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Estimation of Actin and 18S rRNA as Internal Controls of RNA in Hyacinth (*Hyacinthus orientalis* L.)

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The potential of different housekeeping genes for their use as internal controls of gene expression under changing environmental conditions was investigated in hyacinth (*Hyacinthus orientalis* L.) bulbs during storage at low (5 °C) and room temperatures (25 °C) and culture period at 25 °C. Proper annealing temperatures for the expression analysis of genes for actin were between 50 and 63 °C, and proper PCR cycles for actin and 18S rRNA were 30 and 25, respectively. The expression of actin increased during cold storage period and unstable during culture, whereas that of 18S rRNA was kept unchanged throughout the storage and culture periods. These results indicated that 18S rRNA was more reliable than actin as an internal control for semi-quantitative RT-PCR of total RNA in hyacinth. This is the first report on the establishment of the internal standard for the studies on expression levels of genes in bulbous plants.

INTRODUCTION

Reverse transcription followed by reverse transcription–polymerase chain reaction (RT–PCR) is useful for the measurement of plant physiological changes in gene expression. It is recommended to use non-regulated housekeeping genes such as actins, tublins, ubiquitins and 18S rRNA as a reference. However, some studies have shown that these genes fluctuate under experimental conditions (McDowell *et al.*, 1996; Huang *et al.*, 1997; Zhong and Simons, 1999; Schmittgen and Zakrajsek, 2000). It is, therefore, important to ensure that the expression of the selected housekeeping gene is stable under any experimental condition.

Hyacinth (*Hyacinthus orientalis* L.), an autumn–planting bulbous plant, needs a cold period for proper shoot elongation under suitable growth conditions. Since the manipulation of the bulbs for forcing is very important for horticultural industry, conditions of cold treatment have been studied extensively. However, it has little been cleared what occurs in the bulbs during cold period. Gene expressions through cold storage and growing periods in bulbous plants including hyacinth are now being conducted in our laboratory, but there are few reports available on internal controls of them.

Actin is a major component of the plant cytoskeleton. It is well known that expres-

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sion of 18S rRNA is correlated with that of total RNA. Both of them have been often used as internal controls in many plants (Hsu *et al.*, 2003; Kiba *et al.*, 2004; Kinoshita *et al.*, 2004). Only the sequence of cDNA for the gene of 18S rRNA in hyacinth has been registered at the Genbank (DDBJ accession number AF168853), but no reports on its expression levels are available. There were no reports on actin in hyacinth. Relative expressions of genes for actin and 18S rRNA under various growth stages in hyacinth were investigated in this study to ensure proper internal control genes at semi-quantitative RT-PCR analysis.

MATERIALS AND METHODS

Plant materials and culture conditions

Bulbs of hyacinth cv. Delft Blue were dry-stored at 5°C (pre-cooled) or 25°C (non-cooled) for 12 weeks and grown at 25°C by hydro-culture under natural daylength for 5 weeks in the phytotron (RH 70%) of Kyushu University. Storage organs (scales) of the bulbs were sampled at 0, 7, 12, 13, 15 and 17 weeks after the start of storage and frozen until use.

Isolation of the cDNA clone

The frozen samples were ground in liquid nitrogen and used for total RNA extraction. The extraction was conducted by Phenol/SDS Method (Ausubel *et al.*, 1997) and then the total RNA was treated with RNase-free DNase I (Roche) to remove the contamination of genomic DNA. The synthesis of cDNA was performed from 900 ng of the total RNA using RNA PCR Kit (AMV) Ver.2.1 (TaKaRa). Random 9 mers and Oligo dT-Adaptor Primer were mixed at a one-four ratio. To isolate cDNA fragments of hyacinth actin, degenerate PCR primers; *HyAct1*: 5'-TGA YAT GGA RAA RAT YTG GCA TC-3' and *HyAct2*: 5'-GTC GTG KAA GGT YGT CTA CAC CT-3' were designed based on the sequences of conserved regions in *Stevia rebaudiana* (DDBJ accession number AF548026), *Oriza sativa* (DDBJ accession number NM_189383), *Hordeum vulgare* (DDBJ accession number AY145451), *Helianthus annuus* (DDBJ accession number AF282624), *Gossypium hirsutum* (DDBJ accession number AY305724) and *Arabidopsis thaliana* (DDBJ accession number NM_112764). Thermal cycling was carried out for 30 cycles of denaturation (94°C, 30s), annealing (55°C, 30s) and extension (72°C, 1 min 30s). The primers for 18S rRNA were designed based on *Hyacinthus orientalis* (DDBJ accession number AF168853), *Oriza sativa* (DDBJ accession number AF069218), *Hordeum vulgare* (DDBJ accession number AY552749), *Zea mays* (DDBJ accession number AF168884), *Arabidopsis thaliana* (DDBJ accession number X16077), *Pisum sativum* (DDBJ accession number U43011) and *Solanum tuberosum* (DDBJ accession number X67238). The sequence of 18S rRNA forward primer (*Hy18S1*) was 5'-CAT TCG TAT TTC ATA GTC AGA GGT GAA ATT C-3' and that of the reverse primer (*Hy18S2*) was 5'-TTA ACC AGA CAA ATC GCT CCA CCA ACT AAG-3'. After amplification, the reaction products were analyzed by agarose gel electrophoresis. The products were subcloned into pGEM-T Easy-vector (Promega) and sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan Inc.). The cDNA obtained from hyacinth was designated as *HoACT*.

Expression analysis of *HoACT* and 18S rRNA in the scales of hyacinth

Reverse transcription was performed using 900 ng of total RNA. Each cDNA was diluted to 1/50 concentration and used as a template for PCR reaction. The sequence of forward primer (*HyAct3*) was 5'-CTT CCT CAT GCT ATC CTT CGT CTG GAT CTT-3' and that of the reverse primers (*HyAct4*) was 5'-CAC ACT TCA TGA TCG AGT TGT ATG TCG TC TC-3'. Investigations of proper annealing temperature and PCR cycles were performed. Thermal cycling was carried out for 20, 25, 26, 28, 30 and 32 cycles of denaturation (94 °C, 30s), annealing (57 °C, 30s) and extension (72 °C, 1 min 30s). After amplification, the amount of the reaction products was analyzed by Molecular Imager FX (BIO-RAD).

RESULTS AND DISCUSSION

The nucleotide sequence of a cDNA (*HoACT*) is shown in Fig. 1. *HoACT* shared high

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      10      20      30      40      50      60
TGACATGGAG AAGATCTGGC ATCACACCTT TTACAATGAG CTCCGTGTTG CCCCTGAGGA

      70      80      90     100     110     120
ACACCCGTGTG CTGCTTACCG AGGCTCCACT CAACCCCAAG GCAAACAGAG AGAAAATGAC

      130     140     150     160     170     180
CCAGATCATG TTTGAATCCT TTAATGTTC GGCATGTAT GTTGCCATCC AAGCCGTCTT

      190     200     210     220     230     240
CTCCCTGTAC GCCAGTGGG GTACAAC TGG CATTGTGCTG GATTCCGGTG ACGGTGTCAG

      250     260     270     280     290     300
CCACACTGTC CCGATTACG AGGGATATGC CCTTCCTCAT GCTATCCTTC GTCTGGATCT

      310     320     330     340     350     360
TGCCGGACGT GACCTCACCG ATGCATTGAT GAAGATCCTT ACCGAGAGAG GGTATTCATT

      370     380     390     400     410     420
CACTACTACT GCAGAACGGG AAATTGTTAG AGACGTGAAG GAGAAGCTCG CTTATGTGTC

      430     440     450     460     470     480
ACTAGACTAC GAGCAGGAGC TGGAAACCG CAAGAGCAGT TCCTCTGTGG AGAAGACCTA

      490     500     510     520     530     540
TGAGCTTCCT GATGGCCAGG TGATCACAAT TGGGGCTGAG AGGTTTCAGGT GCCCAGAGGT

      550     560     570     580     590     600
CCTCTTCAG CCATCGCTCG TCGGTATGGA AGCTGCAGGC ATCCATGAGA CGACATACAA

      610     620     630     640     650     660
CTCGATCATG AAGTGTGATG TTGATATCAG GAAAGACTTG TATGGCAACA TTGTGCTCAG

      670     680     690     700     710     720
CGGAGGATCA ACCATGTTCC CTGGCATTGC CGATCGTATG AGCAAGGAGA TCACCCGCCCT

      730     740     750     760     770     780
CGCGCCGAGC AGCATGAAGA TTAAGTTGT TGCTCCCCCG GAAAGGAAGT ACAGCGTCAG

      790     800     810     820     830
GATTGGAGGG TCGATCCTTG CCTCCCTCAG CACCTTCAA CAGATGTGGA

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Fig. 1. Nucleotide sequence of *HoACT* cDNA. *HyAct3* and *HyAct4* are indicated by an underline and a double underline, respectively.

homology with other plants; *Hordeum vulgare* (DDBJ accession number AY145451) (81.8%) and *Oryza sativa* (DDBJ accession number NM_189383) (81.4%). We designed specific forward primer (*HyAct1*) and reverse primer (*HyAct2*) based on the sequence of *HoACT* for expression analysis using RT-PCR (Fig. 1).

T_m values of *HyAct3* and *HyAct4* calculated by Breslauer *et al.* (1986) were 70 and 69.5°C, respectively. PCR products were detected from 57 to 63°C at a similar level, but not at 69°C (Fig. 2). The products were also obtained at annealing temperatures from 50 to 60°C at a similar level. This finding indicates that an annealing temperature of PCR reaction with *HyAct3* and *HyAct4* should be set between 50 and 63°C.

Among the relative amounts of the PCR products measured at 20, 25, 26, 28, 30 and 32 cycles, amplified products were not detected at 20 and 25 cycles (data not shown). The amount of PCR products increased semi-logarithmically in the range from 26 to 32 cycles (Fig. 3), but non-specific bands appeared at 32 cycles, indicating that it is proper

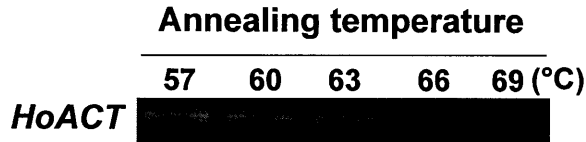


Fig. 2. RT-PCR of *HoACT* in hyacinth bulbs at various annealing temperatures.

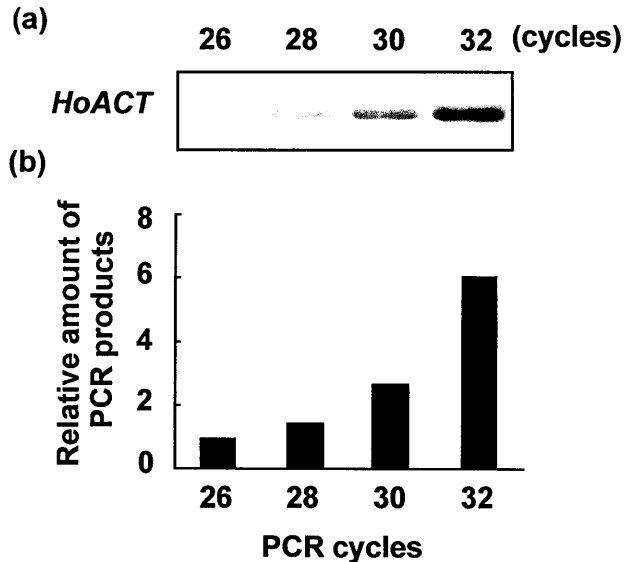


Fig. 3. RT-PCR of *HoACT* in hyacinth bulbs at various PCR cycles.

to perform the analysis at less than 32 cycles. Likewise, cycles for 18S rRNA were proper at less than 26 (data not shown).

The accumulation of transcripts of *HoACT* and 18S rRNA was kept unchanged during 25°C storage and culture periods in non-cooled bulbs (Fig. 4a). In pre-cooled bulbs, the expression of *HoACT* increased during cold storage period and was unstable during culture, whereas the transcription levels of 18S rRNA were kept unchanged throughout the storage and culture periods (Fig. 4b). These results indicate that 18S rRNA is more suitable than *HoACT* as an internal control in hyacinth.

It has been reported that actin enhanced the activity of hexokinase, an enzyme related to sugar metabolism (Aon *et al.*, 1999). Starch hydrolysis and sucrose synthesis occurred in the scales of hyacinth bulbs during cold storage period (unpublished data). It is considered that an increase in actin induced by low temperature enhances the ability of sucrose synthesis. It is, therefore, concluded that actin is improper as an internal control in the experiments that have possibility of the changes in sugar metabolism.

In almost all plants, 18S rRNA is highly expressed. *Hy18S1* and *Hy18S2* were designed by referring the conserved sequences of many monocotyledons and dicotyledons. Therefore, these two primers have possibility to be useful for PCR in many other

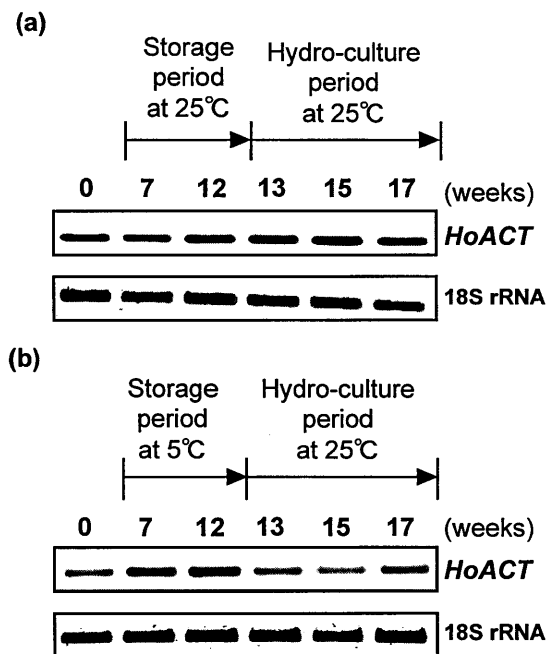


Fig. 4. Semi-quantitative RT-PCR analysis of *HoACT* and 18S rRNA in the bulbs of hyacinth stored at 5°C (pre-cooled) or at 25°C (non-cooled) for 12 weeks during storage and hydro-culture periods. PCR cycles for *HoACT* and 18S rRNA were set at 30 and 25, respectively.

plants as well as in hyacinth (this study) and tulip (unpublished data). When PCR productions of target genes are not obtained in plants, that have been little investigated before, it is difficult to judge whether the target genes were not expressed in the samples or RT-PCR reactions did not work because of contamination with PCR inhibitor. We suppose that *Hy18S1* and *Hy18S2* are helpful to check whether total RNA extraction and RT-PCR reaction are successful or not.

In conclusion, 18S rRNA is an excellent internal control gene for semi-quantitative RT-PCR in hyacinth bulbs, and *Hy18S1* and *Hy18S2* are useful as universal primers to check experimental methods in molecular experiments with various plants.

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