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Differential Expressed Protein in Developing Stages of *Nepenthes gracilis* Korth. Pitcher

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Abstract: *Nepenthes gracilis* Korth. is a member of carnivorous plants in family Nepenthaceae. The plants have beautiful and economically important pitchers. It is interesting to study the protein(s) correlated with the pitcher. Crude proteins were extracted from leaf, leaf with developing pitcher and developed pitcher of the same plant and analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Two protein bands with molecular weights of 42.7 and 38 kDa were obtained from young leaf and leaf with developing pitcher, respectively. The 42.7 kDa protein was identified as phosphoglycerate kinase (PGK) by Liquid Chromatography Mass Spectrometry (LC-MS/MS), but the 38 kDa band is an unknown protein. Both proteins were differentially expressed in each developing stage of the pitcher, thus may be powerful candidates play role in development pathway of leaf and pitcher.

Key words: Nepenthaceae, phosphoglycerate kinase, pitcher plant, protein markers, Thailand

INTRODUCTION

Nepenthaceae is represented by a single genus *Nepenthes* which is commonly known as the tropical pitcher plant. It consists of about 85 species (Clarke, 2002) originating from parts of Southeast Asia, Madagascar and Australia. The islands of Sumatra and Borneo contain the largest number of endemic species. The main interesting point of the *Nepenthes* species is their pitchers. The pitcher forms from a swelling at the tip of the leaf mid-vein varying in shape and size. On account of their fascinating beauty, wild *Nepenthes* species are often collected from the forest and sold in the market. Collectors may further breed hybrids to produce a diversity of pitcher characters. Natural hybrids are possible. However, hybrid offsprings rarely succeed to develop into a wild population (Clarke, 2002). As a result, it has become difficult to find *Nepenthes* species growing in the wild. Due to their interesting characteristic as carnivorous plants with attractive pitchers, these plants have high economic importance as ornamentals. Wherever it grows, *Nepenthes* rarely fails to excite the interest and curiosity of people.

Because of their pitcher uniqueness, scientists have interested to study their native, genetics and evolution such as ammonium and amino acid transporter genes (Schulze *et al*., 1999), genetic diversity (Chaveerach *et al*., 2006), species identification and sex determination (Mokkamul *et al*., 2007) and the proteins in pitcher fluid (Hatona and Hamada, 2008). However, the genetic mechanism of the pitcher development has not been revealed.

This research aims to find a possibly candidate somehow involve in the pitcher development of *Nepenthes gracilis* by studying protein profile from three developing stages of pitcher. The early stage is young leaf, the developing stage is leaf with developing pitcher, and the mature stage is developed pitcher. Theoretically, pitcher is a part of a leaf; therefore these three stages should show similar protein profile. The hypothesis is that differentially expressed protein(s) in each sample may involve in development of pitcher.

MATERIALS AND METHODS

Plant materials: Three samples, young leaf, leaf with developing pitcher and developed pitcher (Fig. 1) of an individual *Nepenthes gracilis* were collected since May 2006 from Phu Wua Wildlife Sanctuary, Nong Khai Province in Northeastern Thailand.

Protein analysis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Plant tissue (0.5 g) was ground in 1 mL of extraction buffer

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(50 mM Tris pH 8.0, 8 M urea, 10% SDS) with mortar and pestle. The homogenate was centrifuged at 12000 x g for 10 min. Supernatant was mixed with an equal volume of solubilizing solution (100 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) and heated in boiling water for 2 min. The protein mixtures (20 µL) were subjected to SDS-PAGE with a continuous gradient of 6-18% acrylamide. The gel was stained with Coomassie Brilliant Blue and photographed.

Protein identification by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and data base search: The differentially expressed protein bands were excised from the gel. Trypsin was used for in-gel digestion. The peptide fragments were then analyzed by LC-MS/MS (LTQ Linear Ion Trap Mass Spectrometer, ThermoFinnigan, USA). Based on LC-MS/MS results, a search in nr.FASTA by Biowork™ 3.1 SR1 (ThermoFinnigan) was performed to identify the protein. The protein sequences of *N. gracilis* were analyzed using MEGA software version 4.0 (Tamura *et al*., 2007).

RESULTS

Protein patterns of *N. gracilis*: The three studied samples representing three developing stages of pitcher from *N. gracilis* showed similar protein profiles on SDS-PAGE. However, two differentially expressed protein bands at 42.7 and 38.0 kDa were found in young leaf and leaf with developing pitcher, respectively, but they were in developed pitcher (Fig. 1). In addition, most of the bands in young leaf and leaf with developing pitcher were more intense in the developed pitcher.

Protein identification by LC-MS/MS with database search: The 42.7 and 38.0 kDa protein bands isolated from young leaf and leaf with developing pitcher, respectively, were excised from the gel and were then analyzed by LC-MS/MS as described above. Based on LC-MS/MS results and a search in nr.FASTA by Biowork™ 3.1 SR1 (ThermoFinnigan), the 42.7 kDa protein sequence has considerable homology to phosphoglycerate kinase (PGK) from many plants and other organisms, for example *Arabidopsis thaliana*, *Medicago truncatula*, *Solanum tuberrosum* as shown in Table 1, but the 38.0 kDa protein is unknown. The PGK sequences of *N. gracilis* and the other organisms were aligned and result in 484 characters (Fig. 2). From this result, the PGK of *N. gracilis* shows high correspondence with various plant species.

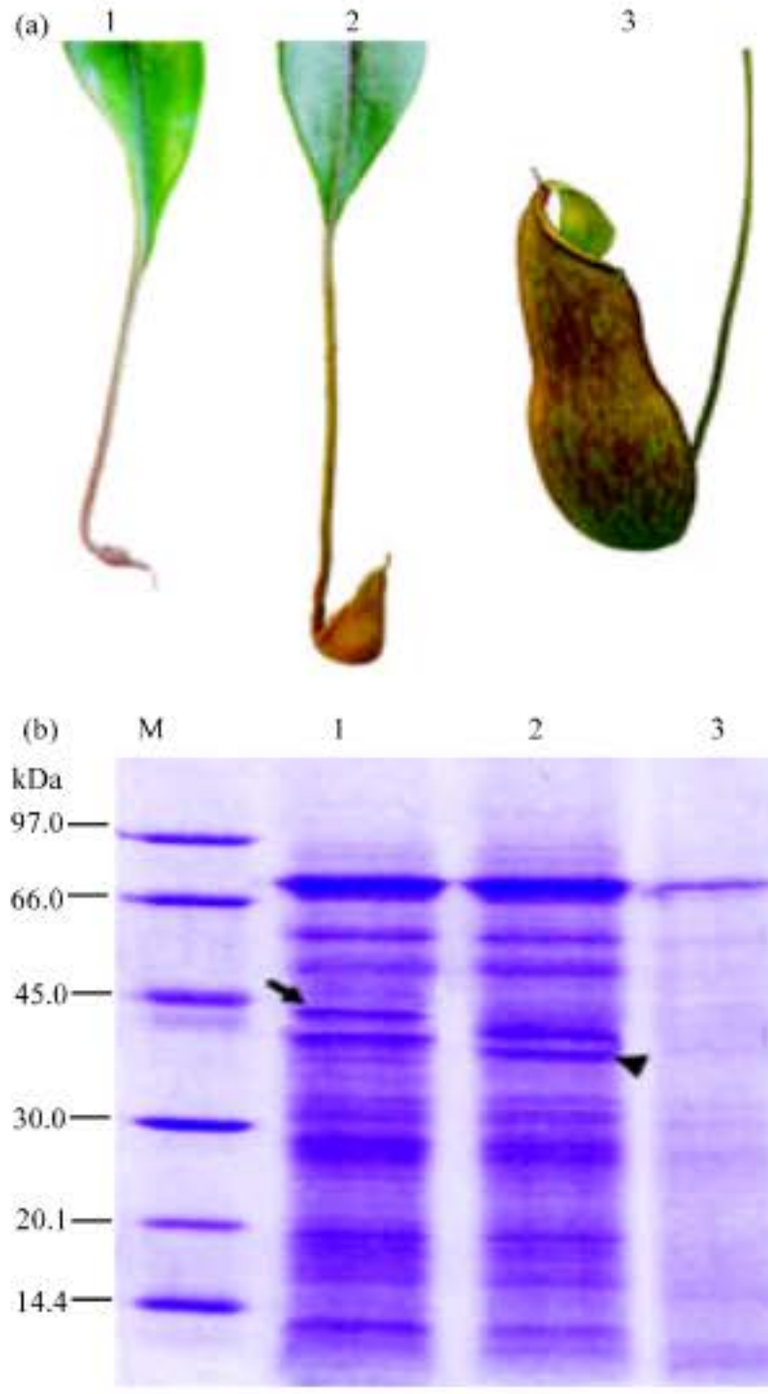

Fig. 1: Three developing stages of pitcher development (A) protein profiles and (B) *Nepenthes gracilis* from young leaf, leaf with developing pitcher and developed pitcher. The 42.7 and 38 kDa bands differentially expressed were indicated by arrow heads. M: Marker; 1: Young leaf; 2: Leaf with developing pitcher and 3: Developed pitcher

Table 1: Identical amino acid fragments of the 42.7 kDa protein from *Nepenthes gracilis* with phosphoglycerate kinase (PGK) fragments from other species

Fragments of 42.7 kDa protein from *Nepenthes gracilis*	Identical PGK fragments from other species
ADLNVPLDDSQNITDDTR	*Spinacia oleracea*
VILSSHLGR	*Medicago truncatula*
LVASLPEGGVLLLENVR	*Medicago truncatula*
FYKEEEKNEPDFAK	*Arabidopsis thaliana*
LASNADLYVNDAFGTAHR	*Volvox carteri* f. *nagariensis*
YLKPSVAGFLLQK	*Medicago truncatula*
ELDYLVGAVSSPK	*Medicago truncatula*
RPFAAIVGGSK	*Solanum tuberosum*
GVSLLLPTDVVVADK	*Arabidopsis thaliana*
FAVGTEAVAK	*Hordeum vulgare* subsp. *vulgare*
KGVTTIIGGGDSVAAVEK	*Arabidopsis thaliana* and *Solanum tuberosum*
VGVAEAMSHISTGGGASLELLEGK	*Spinacia oleracea* and *Synechococcus elongates*

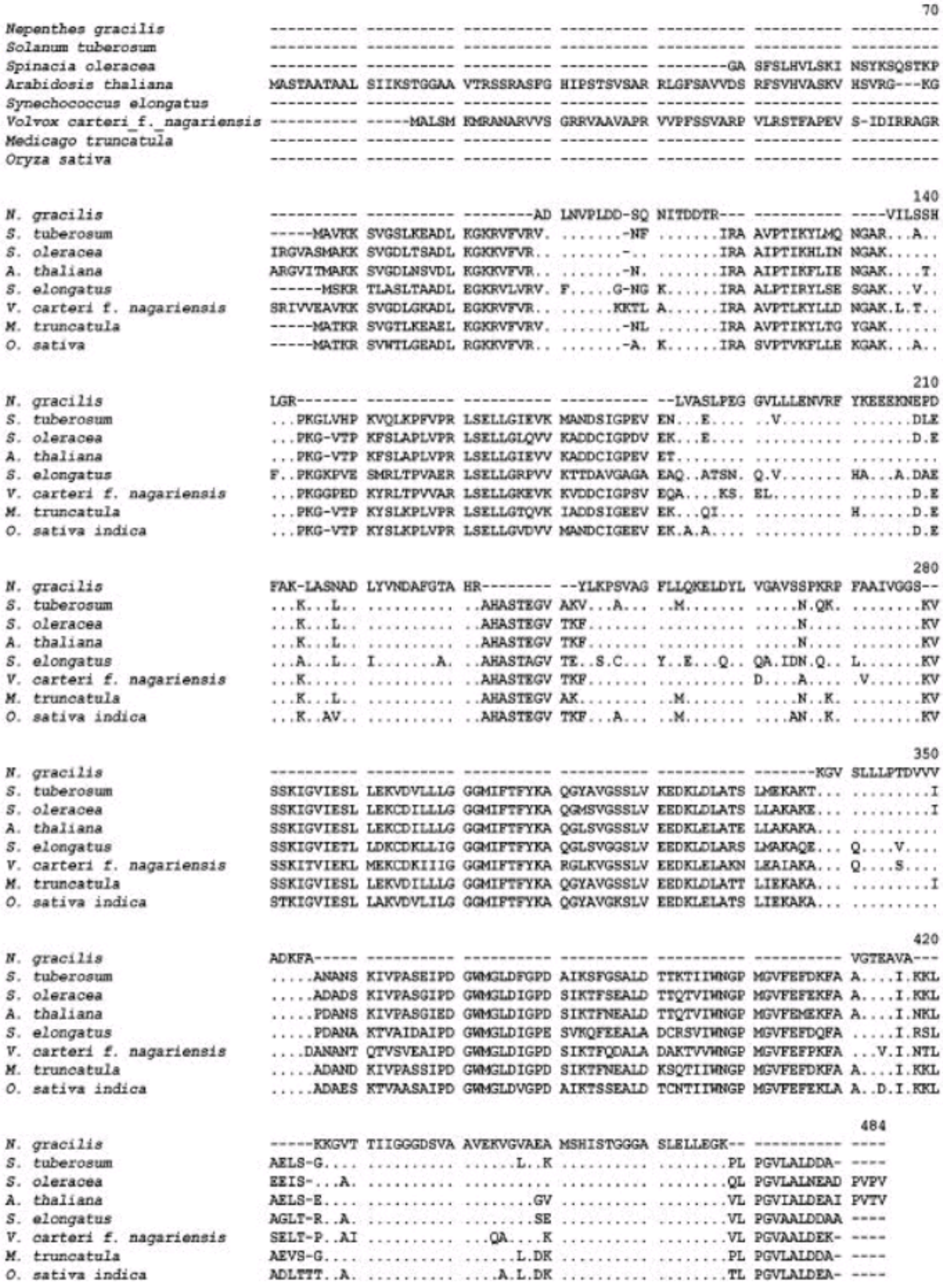

Fig. 2: Sequence alignment of phosphoglycerate kinase from *N. gracilis* and other organisms. Dots (.) indicate the same amino acids and dashes (-) are introduced to maximize homology

DISCUSSION

Although transporter genes of ammonium, amino acid and peptide (Schulze *et al*., 1999) and proteins in pitcher fluid (Hatano and Hamada, 2008) in *Nepenthes* have been published, proteins correlated with pitcher construction are still secret. This research is the first report on protein profile from different developing stages of leaves and pitchers of *N. gracilis* to propose a possibly candidate somehow involve in the pitcher development. Protein profiles from three developing stages of pitcher development were analyzed by SDS-PAGE. The three plant extracts showed similar protein profiles but protein expression level in developed pitcher was lower than the other two stages (Fig. 1). The results indicated that genes correlated with the pitcher formation may be expressed in the leaf. Also, the similar protein profile results supported that pitcher is a part of the leaf. However, there were a few different protein profiles in each sample. Two differentially expressed bands at 42.7 and 38.0 kDa were found in extracts from young leaf and leaf with developing pitcher, respectively, but they were absent in extract from developed pitcher. Based on LC-MS/MS results and a database search, the 42.7 kDa protein is identified as phosphoglycerate kinase (PGK) with identical amino acid fragments of PGK from many other plants (Table 1). This result is supported by the protein sequence alignment of PGK of other plants (Fig. 2). The predicted protein sequence of the *N. gracilis* PGK shows considerable homology to PGK from many plants, pointing toward a high degree of conservation of PGK sequences in organisms, especially dicotyledonous plants. The 38.0 kDa protein needs further study for type of protein.

PGK is an important enzyme presenting in higher plants. There are two isoforms which can be separated on the basis of their isoelectric points, one of which is located in the cytosolic compartment and functions in photosynthetic carbon metabolism and the other of which is localized in the stroma of chloroplasts and takes part in glycolysis and gluconeogenesis (McMorrow and Bradbeer, 1990; Bertsch *et al*., 1993). Role of PGK within *Nepenthes* nucleus has not been clearly established. However, the data from earlier studies suggested that PGK acts as an accessory protein to DNA polymerase-α, a situation that has also been reported for certain mammalian cells (Brice *et al*., 2004). Thus, PGK is a potential candidate for a regulatory element in DNA replication. Moreover, PGK may be a direct link between the clock and the control mechanisms of the cell cycle in *Chlorella* (Walla *et al*., 1994).

The data suggest that it should be feasible to screen *Nepenthes* cDNA and genomic libraries with oligonucleotides based on the *Nepenthes* PGK protein sequence presented here or PGK gene probes derived from other plants in order to isolate full-length clones of the PGK gene of *Nepenthes*. Information derived from such clones, such as the physical organization and sequence of transcriptional control elements, will provides clues as to understand the mechanism of pitcher construction. Additionally, it is at least theoretically feasible that, with the appropriate molecular genetic manipulations, one might specifically modify or delete chromosomal copies of the PGK and thereby directly investigate its purported role in the pitcher plant mechanism.

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