Table 3-1. Primers used for RACE and RT-PCR analysis of gene expression.

Primer name[a]	Sequence
Primers for RACE: *NuBP*	
5' GSP1	5' CAA TTGAC GTCC TTCAG GAAC
5' GSP2	5' CAG GTTGC TCTGA TGGA TGTC
5' GSP3	5' CAA CCTGG TAGTCCT TGCTTG
5' GSP4	5' GCA GTAGCA CATA TTTGC TG
3' GSP1	5' G TTGA TTCC AATCTT GGTGTG
3' GSP2	5' G TTCC TGAAG GACG TGAA TTG
Primers for RACE: *Tps*	
5' GSP1	5' GA GGA TAGCAC TGA TGCA TTC
5' GSP2	5' CA TTTGC AAGT ACACTC TCAAG
5' GSP3	5' GG ATCC ATCA GTCG ATTCTG
5' GSP4	5' CT CGTG AAGT ATCC ATGCTC
5' GSP5	5' AG GCA TTTGT GGCA TCA TCG TG
5' GSP6	5' AG TGGTCC TTGCA TGTCC TCTC
5' GSP7	5' G TGA TTACC TCAA TGACCT TC
3' GSP1	5' CAG AAACCGA GCAA AAGC AAG
Primers used for RT-PCR analysis of gene expression	
Tps Fwd	5' GA AAGAGA GTGCA TTGGTGTG
Tps Rev	5' GC TTTTGC TCGG TTTCTGTC
NuBP Fwd	5' G TAGAGTTTGAGA TGGAGAATG
NuBP Rev	5' CACC ACTAGA TAGTCAATCTC
Act Fwd	5' GG AYGAYA TGGARA ARA TYTGGC
Act Rev	5' ACV ACCTTRA TYTTCA TRCT GC

[a] Primer names reflect, for RACE-PCR, the end of the gene being amplified (5' or 3') and the number of the gene specific primer (GSP). For RT-PCR analysis of gene expression, the name reflects which gene is being amplified trehalose-6-phosphate synthase/phosphatase (*Tps*), nucleotide binding protein (*NuBP*) or actin (*Act*) and whether it is used in the forward (Fwd) or reverse (Rev) direction. All primers were designed manually based on sequences of genes and were custom made by Invitrogen Life Sciences.

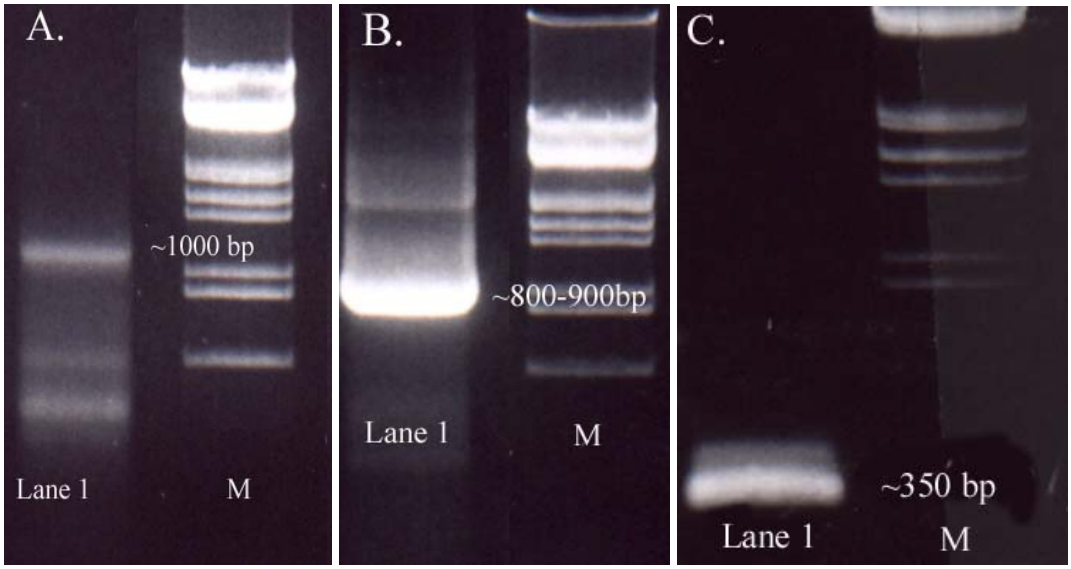

Figure 3-1. RACE PCR to clone 3' ends of *NuBP* and *Tps*.
RNA from roots of *Cypripedium parviflorum* was reverse transcribed using an oligo dT primer with a 5' extension. PCR was performed using a gene specific primer (GSP) and a primer specific to the 5' extension of the oligo dT primer. Amplified DNA was separated on a 1% agarose gel and visualized with ethidium bromide and UV light. In cases, where multiple fragments were found, PCR was performed a second time with a GSP closer to the 3' end.

A. First round RACE PCR of *NuBP* following RT reaction with 3' GSP1 at 55°C annealing temp. Lane 1, fragments from RACE, upper band is expected size of 1000bp. M: molecular size marker.
B. Second round RACE PCR of *NuBP* with 3' GSP2 at 58°C. Lane 1, fragment from RACE PCR, band is expected size of ~800-900bp. This fragment was cloned, sequenced and confirmed to be *NuBP* using a BLAST search against GenBank. M: molecular size marker.
C. First round RACE PCR of *Tps* with 3' GSP1 at 55°C. Lane 1, fragment from RACE, band is expected size of ~350bp. This fragment was cloned, sequenced and confirmed to be *NuBP* using a BLAST search against GenBank. M: molecular size marker.

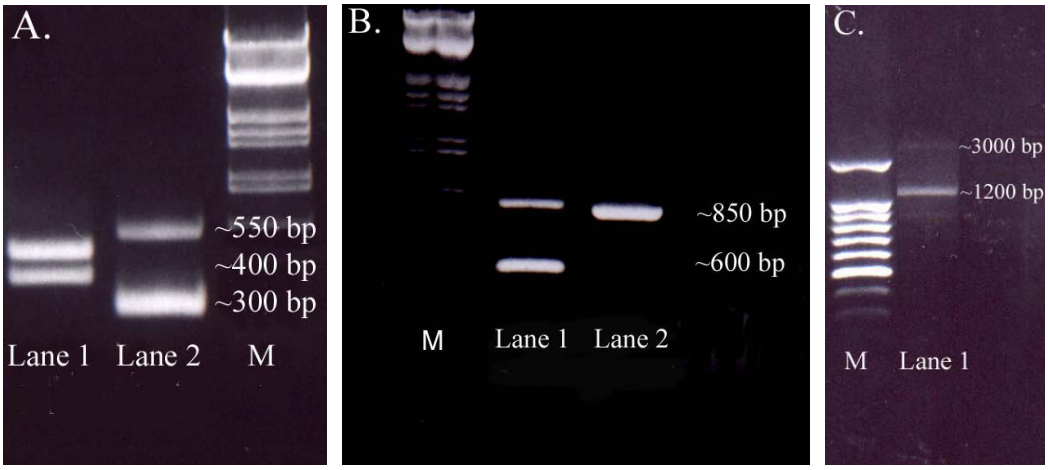

Figure 3-2. RACE PCR to clone 5' ends of *NuBP* and *Tps* (representative gels). RNA from roots of *Cypripedium parviflorum* was reverse transcribed using a gene specific primer (GSP). A poly C region was added to the 5' end of mRNAs with terminal deoxynucleotide transferase and dCTP. PCR was performed using a second gene specific primer (GSP) and an adapter primer designed to bind to the added poly C region.. Amplified DNA was separated on a 1% agarose gel and visualized with ethidium bromide and UV light. In cases, where multiple fragments were found, PCR was performed again with GSPs closer to the 5' end.

A. RACE PCR of *NuBP* and *Tps* using 5' GSP2 at 55°C annealing temperature. Lane 1: fragments generated for *NuBP*, the two fragments generated (~400 and 500 bp) were smaller than expected and RT was redone with a second GSP followed by PCR with a third GSP. Lane 2: fragments generated for *Tps*, the two fragments generated were smaller than expected and RT and PCR was redone as above. M: molecular size marker.
B. RACE PCR of *NuBP* and *Tps* using 5' GSP3 at 57°C annealing temperature. Lane 1: fragments generated for *NuBP*, the fragment generated (~850 bp) was the expected size and was cloned, sequenced and confirmed to be *NuBP* using a BLAST search against GenBank. Lane 2: fragments generated for *Tps*, the two fragments generated were smaller than expected and RT was redone with a fourth GSP followed by PCR with a fifth GSP. M: molecular size marker.
C. RACE PCR of *Tps* using 5' GSP7 and Gene Racer RACE protocol. After removal of the 5' methyl-G cap, an RNA adpater was ligated to mRNAs which were then reverse transcribed using a gene specific primer. PCR was performed with another GSP (GSP7) and a primer specific to the RNA adapter. Lane 1, fragments generated from Gene Racer RACE, the smaller of the two fragments (~1200bp and ~3000bp) was cloned, sequenced and confirmed to be *Tps* using a BLAST search against GenBank. M: molecular size marker.

Figure 3-3. Nucleotide and deduced amino acid sequence of *NuBP*. (p 44)
The full length cDNA of *NuBP* was cloned and sequenced as described in Fig. 3-1 and 3-2. The amino acid seqeunce was deduced from the longest open reading frame. Conserved motifs were identified through comparisons with known nucleotide binding proteins. The ATG initiation codon and TAG stop codon are in boldface type. The four conserved cysteines in the n-terminal cysteine motif are italicized. The ATP binding motif (motif A, LVLSGKGGVGKST) is underlined. Motif A' (DYQVGLLDIDIC) is underlined and italicized. Motif B (DYLVVD) is underlined and in boldface.

Figure 3-4. Protein sequence alignment of NuBP. (p. 45)
The deduced amino acid sequence of NuBP was aligned with other nucleotide bindng proteins using the CLUSTALW program at the San Diego Supercomputer (SDSC) website Biology Workbench (http://workbench.sdsc.edu). *Cypripedium parviflorum* (CyPP, line 4) was aligned with NuBP from human (Hsap-NBP), mouse (Mus-NBP), *Arabidopsis* (At-NBP) and yeast (Sac-NBP). Completely conserved residues are highlighted in green, residues identical to CyPP are in yellow and conserved residues are in blue.

Figure 3-5. Nucleotide and deduced amino acid sequence of *Tps*. (p.46)
A 2200 bp fragment of *Tps* was cloned and sequenced. The amino acid sequence was deduced from the start of the nucleotide sequence to the TAA stop codon. The TAA stop codon (nt 1229-1231) is in bold. Conserved phosphatase boxes (LDYDGTMM and GDDRSH/D) were identified by comparisons with known Tps proteins and are underlined.

Figure 3-6. Protein sequence alignment of Tps. (p.47)
Alignment was performed using CLUSTALW at SDSC Biology Workbench. *Cypripedium parviflorum* (CyPP) Tps was aligned with Tps from rice (Osat Tps), *Arabidopsis* (AtTps) and potato (Stub Tps). Color coding is as described in Fig. 3-4.