MATERIALS AND METHODS

Mangosteen (*Garcinia mangostana* Linn.) fruit used in this study were obtained from a commercial orchard in Chanthaburi province at normal harvest time; May to July. The fruit were divided into 2 groups based on their surface color development; reddish brown (Figure 2A) and reddish purple (Figure 2B) (Kosiyachinda, unpublished data; Tongdee and Suwanagul, 1989). Fruit were carefully harvested to minimize possible mechanical damage, packed in 10 kg plastic baskets, and transported to the Postharvest Research Unit, Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom within 1 day of harvest by a refrigerated truck. Then fruit were selected for uniformity of color and size for experimentation.

Experiment 1 Study on physico-chemical changes of mangosteen pericarp stored at low temperature

1.1. Effect of maturity stages on pericarp hardening of mangosteen fruit during and after low temperature storage

Reddish brown and reddish purple fruit (330 fruit in each stage) were packed in 10 kg plastic baskets and stored at 6 and 12°C (87 and 83.5%RH) for 15 days. At 3-day intervals, 30 fruit in each stage (3 replications) were taken randomly from each storage temperature to analyze immediately physical and biochemical changes of fruit pericarp and the other group (30 fruit in each stage) was transferred to room temperature (29.5^{o}C, 74%RH) for 3 days. After transfer, the second group from 6 and 12°C were held at room temperature, physical and biochemical changes of the fruit pericarp were then determined in the same manner as the first group. The two opposite sides on each fruit were determined for firmness and two other sides from the same fruit were selected and bulked in 3 replications for analysis of total phenolics and lignin contents as follows:

Figure 2 Mangosteen fruit at reddish brown (A) and reddish purple (B) stages were used in this study.

1.1.1 Pericarp firmness

Firmness was determined on fruit without peeling using a Effegi firmness tester (FT011, Italy), equipped with cylinder plunger head 0.1-0.5 cm in diameter, and pressed 0.5 cm deep into the pericarp at 2 positions on opposite sides of each fruit. The force was recorded in newtons (N).

1.1.2 Lignin contents

Lignin contents were determined as lignin thioglycolic acid by preparation of alcohol insoluble residues (AIR), according to Bruce and West (1989). Five g of pericarp was homogenized with 100 ml methanol for 1 min and vacuum-filtered using Whatman GF/A. The residues were dried at 60°C for 24 h. Ten mg of dried AIR were placed in a screw-cap tube, and then mixed with 5 ml of 2 N HCl and 0.5 ml thioglycolic acid. The solutions were heated at 100°C for 4 h with gentle agitation, using a horizontal shaker (GFL 3017 shaker, Germany). After that, the samples were cooled on ice and centrifuged at 18,000 x g for 30 min at 4°C. The pellet was re-suspended in 5 ml of 0.5 N NaOH, sealed with parafilm and agitated gently at 25°C for 18 h to extract the lignin thioglycolate. The samples were centrifuged again at 18,000 x g for 20 min and supernatants were transferred to test tubes. One ml of concentrated HCl was added to each sample and lignin thioglycolic acid was allowed to precipitate at 4°C for 4 h. After centrifugation at 10,000 x g for 15 min, the orange-brown pellet was dissolved in 10 ml of 0.5 N NaOH and the absorbance was measured at 280 nm.

1.1.3 Total free phenolics contents

Total free phenolics were extracted from fruit pericarp using the method of Zhang *et al*. (2000). Five g of pericarp was homogenized with 100 ml of extraction buffer (65% ethyl alcohol and 0.5% sodium metabisulfite, pH 5.25). The homogenate was gently shaken for 30 min and filtered through 4 layers of cheesecloth. The remaining residue was twice re-extracted with 50 ml of extraction solution, as

above. The combined extracts were centrifuged at 12,600 x g for 15 min (4°C). Extraction solution was removed from the supernatant under vacuum at 40°C for 15 min or until 1/3 supernatant left, using a rotary evaporator (Buchi Rotavapor R-205, Switzerland). The remaining pigments in the supernatant were removed by two re-extractions with petroleum ether (2:1 v/v). Anhydrous ammonium sulfite (20%) and 2% metaphosphoric acid were added to the solution. Phenolic compounds were extracted 3 times by ethyl acetate (1:1 v/v), then evaporated and dried under vacuum at 40°C. The residues were dissolved in 5 ml methanol (HPLC grade). Solutions were stored at -20°C before analysis of phenolic compounds. Total phenolic contents were determined according to Singleton and Rossi (1965) and individual phenolic compounds were identified by high performance liquid chromatography (HPLC). One ml of the above extracted solutions was filtered through 0.45 μm Nylon Acrodisc® Filters (Orange Scientific, Belgium). Ten μl of sample was analyzed by Shimadzu analytical HPLC CLASS-VP (Shimadzu, Japan) with SCL-10A VP system controller, LC column oven GL Sciences and SPD 10A VP UV-VIS detector. The chromatograms were recorded and integrated with SCL-10A VP system processor. Separations were achieved on a Shim-pack CLC-ODS, LC column (C18 column, 150 x 6.0 mm, 5 mm size particle) using a mobile phase containing 10% acetronitrile, 15% methanol and 2% acetic acid. The flow rate of mobile phase was 0.75 ml/ min controlled by LC-10AD VP pump. Quantification of individual phenolic contents was done by comparison with individual standards at 280 nm. The retention time for *p*-coumaric and sinapic acid peaks was 12.10 and 18.50 min, respectively.

1.1.4 Histochemical tests

Non-chilled and chilled of reddish purple pericarp with a size of 0.25x0.25x0.30 cm (WxLxD) were fixed in 50% formalin-acetic alcohol (FAA) containing with 70% C_2H_5OH, formaldehyde, glacial acetic acid and distilled water (10:2:3:5, v/v/v/v) before keeping at room temperature at least 12 h. Pericarp tissues were washed 3 times in 50% ethanol. After that, the samples were replaced with tertiary butyl alcohol (TBA) using a series of 50, 70, 85, 95 and 100% concentrations, 24 h in each. The samples were then replaced 3 times in absolute

TBA each for 12 h. To infiltrate paraffin oil into the pericarp tissues, the samples were transferred into TBA and paraffin oil (1:1, v:v) at 60°C for 12 h. Hard paraffin (paraplast) was then added into the samples at 60°C (1:1:1, TBA: paraffin oil: paraplast). Then pure paraplast was added 3 times for at least 12 h each at 60°C. Embedded samples in the plastic block were then left at room temperature until the paraffin became hard before sectioning using Microm HM 335E rotor microtome (Microtom Laborgerate GmbH, Germany). The slides with paraffin ribbons were stained for lignin with toluidine blue and safranin fast-green. The slides were then covered with cover slips and observed under a light microscope (Carl Zeiss Light Microscope, Germany) using a 4x objective lens (Carl Zeiss / Axiostar plus microscope) and digitalized by a camera (Canon Powershot G5, Japan).

1.2 Effect of low temperature on pericarp hardening of mangosteen fruit after transfer to room temperature

This experiment only used fruit at the reddish purple stage stored at 6°C (84%RH) since their hardening symptoms were greater than those at the reddish brown stage and in 12°C storage (results from Experiment 1.1). Two hundred and sixteen fruit were stored at 6°C for 0, 6 and 12 days and then transferred to room temperature (30°C, 71.5%RH) for 0, 1, 2 and 3 days. Six fruit represented 1 replication and 3 replications were used for each treatment. Physical and biochemical changes of the fruit pericarp were determined in the same manner as the Experiment 1.1.

Experiment 2 Study on the effect of low O_2 on pericarp hardening and some enzyme activities involved in lignin biosynthesis of mangosteen fruit

2.1 Effect of low O_2 treatment on pericarp hardening and some enzyme activities involved in lignin biosynthesis of mangosteen fruit applied during low temperature storage

Six reddish purple fruit were placed in a 4-l plastic jar at 6°C (84%RH) with and without O_2 (normal air and N_2 atmosphere, respectively) for 0, 3, 6, and 9 days. Atmospheric conditions were set up using a flow system with air and N_2 in compressed tanks (OFN grade, 99.99% N_2). The concentration of O_2 was measured by a gas chromatograph equipped with thermal conductivity detector (Shimadzu GC-14A, Shimadzu, Japan). Two sets of fruit (6 jars) were taken randomly from each condition. The first set was immediately analyzed for physical and biochemical changes of the fruit pericarp and another set was transferred to room temperature (30°C, 71.5%RH) for 3 days, and analyzed thereafter. Pericarp firmness, lignin contents and total free phenolics contents were determined as described for Experiment 1.1.

Enzyme activities involved in lignin biosynthetic pathway were measured as follows. Two g of pericarp was added to 20 ml of extraction buffer (100 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.05% triton X-100, 2.5 mM dithiothreitol) and 0.2 g of polyvinyl polypyrolidone (PVPP). Then the mixture was homogenized using a Polytron PT 2100 (Kinematica AG, Switzerland, 1.8 mm diameter head) at speed 15 for 1 min. The homogenate was centrifuged at 18,000 x g at 4°C for 30 min. The supernatant was used for analyzing enzyme activities and protein content. The extract was analyzed within 12 h and kept at 5-10°C to avoid gel formation.

1. Phenylalanine ammonia lyase (PAL)

PAL activity was determined using a modification of the method of Camm and Towers (1973). The reaction of PAL comprised of 0.1 ml of enzyme extract and 2.4 ml of L-phenylalanine solution (10 mg/ml Tris buffer, pH 8.5). The reaction was incubated in a water bath at 37°C for 1 h and stopped by adding 0.5 ml of 5 N HCl. The sample was measured for PAL activity using a spectrophotometer at 290 nm. PAL activity was expressed as micromoles of *trans*-cinnamic acid per mg protein.

2. Cinnamyl alcohol dehydrogenase (CAD)

CAD activity was assayed using the oxidation reaction with coniferyl alcohol as a substrate (Goffner *et al*., 1992). The reaction mixture contained 1 ml of 100 mM Tris-HCl, 24 μl of 0.2 mM NADP, 10 μl of 0.1 mM coniferyl alcohol and 2 ml of H_2O with 100 μl enzyme extract. The reaction was measured within 3 min, using a spectrophotometer at 400 nm. One unit of CAD activity was defined as the amount of the enzyme that caused a change in absorbance per min.

3. Peroxidase (POD)

POD activity was determined using a modification of the method of Morita *et al*. (1988). The reaction mixture contained 600 μl of extraction buffer, 800 μl of 100 mM 4-aminoantipyrine, 800 μl of 100 mM phenol, 20 μl of 100 mM H_2O_2 and 100 μl of enzyme extract. The reaction was measured within 4.5 min, using a spectrophotometer with absorbance at 512 nm. One unit of POD activity was defined as the amount of the enzyme consuming 1 μM of 4-aminoantipyrine or H_2O_2, under the assay conditions.

4. Protein content

Protein content of samples was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/ml). Specific activity of the enzyme was expressed as units per mg protein.

2.2 Effect of low O_2 treatment on pericarp hardening and some enzyme activities involved in lignin biosynthesis of mangosteen fruit after low temperature storage

One hundred and eighty fruit at the reddish purple stage were stored in normal air at 6°C (84%RH) for 9 days, and then transferred to room temperature

($30^{o}C$, 71.5%RH) in conditions with and without O_2 (normal air and N_2 atmosphere, respectively) for 0, 1, 2 and 3 days. Six fruit represented 1 replication and 3 replications were used for each treatment. Atmospheric conditions were set up using a flow system and physical and biochemical changes were determined as described for Experiment 2.1.

2.3 Gene expression of the lignified biosynthetic pathway involved in pericarp hardening of mangosteen fruit

This study consisted of an investigation into gene expression of the two enzymes in lignification biosynthetic pathway, PAL and lignin peroxidase (LgPOD). The gene expression of these enzymes was carried out using the northern blotting technique, semi-quantitative RT-PCR and real-time PCR.

2.3.1 Total RNA extraction

This protocol was modified from Lopez-Gomez and Gomez-Lim (1992) to make it suitable for RNA extraction from mangosteen pericarp. The pericarp tissues were ground using Mixer Mill MM 301 (Retsch, Germany) under liquid N_2, kept in the RNAse-free Falcon tubes and then stored at $-70^{o}C$ until use (for 1-2 weeks). One g of ground pericarp tissue was added to 15 ml of Gomez lysis buffer containing 2% SDS, 50 mM EDTA, 150 mM Tris base with pH adjusted to 7.5 with 1 M boric acid, 0.5 g of PVPP and 300 µl of β-mercaptoethanol in the RNAse-free Falcon tubes, and the contents vortexed until well mixed. The mixture was homogenized using a Polytron PT 2100 (Kinematica AG, Switzerland, 1.2 mm diameter head) at speed 12 for 1 min. Then 1.5 ml of 5 M potassium acetate and 4 ml of chilled absolute ethanol were added to the mixture, and vortexed. Then 20 ml of chloroform/isoamyl alcohol (CI, 24:1) was added, the mixture vortexed, and then centrifuged at 7,000 x g for 15 min. The top aqueous phase was transferred to a new RNAse-free Falcon tube, 10 ml of phenol and 10 ml of CI added, the solution mixed and then centrifuged at 7,000 x g for 15 min. The top aqueous phase was again transferred to a RNAse-free Falcon tube, and 20 ml of CI added, the solution mixed

and centrifuged at the same speed. The top aqueous phase was then transferred to a RNAse-free Oakridge tube and the volume recorded. RNA was then precipitated with 8 M lithium chloride (LiCl) to obtain a 3 M final concentration at $4^{o}C$ overnight. After centrifugation (20,000 x g for 15 min at $4^{o}C$), the supernatant was carefully poured off and the pellet was washed with 3 ml of 3M LiCl. The RNA was then re-suspended in 350 µl of DEPC-water and transferred to an autoclaved microtube, and then 35 µl of 5 M potassium acetate and 962.5 µl of chilled absolute ethanol were added. The RNA was precipitated at $-70^{o}C$ for 1 h. The RNA was pelleted by centrifugation (15,000 x g for 30 min at $4^{o}C$), washed once with 500 µl of chilled 70% ethanol, and re-suspended in 50 µl of chilled DEPC-water. After that RNA was quantified spectrophotometrically and qualitatively checked using gel electrophoresis on a 0.8% agarose gel.

2.3.2 PCR

The first strand cDNA was synthesized from 1 µg of total RNA using Omniscript RT Kit (Qiagen, Germany) for PAL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, and SuperScript^{TM} III Reverse Transcriptase kit (Invitrogen, USA) for LgPOD and 18S ribosomal RNA (18S rRNA) genes as described by the supplier's instructions. The synthesized cDNA was used as a template to amplify the targeted genes by PCR. Two degenerate primers with designs based on two highly conserved amino acid sequences in the PAL and LgPOD genes, which are the key enzymes in the lignification pathway, were used as forward and reverse primers. The sequences of the primers are described in Table 1.

Table 1 Upstream (forward) and downstream (reverse) primers for PCR reactions

Gene	Forward primer	Reverse primer
PAL	GYDATYTTYGCWGARGTBATG	AGATTNGAHGGYAABCCRTTGTTG
LgPOD	CATTTYCAYGATTGYTTYGTYVAHGGTTGTG	GTKGYGTTSADWGTTGGRTCWGG
18S rRNA	CGGGGAGGTAGTGACAATAAATA	TAATGAAAACATCCTTGGCAAAT
GAPDH	GGGTACAATGACAACTACTCACTCTT	CTTAACCATATCATCTCCCATGACC

Degenerate primers were designed to the conserved amino acid sequences of PAL from GenBank accession numbers AF299330, AF401636, D78640, M29232.1, AY036011, D26596.1 and AB042520.1 and LgPOD from GenBank accession numbers L78163.1, X71593.1, J02979.1 and AY366083.1. Specific primers of 18S rRNA were also designed to the conserved amino acid sequence from GenBank accession numbers D29786.1, U42495.1, DQ341382.1, AY168824.1, AF195622.1, AY179548.1 and AY819057.1. Specific GAPDH primers were obtained from Invitrogen, New Zealand. The amplification reactions were initially denatured at 94°C for 5 min and then subjected to 35 cycles at the following conditions: 94°C for 30 s, 48°C for 30 s (PAL) or 55°C for 30 s (LgPOD and GAPDH) or 55°C for 45 s (18S rRNA), and 72°C for 30 s (PAL, LgPOD and GAPDH) or 45 s (18S rRNA) with a final extension at 72°C for 10 min.

2.3.3 PCR products cloning and DNA sequencing

The amplified PCR fragments from each gene were purified and cloned into pGEM®-T Easy Vector (Promega, USA) as described in the supplier's instructions.

1) Protocol for ligations using the pGEM®-T Easy Vectors

The ligation reactions were set up using 5 µl of 2x Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector (50 ng), 3 µl of PCR product, 1 µl of T4 DNA Ligase and adjusted with deionized water to a final volume of 10 µl. The reactions were mixed by pipetting and then incubated overnight at 4°C.

2) Protocol for transformations using the pGEM®-T Easy Vector Ligation Reactions

DH5-α competent cells were used for the transformations. Two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction were prepared, and equilibrated at room temperature prior to plating. The 100 µl frozen DH5-α tube(s)

were removed from –70°C storage and placed in an ice bath until just thawed (about 10 min). The ligation reaction (10 µl) was carefully put into each competent cell. The tubes were gently flicked to mix and then placed on ice for 20 min. The cells were heat-shocked for 45 s in a water bath at exactly 42°C without shaking and immediately returned to ice for 2 min. Then 1 ml SOC medium was added to the tubes and incubated for 1.5 h at 37°C with shaking (~150 rpm). Each transformation culture (100 ml) was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates and incubated overnight (16–24 h) at 37°C. White colonies would be selected for the next step.

3) Protocol for Purification of plasmid DNA using QIAprep® spin Miniprep Kit (Qiagen, USA)

Single white colonies containing the inserted genes were cultured in 5 ml LB medium overnight. Cells were centrifuged at 14,000 x g for 3 min and only the pellet cells collected. The pellet cells were re-suspended in 250 µl of re-suspension buffer and transferred to a 1.5 ml microtube. Then, 250 µl of lysis buffer was added and the tube gently inverted to mix. Three hundred and fifty µl of neutralization buffer were added and the tube inverted immediately and then centrifuged at 14,000 x g for 10 min. The supernatant was transferred to the spin column by pipetting. The tube was then centrifuged for 1 min and the flow-through discarded. The spin column was washed by adding 750 µl of washing buffer and centrifuged for 1 min, the flow-through discarded, and centrifuged again to remove the residual washing buffer. DNA was eluted by adding 50 µl of elution buffer and centrifuging for 1 min. DNA was kept at -20°C until used for cutting with the EcoRI restriction enzyme (New England Biolabs, Inc., USA) and the insert size was checked by gel electrophoresis before sequencing.

4) DNA sequencing and analysis

The sequence analysis of the clone was conducted by automatic sequencing using the ABI PRISM® 377 DNA sequencer (Applied

Biosystems, USA). The sequences of amplified PAL, LgPOD, 18S rRNA and GAPDH fragments were compared with the genes in the Genbank database using the BLAST program from NCBI (National Centre for Biotechnology Information).

2.3.4 Northern analysis using digoxygenin-11-UTP (DIG) labeling probes

1) Quantification of RNA

Total RNA was quantified using a Genesys 10 UV spectrophotometer (Thermo Spectronic, USA) at absorbances of 240, 260 and 280 nm. An absorbance of 1 unit at 260 nm corresponds to 40 μg of RNA per ml. The ratio of readings at 260 and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA. Pure RNA has an A_{260}/A_{280} ratio of 1.8-2.0. Total yield of RNA was calculated from the concentration of RNA as follow:

$$\text{Concentration of RNA sample } (\mu g/\mu l) = \frac{40 \times A_{260} \times \text{dilution factor}}{1000}$$

$$\text{Total yield} = \text{concentration} \times \text{volume of RNA}$$

2) DIG labeling (DNA probe) using PCR DIG Probe Synthesis Kit (Roche, USA)

Plasmid DNA of PAL, LgPOD and 18S rRNA clones (10 pg) were used as template for amplification with the specific upstream and downstream primers. The sequences of specific primers described as Table 2. Templates were used for the PCR reaction compared with DIG labeled probe and unlabeled DNA control as Table 3. The PCR mixture was initially denatured at 94°C for 5 min and then subjected to 30 cycles at the following condition: 94°C for 30 s, 48°C (PAL) or 55°C (LgPOD and 18S rRNA) for 30 s, and 72°C for 40 s with a final extension at 72°C for 7 min. PCR products of PAL, LgPOD and 18S rRNA clones (380, 227 and

512 bp, respectively) were quantified by comparing with DNA low mass marker (Invitrogen, USA).

Table 2 Specific forward and reverse primers for PCR DIG labeling using PCR reactions

Gene	Forward primer	Reverse primer
PAL	CTGACCACTTGACTCACAAA	AACTCATGTTTGCTCAATTC
LgPOD	GGAACGTGCAACTAGGGAGA	GTCAGGGTTTTCGGTGTTGT
18S rRNA	CGGGGAGGTAGTGACAATAAATA	TAATGAAAACATCCTTGGCAAAT

Table 3 The components of PCR reactions with DIG labeling

Components	DIG labeled probe	Unlabeled DNA control
Sterile H_2O	variable	variable
10x PCR buffer with $MgCl_2$	5 µl	5 µl
PCR DIG labeling mix	5 µl	-
dNTP stock solution	-	5 µl
Forward primer	1 µl	1 µl
Reverse primer	1 µl	1 µl
Expand enzyme mix	0.75 µl	0.75 µl
Template (10 pg)	variable	variable
Final volume	50 µl	50 µl

3) Electrophoresis and blotting

Total RNA (30 µg) isolated from mangosteen pericarp was separated by electrophoresis on 1.0% MOPS-formaldehyde agarose gel at 55 volts for 2.5 h under a fume hood. After running the gel with 1x MOPS buffer, the gel was pre-equilibrated in 10x SSC with gentle shaking for 10 min to remove any remaining formaldehyde and the RNA transferred to a positively charged nylon membrane (Roche, Germany) with overnight blotting using 10x SSC (Sambrook *et al.*, 1989).

The membrane was soaked in 2x SSC to remove 10x SSC and then baked in a hybridization oven (Thermo Hybrid, UK) at 80°C for 2 h. The membrane was packed in a plastic bag and stored at 4°C for later use or used directly for pre-hybridization.

4) Pre-hybridization, probe hybridization, post-hybridization washing and chemiluminescent detection

Membranes (15 x 10 cm) were pre-hybridized in 10 ml hybridization buffer (7% SDS, 50% formamide, 5x SSC, 0.1% N-lauroylsarcosine, 2% blocking solution, and 50 mM sodium phosphate, pH 7) at 42°C (PAL) or 45°C (LgPOD) for 45 min, with gentle agitation. Denatured probe was added to the 10 ml pre- hybridized buffer to achieve a final probe concentration of 20 ng/ml of hybridization buffer. Following hybridization at 42 or 45°C overnight, the membrane was washed twice with 2x SSC containing 0.1% SDS for 10 min at room temperature and 0.5x SSC containing 0.1% SDS for 15 min at 42 or 45°C (pre-warmed). The hybridized membrane was equilibrated in 1x maleic acid washing buffer for 5 min, and then blocked with 5 ml of 1x blocking solution with gentle shaking for 45 min at room temperature. 0.55 μl was taken from the top of the centrifuged alkaline phosphatase antibody solution (Anti-Digoxigenin-AP, Roche, USA), added to the blocking solution and incubated with gentle shaking for 30 min at room temperature. After being washed twice with 1x maleic acid washing buffer for 15 min at room temperature, the membrane was equilibrated in detection buffer (0.1 M Tris, pH 9.5 and 0.1 M NaCl) for 5 min, and then sealed in a plastic bag and equilibrated in CDP-Star substrate (Roche, USA) for 1 min. The membrane was exposed to radiography film (Kodak Medical X-Ray film) suitable for chemiluminesence.

5) Stripping of the bound probe and re-hybridization with different probes

The membrane was rinsed briefly in RNAse-free water, 5x SSC for 5 min, then stripped in stripping solution. The 0.1% SDS boiled stripping

solution was poured on the membrane and allowed to cool down at room temperature with vigorous agitation. After stripping, the membrane was washed in 2x SSC for 5 min. Now the membrane is ready for pre-hybridization again or was stored in 2x SSC in a sealable plastic bag at 4°C for later use. At last, equal RNA loading was checked by hybridization with mangosteen 18S rRNA probe at 55°C.

2.3.5 Semi–quantitative RT-PCR and real-time PCR

1) Design of specific primers for reference (GAPDH) and target (PAL and LgPOD) genes

This experiment used the GAPDH gene as the reference gene. All specific primers for real-time PCR were designed based on the ABI supplier's instructions and set to the annealing temperature 58-60°C with the amplification size of 100-150 bp. Specific GAPDH, PAL and LgPOD primers were designed from the mangosteen sequence given in the results (Table 6), and described in Table 4.

Table 4 The sequence of specific primers used for reverse transcription followed by semi–quantitative RT-PCR and real-time PCR assays

Gene	Forward primer	Reverse primer
Semi-quantitative RT-PCR		
PAL	CTGACCACTTGACTCACAAA	GAATTGAGCAAACATGAGTT
Mgstpal	TGGAGCACAGTCTTGATGGA	GAGCCATTGAGGTGAAGTCC
LgPOD	GGAACGTGCAACTAGGGAGA	GTCAGGGTTTTCGGTGTTGT
GAPDH	GCCCTTGTCCTTCCAACTCT	CACCTCTTCGGCAAATGTCT
Real-time PCR		
Mgstpal	TGGAGCACAGTCTTGATGGA	GAGCCATTGAGGTGAAGTCC
LgPOD	GGAACGTGCAACTAGGGAGA	GTCAGGGTTTTCGGTGTTGT
GAPDH	GCCCTTGTCCTTCCAACTCT	CACCTCTTCGGCAAATGTCT

2) DNase treatment and cDNA synthesis

Five µg of total RNA were treated with DNaseI enzyme (Fermentas, USA) as described by the supplier's instructions. The reaction mixture (5 µg of total RNA, 5 µl of 10x DNaseI buffer containing $MgCl_2$, 5U of DNaseI enzyme, and adjusted with DEPC-water to 50 µl) was incubated at 37°C for 30 min, an equal volume of PCI (50 µl) and 200 µl of DEPC-water added, and then mixed well by vortexing. After centrifugation (13,000 x g for 5 min at room temperature), the top aqueous phase was transferred to a new 1.5 ml microtube, and 2.5 volumes of chilled absolute ethanol were added. The RNA was precipitated at -70°C for 30 min. The RNA was centrifuged at 13,000 x g for 20 min at 4°C, washed once with 70% chilled ethanol and re-suspended in 10 µl of DEPC-water. To confirm that no any DNA remains, DNased RNA was quantified by PCR amplification with GAPDH primers.

The first strand cDNA was synthesized from treated DNase RNA (10 µl) using M-MuLV Reverse Transcriptase kit (Fermentas, USA) as described by the supplier's instructions and used as a template to amplify the targeted genes for semi-quantitative PCR and real-time PCR.

3) Reaction of semi-quantitative RT-PCR

The PCR mixture was initially denatured at 94°C for 5 min and then subjected to 20-30 cycles (before the saturated band was obtained on the agarose gel) in the following conditions: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min. The reaction components were used for semi-quantitative RT-PCR as shown in Table 5.

Table 5 The components of reactions for semi–quantitative RT-PCR

Components	Volume (µl)
10x PCR buffer	2.5
25 mM $MgCl_2$	2.5
10 µM dNTPs	0.5
10 µM forward primer	0.5
10 µM reverse primer	0.5
Taq polymerase enzyme	0.15
Distilled water	variable
cDNA	variable
Total volume	25

4) Reaction of real-time PCR

The real-time PCR analyses of reference and target gene transcripts in cDNA samples were conducted in an ABI model 7000 thermocycler (Applied Biosystems, USA). A 20-µl PCR reaction was prepared containing 1 µl of template cDNA, 10 µl of QuantiTect^{TM} SYBR$^{®}$ Green PCR master mix (Qiagen, Germany), 1 µl of 10 µM of each primer and 7 µl of H_2O. All samples were amplified under the following conditions: 95°C for 10 min for 1 cycle, followed by 40 cycles of 95°C for 15 s and 1 min at 60°C. The PCR products for each primer set were also subjected to melting curve analysis. The melting curve analysis was done from 60-95°C to ensure the resulting fluorescence originated from a single PCR product and did not represent the primer dimers or nonspecific product. No-template controls were also included to detect any signals from the amplification of any DNA contamination or the primer dimer formed during the reaction. Results were analyzed with the sequence detection software SDS version 2.0 (Applied Biosystems, USA).

Direct detection of the PCR product was measured by monitoring the increase in fluorescence caused by the binding of SYBR® green dye to double-stranded DNA. A fluorescence threshold was set manually to ΔRn on the log fluorescence scale to determine the fractional cycle number (Ct value) at which the fluorescence passed the detection threshold. For each cDNA sample, relative expression levels of each protein coding gene were normalized by reference to the rRNA gene assay. The transcript abundance ratio of target gene to reference gene was determined by the following equation: relative expression = $(E_{ref})^{Ctref}/(E_{target})^{Cttarget}$, where E_{ref} and E_{target} are the efficiencies of the primers for the reference and target gene, respectively, and Ct_{ref} and Ct_{target} are the mean Ct value of reference and target genes, respectively.

Statistical analysis

Using a SAS package, data were analyzed statistically by ANOVA and the significance of the differences between means was estimated by Duncan's new multiple range test (DMRT). Data were the average of three or four replications ± standard deviation (S.D.). All experiments were repeated once or twice.

THE EXPERIMENTAL TIME AND PLACES

The experiments were carried out during June 2002-February 2007 and the research was conducted at the places as described below:

1. Postharvest Research Unit, Central Laboratory Greenhouse Complex (CLGC), Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom, Thailand.

2. The Horticulture and Food Research Institute of New Zealand (HortResearch, Mt Albert Research Centre), Auckland, New Zealand.