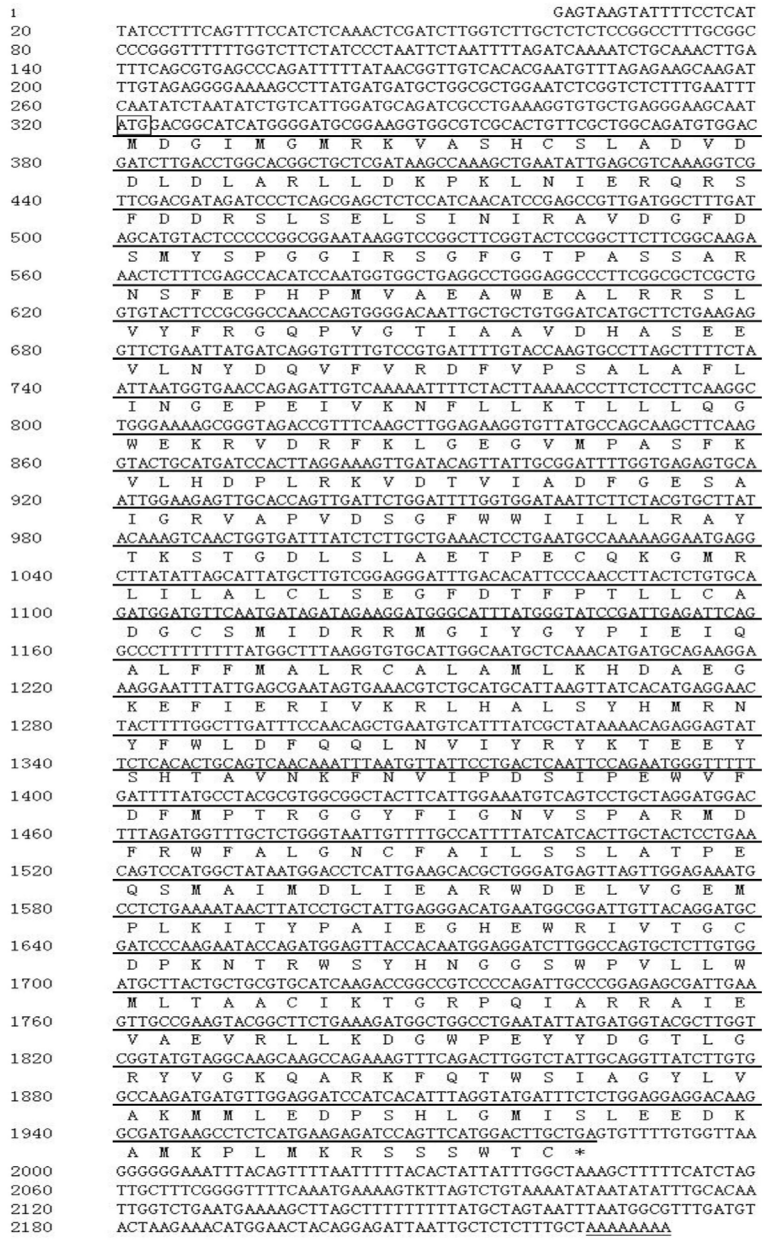

Figure 1. *Dendrobium officinale* alkaline/neutral invertase **(***DoNI*) nucleotide sequence and its deduced amino acid sequence. Initiation and termination codons are boxed and open reading frames are underlined.

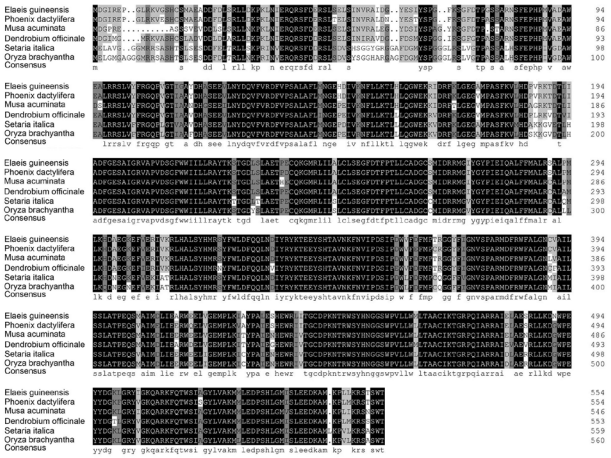

Figure 2. Multiple alignments of *Dendrobium officinale* alkaline/neutral invertase **(**DoNI) and alkaline/neutral invertases (NIs) from other plant species that were obtained from GenBank. NI sequences from the following species were used: *Dendrobium officinale*, *Elaeis guineensis* (XP_010911894), *Phoenix dactylifera* (XP_008783399), *Cucumis melo* (XP_008461922), *Citrus sinensis* (XP_006473179), *Eucalyptus grandis* (KCW69034), *Beta vulgaris* (XP_010679425), and *Vitis vinifera* (XP_002271919).

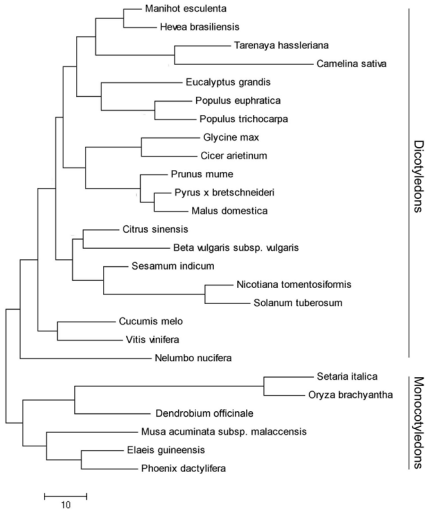

Figure 3. Phylogenetic analysis of *Dendrobium officinale* alkaline/neutral invertase (DoNI) and alkaline/neutral invertase (NI) proteins from other plant species. Almost all of the NI amino acid sequences were obtained from GenBank, and included *Elaeis guineensis* (XP_010911894), *Phoenix dactylifera* (XP_008783399), *Cucumis melo* (XP_008461922), *Citrus sinensis* (XP_006473179), *Eucalyptus grandis* (KCW69034), *Beta vulgaris* (XP_010679425), *Vitis vinifera* (XP_002271919), *Manihot esculenta* (ABA08442), *Glycine max* (XP_006606227), *Musa acuminata* (XP_009410670), *Prunus mume* (XP_008218919), *Hevea brasiliensis* (ADC68261), *Pyrus* x *bretschneideri* (XP_009379235), *Populus euphratica* (XP_011009346), *Malus domestica* (XP_008339170), *Sesamum indicum* (XP_011096585), *Cicer arietinum* (XP_004496345), *Nicotiana tomentosiformis* (XP_009624723), *Solanum tuberosum* (XP_006350339), *Populus trichocarpa* (EEF00365), *Nelumbo nucifera* (XP_010256464), *Tarenaya hassleriana* (XP_010521142), *Setaria italica* (XP_004952843), *Oryza brachyantha* (XP_006647359), and *Camelina sativa* (XP_010498746).

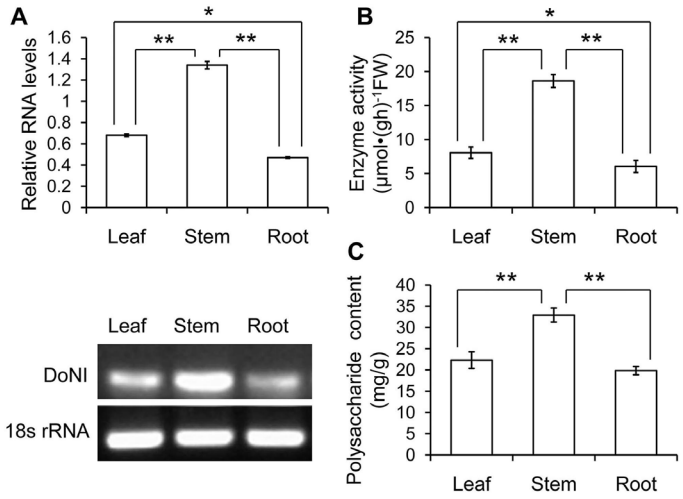

Figure 4. Relationship between *Dendrobium officinale* alkaline/neutral invertase (*DoNI*) expression and polysaccharide accumulation. A. *DoNI* expression in *Dendrobium officinale* leaves, stems, and roots. B. Alkaline/neutral invertase activity in *D. officinale* leaves, stems, and roots. C. Polysaccharide content of *D. officinale* leaves, stems, and roots. Means ± SEMs are presented from three independent experiments; **P < 0.01, *P < 0.05.

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