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Analysis of the PaGAMYBBP gene in *Phalaenopsis amabilis*

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Abstract

An EST encoding the GAMYB binding protein (GAMYBBP) of *Phalaenopsis amabilis* subsp. *formosa* was isolated from a cDNA library constructed using the PCR-select cDNA subtraction method. Gibberellin (GA) signaling plays important roles in plant growth and development. The full length cDNA was 2552 bp obtained with RT-PCR and 5'RACE and designated *PaGAMYBBP*. The predicted coding region of *PaGAMYBBP* contains 1899 nucleotides. The deduced peptide sequence of GAMYBBP has a conserved SKIP/SNW domain from residues 205 to 368. The SKIP (Ski interacting protein) is a nuclear protein exists in chromatin and functions as a transcriptional regulator in *Drosophilia.* The expression of *PaGAMYBBP* was inhibited by GA at concentration of 10^{-4} M. Abnormal temperature that caused heat stress increased the expression of *PaGAMYBBP* but cold stress did not affect the expression. We hypothesize that the PaGAMYBBP binds to GAMYB with the SKIP/SNW and thus regulate the expression of GA-responsive genes to prevent the precocious expression of downstream hydrolase gene under insufficient GA signal.

關鍵詞：*Phalaenopsis amabilis,* Gibberellin, GAMYB binding protein

Analysis of the PaGAMYBBP gene in *Phalaenopsis amabilis*

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Introduction

Phalaenopsis is the most beautiful one among all orchids, and distributes from subtropics to tropic areas. The classification hierarchy of *Phalaenopsis* is as follows, Angiospermae, Monocotyledoneae, Gynandrae, Orchidaceae, Epidendroideae, Subtribe Vandeae, and *Phalaenopsis*. *Phalaenopsis amabilis* var. *formosa* Shimadzu and *Phalaenopsis equestris* are native in Taiwan (陳, 2002).

Phalaenopsis fixed CO_2 *via* CAM (Crassulacean acid metabolism) pathway (Neales and Hew, 1975; Arditti, 1992). CAM is an adaptation of photosynthesis to limited availability of water or CO_2 (Cushman and Bohnert, 1999). In CAM plants, CO_2 is captured at night by PEP carboxylase in cytosol and the malic acid is stored in vacuole. CAM plants close stomata during the day, and the storage malic acid is released from vacuole to chloroplast and decarboxylated by NADP-malic enzyme to release CO_2. The released CO_2 is fixed by Calvin cycle. The CAM mechanism enables plants to improve water use efficiency which is achieved by closing stomata during the hot, dry day to minimize water loss (Taiz and Zeiger, 2002). Thus, CAM is associated with plants that inhabit extremely arid environment like deserts (Cushman, 2001).

Gibberellins (GAs) control growth and development throughout the life cycle of the plant. GAs promote stem and leaf growth, induce seed germination, modulate flowering time and the development of flowers, fruits, and seeds (Thomas and Sun, 2004; Sun and Gubler, 2004). There is much evidence that environmental stimulation, including light and temperature can affect plant growth by either changing the GA concentration and/or altering the GA response (Olszewski *et al*., 2002). The biosynthesis of GA in higher plants can be divided into three stages: (1) biosynthesis of *ent*-kaurene in proplastids; (2) conversion of *ent*-kaurene to GA_{12} via microsomal cytochrome P450 monooxygenases; and (3) formation of C_{20}- and

C_{19}-GAs in the cytoplasm (Olszewski *et al*., 2002). Low temperature alters GA biosynthesis. A subset of genes involving in GA biosynthesis were up-regulated in response to low temperature, including *AtGA3ox1* and *AtGA3ox3* (Yamauchi *et al*., 2004). Induction of seed germination or flowering (vernalization) by exposure to low temperature is related to GA signaling (Hedden and Kamiya, 1997).

The GA-inducible expression of the key hydrolytic enzyme, α-amylase, has been intensively studied GA-responses in aleurone cell (Woodger *et al*., 2004). GARE (GA responsive element) has been defined in the promoters of α-amylase genes and candidates for transcription factors which bind to these regulatory element have also been isolated (Woodger *et al*., 2003).

A GA-inducible MYB-transcription factor known as GAMYB functions as a transcriptional activator of α-amylase gene expression in barley aleurone (Gubler *et al*., 1995). HvGAMYB was the first identified MYB transcriptional activator expressed in barley (*Hordeum vulgare*) aleurone which is induced by GA (Murray *et al*., 2003). GAMYB binds to the GARE to trans-activate a number of hydrolase gene promoters. The N-terminal region of HvGAMYB contains a typical R2/R3-MYB DNA-binding domain, consisting of two helix-loop-helix repeats, and *in vitro* binding studies had shown that HvGAMYB binds specifically to GARE (Woodger *et al*., 2003). GAMYB also appears to function in tissues out of the aleurone layer. GAMYB implicates in floral organ development, over-expression of GAMYB led to aberrant anthers and reduced fertility in barley (Murray *et al*., 2003). Rice GAMYB loss-of function mutants have defects in pollen development (Kaneko *et al*., 2004)

Four GAMYB binding proteins were found in barley through yeast two-hybrid system including KGM and GMPOZ (Woodger *et al*., 2003). KGM (Kinase associated with GAMYB) is a Mak-like kinase with ser/thr kinase activity. Transient expression of KGM blocks HvGAMYB transcription regulation of barley *α-Amy 1* promoter, indicating that KGM acts as a repressor of GAMYB (Woodger *et al*., 2003). The repressor activity of KGM depends on a conserved Tyr_{158} residue in the activation loop of KGM. Phosphorylation of GAMYB through KGM has been proposed as a potential mechanism for regulation of GAMYB function in aleurone cells (Woodger *et al*., 2003; Sun and Gubler, 2004). Another GAMYB-binding protein GMPOZ (GAMYB-associated *POZ* protein) which

contains a BTB/POZ domain is a GA-inducible activator of α-amylase promoter activity (Woodger *et al*., 2004).

The predicted amino acid sequence of an EST (H136) previously isolated from *P. amabilis* was found to be homologue to the SKIP/SNW GAMYB-binding protein. SKIP was originally discovered as Ski interacting protein, which interacts with ski protein through its conserved SNW domain (Prathapam *et al*., 2001). *Ski* was first identified in Sloan-Kettering avian retroviruses and v-ski was found to transform chicken embryo fibroblasts (Li *et al*., 1986). Skip was found to interact with a highly conserved region of Ski required for its transforming activity, suggesting that this interaction was important for the ability of Ski to transform cells (Prathapam *et al*., 2001). An EST clone of 413 bp was isolated from our PCR-select subtraction library based on heat subtraction in a previously study in our lab (Houng, 2004).

In this study, a full length of cDNA of 2552 bp encoding the *P. amabilis* GAMYBBP was completed and designated *PaGAMYBBP*. We examined the *PaGAMYBBP* mRNA expression level under gibberellin treatment and temperature stress. Our results indicated that the expression level of *PaGAMYBBP* was inhibited at 10^{-4} M GA concentration and induced by heat treatment.

Materials and Methods

I.Plant Material, Gibberellin Treatment and Temperature Stress Treatment

The *Phalaenopsis amabilis* (TS97) were purchased from Taiwan Sugar corporation agri-business division. Plants contained at least four leaves and the length of leaf-span was over 30 cm. Plants were grown in 6 inch pots with sphagnum moss in a 25°C growth chamber with 14 hr light: 10 hr dark, 75% relative humidity, and 150 mol m^{-2} s^{-1} of illumination provided by fluorescent lights. Plants were watered weekly with half strength Hoagland solution. Intact plants were treated with 5 ml of 1×10^{-4} M, 1×10^{-5} M or 1×10^{-6} M GA_3, respectively, for 6 hr before harvesting. The hormone solution (containing 0.2% ethanol, 0.1% Tween® 20, pH 7.5-8.0; Cox *et al*., 2004) was applied to the growth point on the top of the stem. The mock solution without GA_3 but containing 0.2% ethanol, 0.1% Tween® 20 was used as control. For high or low temperature stress treatments, plants grown in a 25°C growth chamber with normal light cycle, were transferred to high (37°C) or low (4°C) temperature condition for the desired time.

II.cDNA Library Screening

TS97 cDNA library was constructed by Lee H. C. following the description in cDNA Synthesis Kit instruction manual (STRATAGENE).

III.Total RNA Extraction

All solutions and de-ionized water must be treated with DEPC. Tissue of 2-2.5 g was ground with pre-chilled mortar and pestle in liquid nitrogen. The fine ground tissue was added to pre-warmed 15 ml RNA extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl and 0.5 mg/ml spermidine) promptly and mixed well by inverting and vortexing, then the sample was heated at 65°C in water bath for 3 min. The sample was extracted two times with 15 ml of chloroform: isoamyl alcohol, 24:1 (CI), mixed well by shaking for 15 min at room temperature and the phases were separated by centrifugation at room temperature for 30 min at 10,000 × *g*. A 1/4 volume of cold 10 M lithium chloride was added to the supernatant and mixed well by inverting slowly. RNA was precipitated overnight at 4°C and harvested by centrifugation at 10,000 × *g* for 30

min. The pellet was washed in 500 µl 80% ethanol and centrifugated at 10,000 × *g* for 30 min in microcentrifuge tube at 4°C. After drying by vacuum, the pellet was dissolved in 400 µl DEPC-ddH_2O. After extraction two times with 400 µl of CI, mRNA was precipitated in 3 times volume of 95% ethanol and 1/10 volume of 3 M sodium acetate at -70°C for an hour and centrifugated at 10,000 × *g* for 30 min. The pellet was washed with 80% ethanol, vacuum dried, and dissolved in appropriated volume of DEPC-ddH_2O. RNA was quantified with a spectrophotometer and then stored in 3 times volume of 95% ethanol and 1/10 volume of 3 M sodium acetate at -70°C.

IV.Full Length cDNA Synthesis

4.1 Reverse Transcription

Total RNA of 20 µg was dissolved in 7.5 µl DEPC-ddH_2O, and add 1 µl of 100 µM 18-mer oligo-dT primer was added. Denaturing was done at 70°C for 5 minutes to melt RNA secondary structure. Four microliters of M-MLV 5 × reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, and 50 mM DTT), 2 µl of 10 mM dNTPs, 2 µl of 100 mM DTT, 2 µl of 10 × BSA (1 mg/ml), and 0.5 µl of RNasin were added to the mixtures respectively. After incubation at 37°C for 10 min, M-MLV reverse transcriptase (200 U/µl, Promega) of 1 µl was added, mixed well by gently pipeting and incubated at 37°C for 90 min. The sample were heated at 95°C for 10 min to inactivate M-MLV reverse trancriptase and stored at -70°C.

4.2Polymerase Chain Reaction

The reaction was carried out in 50 µl mixture containing 4 µl of reverse transcription products, 5 µl of 10 × PCR buffer, 6 µl of 5 µM primers respectively, 6 µl of 1.25 mM dNTPs, 1 µl of *Taq* polymerase and 28 µl of ddH_2O. Reaction was carried out with PCR program with 1 cycle at 95°C for 5 min, followed 35 cycles of 95°C for 50 sec, annealing for 50 sec (the annealing temperature is depending on the Tm of the primers) and 72°C for 90 sec and held at 72°C for 10 min.

4.35'-Rapid Amplification of cDNA Ends (5'-RACE)

BD SMARTTM RACE cDNA Amplification Kit (BD Bioscience Clontech) was used to amplify the 5' end of a specific gene (appendix 1). First strand cDNA was synthesized from 20 µg total RNA extracted from 37°C treatment of *P. amabilis* leaves with 5'-CDS primer. The PCR mix for 5'-RACE PCR reaction contained

34.5 μl PCR grade water, 5 μl of 10 × BD advantage 2 PCR buffer, 1 μl of 10 mM dNTP mix, 1 μl of 50 × BD advantage 2 polymerase mix, 2.5 μl of 5'-RACE-Ready cDNA, 5 μl of 10 × universal primer mix (UPM) and 1 μl of 10 μM gene specific primer (GSP). Reactions were carried out with 5 cycles of 94°C for 30 sec, 72°C for 3 min, followed 5 cycles of 94°C for 30 sec, 70°C for 30 sec, 72°C for 3 min, and 30 cycles of 94°C for 30 sec, 68°C for 30 sec, 72°C for 3 min.

PCR products were purified with the Gel-M™ Gel Extraction System (Viogene). The excised gel slice containing the DNA fragment (50-200 mg) was added into 500 μl of GEX buffer and incubated at 60°C until the gel was completely dissolved. A Gel-M™ column was placed onto a collection tube and centrifuged for 30-60 sec. The flow through was discarded and the column was washed with 500 μl WF buffer and 700 μl WS buffer with centrifugation for 30-60 sec, respectively. The column was centrifuged at 10,000 × *g* for additional 3 min to remove residual ethanol. The column was placed on a new 1.5 ml microcentrifuge tube and 30 μl of Elution buffer was added on the center of the membrane. The column was stood for 1-2 min and centrifuged at 10,000 × *g* for 3 min to elute DNA, then stored at -20°C. The 5'-RACE product was cloned into pGEM®-T easy vector and sequencing.

4.4Ligation

The ligation reaction was performed at 14°C for 20 hr in the presence of 100 ng vector DNA, 160-600 ng insert DNA (1:3-20 molar ratio of vector:insert), 3 U T4 DNA ligase (3 U/μl, Promega), and 1 × ligase buffer (30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP).

4.5Competent Cell Preparation

A single colony of *E. coli* XL1-Blue MRF' strain was incubated in 3 ml of LB medium on an orbital shaker at 37°C overnight with 240 rpm shaking. The culture was diluted in 50 ml LB and grown until OD_{600} reached 0.5-0.6. The cells were centrifuged at 3,000 × *g*, 4°C for 10 min and suspended in 25 ml cold 100 mM $CaCl_2$. After sitting on ice for 30 min, the cells were centrifuged at 3,000 × *g*, 4°C for 10 min. The precipitated cells were suspended in 5 ml cold 100 mM $CaCl_2$/50% glycerol and then put on ice for 20 min. Finally, the cells were aliquoted into 1.5 ml microcentrifuge tubes (300 μl each), frozen in liquid nitrogen and then stored at -70°C.

4.6Transformation

Ten microliters of ligation product were added to 300 μl of competent cell, and put on ice for 45 min. The mixture was heated at 42°C for 90 sec and put on ice for 2 min. LB medium of 500 μl was added and incubated on an orbital shaker at 37°C for 30 min. The transformed cells were centrifuged at 4,000 × *g*, 4°C for 5 min, and supernatant of 500 μl were removed. Cells were resuspended and laid on 1.5% LB agar medium containing 50 μg/ml~100 μg/ml ampicillin, 0.5 mM IPTG, and 80 μg/ml X-Gal. The plate was incubated at 37°C for 12-16 hr.

4.7Mini-Preparation of Plasmid DNA

4.8Restriction Enzyme Reaction

The reaction was carried out in 15 μl mixture containing 3 U of restriction enzyme (New England Biolab), 1.5 μl of 10 × buffer, 5 μl (1 μg) plasmid DNA and ddH_2O of 8.2μl and then incubate at 37°C for 12-16 hr.

4.9Double-Stranded DNA Sequencing

DNA sequencing was performed on both strands by cycle sequencing reaction method on double-stranded with fluorescent BigDye Terminators Kit (Applied Biosystems Industries, Foster City, CA, USA). The reaction was carried out in 20 μl mixture including 2 μl of BigDye Terminator ready reaction mix, 300 ng template DNA, 3.2 pmol reverse primer or T7 primer, and 6 μl of 2.5 × sequencing Buffer (200 mM Tris-HCl pH 9.0, and 5 mM $MgCl_2$). Sequencing was carried out with PCR programs of 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. DNA sequence were collected and analyzed in an ABI PRISM^{TM} 3700 automatic DNA sequencer (Applied Biosystems Industries, Foster City, CA, USA).

V.Northern Hybridization

5.1 RNA Electrophoresis

5.1.1 Formaldehyde Denatured Agarose Gel

An agarose gel of 1.2% containing 6.6% formaldehyde and 1 × MOPS was prepared by dissolving agarose in sufficient DEPC-ddH_2O and boiled in a microwave oven. The solution was cooled to 55°C and 10 × MOPS and 37% formaldehyde were added in a chemical fume hood, the gel solution was mixed well and poured into a tank.

5.1.2 RNA Sample Preparation

Each RNA sample of 2 μl, 2 μl of 10 × MOPS, 4 μl of 37% formaldehyde, 10 μl of 100% formamide and 1 μl of 200 μg/ml ethidium bromide was added to a microcentrifuge tube and incubated at 65°C for 10 min. The samples were chilled on ice for 10 min and then centrifuged briefly. Two microliters of 10 × formaldehyde gel-loading buffer (0.25% BB dye, 50% glycerol, and 10 mM EDTA) were added to each sample and sample were put on ice bucket.

5.1.3 Electrophoresis

The gel was installed in a horizontal electrophoresis box with sufficient 1 × MOPS buffer to cover the gel to a depth about 1 mm. The gel was pre-run at 5 V/cm at least 5 min and then RNA samples were loaded into the wells of the gel. RNA samples were resolved at 5 V/cm until the bromophenol blue has migrated about 8 cm.

5.2 Capillary Transfer

The gel was rinsed with DEPC-ddH_2O briefly and soaked the gel in 5 folds gel volume of 10 × SSC (20 × SSC: 0.33 M sodium citrate and 3 M NaCl) for 20 min. The Hybond N^+ nylon membrane (Amersham Bioscience) of the gel size was immersed in 10 × SSC for at least 5 min. A piece of 3 MM paper of the same size was placed under the gel to serve as salt bridge of the buffer. The tank was filled with appropriated transfer buffer (10 × SSC). The gel was placed on the 3 MM paper with surrounding area blocked by parafilm. The wet nylon membrane was put on the gel and two pieces of wet 3 MM paper were placed on the membrane. Any air bubbles were smoothed out with a glass rod. A stack of paper towels (5-8 cm high) were put on the 3 MM paper, and allowed upward transfer of RNA for 6-18 hr. The membrane was washed with 6 × SSC buffer for 5 min. The RNA was crosslinked onto the membrane using the optimal crosslink setting on the SPECTROLINE® UV crosslinker XLE-1000 (120 mJ/cm^2 of UV energy) for 30 seconds.

5.3 DNA Probe Preparation

DIG-labeled probes were generated during PCR. PCR was carried out in 50 μl mixture including 5 μl of 10 x PCR buffer, 100 pg of plasmid DNA, 0.1-1 μM

primers, 1 μl of *Taq* polymerase, 1 μl of 10 mM dATP、dGTP、dCTP respectively, 0.9 μl of 10 mM dTTP, 1 μl of 10 mM DIG-11-dUTP (Roche Applied Bioscience) and variable amount of ddH_2O to make a volume of 50 μl. Labeling was carried out with PCR programs of 3 cycles of 95°C for 3 min, 55°C for 90 sec, 72°C for 1 min, followed 35 cycles of 95°C for 2 min, 55°C for 90 sec, and 72°C for 1 min, and held at 72°C for 10 min. After PCR amplification, product of 5 μl was analyzed on agarose gel with electrophoresis and a specific band was visible. PCR products were purified with PCR-M™ Clean Up System (Viogene). PCR mixture was added to 500 μl of PX Buffer™, transferred to PCR-M™ column and centrifuged at 13,000 × *g* for 1 min. The waste solution was discarded, and the column was washed with 500 μl of WF buffer™, and then 700 μl of WS buffer™ (contain 80% ethanol). Centrifugation at 10,000 × *g* for additional 3-5 min was applied to remove the residual ethanol. 30-50 μl of Elution Buffer™ was added to the column. After standing the column for 3 min, the column was centrifuged at 10,000 × *g* for 3 min. The products were stored at -20°C.

5.4 Hybridization and Detection

5.4.1 Pre-Hybridization

The membrane was placed in a hybridization bag containing pre-warmed 20 ml pre-hybridization buffer (High SDS buffer: 7% SDS, 50% formamide, 5 x SSC, 2% blocking reagent (Roche), 50 mM sodium phosphate, and 0.1% N-lauroylsarcosine, pH 7.0) per 100 cm^2 of membrane surface area. The bag was sealed, and prehybridization was performed at 50°C for 3 hours.

5.4.2 Hybridization and Post-Hybridization Wash

The probe was denatured in boiling water bath for 10 min and diluted in high SDS buffer (probe concentration: 25 ng/ml). The pre-hybridization buffer was discarded and the hybridization buffer containing DIG-labeled probes was added. The bag was sealed, and hybridization was performed at 50°C for 16 hr. The membrane was washed with twice 15 min per wash in 2 × wash solution (2 × SSC and 0.1% SDS) at room temperature to remove unbound probe. The membrane was washed twice with 15 min per wash in 0.5 × wash solution (0.5 × SSC and 0.1% SDS) at 68°C.

5.4.3 Detection

The membrane was equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, and 0.3% Tween® 20) for 1 min and place the membrane in a hybridization bag containing 20 ml blocking solution (1% blocking reagent dissolve in 100 mM maleic acid, 150 mM NaCl, pH 7.5) per 100 cm^2 with gently shaking for an hour. The Anti-Digoxigenin-AP was diluted at 1:10,000 ratio in blocking solution after centrifuged for 5 min (antibody solution). The blocking solution was poured off and the membrane was incubated in antibody solution for 30 min. The antibody solution was discarded and the membrane was washed in washing buffer twice with 15 min per wash. The membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) for two min. After returning room temperature CSPD® was diluted at 1:100 ratio in detection buffer. The membrane was placed between two sheets of plastic page protectors, and 2.5 μl CSPD® per 100 cm^2 membrane area was added on the membrane and incubated for 15 min and then gently wiped to remove bubbles and additional solution to create a liquid seal around the membrane. The membrane was exposed to X-ray film for an hour.

VI. RT-PCR

The reverse transcripted (RT) product was used as PCR template. PCR was carried in 30 μl mixture containing appropriate RT-products, 3 μl of 10 × PCR buffer (10 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin, 1% Triton X-100), 3 μl of 6 μM Reverse and Forward primers, 3 μl of 1.25 mM dNTPs, 1 μl of Home made *Taq* polymerase (5U/μl) and ddH_2O of desired volume. Reaction was carried out with PCR program with 1 cycle of 95°C for 5 min, 22-24 cycles of 95°C for 50 sec, 60°C for 1 min (the annealing temperature is depending on the Tm of the primers) and 72°C for 90 sec and held in 72°C for 10 min. PCR products were analyzed with by electrophoresis on 2% agarose gel.

Results

I. Construction and Analysis of Full Length cDNA of *PaGAMYBBP*

An EST clone of 413 bp was isolated from our PCR-select subtraction library based on heat subtraction in a previously study (Houng, 2004). The EST was compared to the open reading frame of the orthologues of barley and rice (*Oryza sativa*), a lack of about 120 bp at 3' end and 1.4 kb at 5' end was predicted (Fig. 1a). To complete the full length cDNA, we first screened the cDNA library. After cDNA library screening, six clones were screened and sequences. These incomplete clones can be aligned to a clone containing 3' coding region of 561 bp and the 3'-UTR of 425 bp was obtained (Fig. 1b). A pairs of primers KgmP3 and KgmP2 were designed to obtain the 986 bp of the 3' region and 3'-UTR with RT-PCR. This fragment was cloned into pGEM®-T Easy vector, and digested with *EcoR* I to check the size of insert fragment (Fig. 2). The BD SMART™ RACE cDNA Amplification Kit (BD Bioscience Clontech) was used to obtain the 5' region with the gene specific primer (KgmP4) 5'-^{1643}CCAGTATTAGCCATCCCCAGAGC^{1621}-3'. The expecting PCR product of 1.7 kb was cloned into pGEM®-T Easy vector and digested with *EcoR* I to confirm the size (Fig. 3). After sequencing, this clone was verified to contain the 5' region of 1392 bp and 5'-UTR of 252 bp. The full length cDNA of *PaGAMYBBP* is 2552 bp, including 5'-UTR of 252 bp and 3'-UTR of 425 bp. The coding region of *PaGAMYBBP* cDNA is 1875 bp from nucleotide 253 (predicted translation start site ATG) to 2124 (predicted stop codon TGA) and codes for polypeptide of a 624 amino acids.

Alignment search of the deduced PaGAMYBBP amino acids sequence was done to find the orthologues and conserved domain with blastx and CDART programs on the NCBI website. The PaGAMYBBP was predicted to be a chromatin protein containing SKIP/SNW domain (Fig. 4). The deduced amino acid sequence of *PaGAMYBBP* was aligned with its orthologues from rice, barley and Arabidopsis. The PaGAMYBBP shares high similarity with its homologues. A SKIP/SNW domain from amino acid sequence 205 to 368 was found in PaGAMYBBP (Fig. 5).

The orthologue of this gene in Arabidoposis is At1g77180 which is annotated as a chromatin protein containing SKIP/SNW domain but with unknown molecular function and biological process. The search result of At1g77180 with WU-Blast2 to TAIR database indicates that the gene is a unigene in Arabidopsis.

II. The Expression of *PaGAMYBBP* mRNA in Leaf and Floral Bud

The transcription level of the *PaGAMYBBP* was analyzed in leaf and floral bud under normal condition. RNA gel blot was hybridized to a gene specific DIG-labeled probe of DNA fragment containing 1 to 1644 of the full length *PaGAMYBBP* cDNA. The expression level of *PaGAMYBBP* mRNA in leaf was similar to that in floral bud (Fig. 6).

III. The Expression of *PaGAMYBBP* mRNA Under Different Treatments

3.1 Gibberellin Treatment

The transcript level of the *PaGAMYBBP* was analyzed in *P. amabilis* leaves after treated with GA_3 of 1 x 10^{-4} M, 1 x 10^{-5} M and 1 x 10^{-6} M concentrations. Northern blot was analyzed by hybridization to the DIG-labeled gene specific probe. The *PaGAMYBBP* mRNA in leaves under GA_3 concentrations of 10^{-5} M and 10^{-6} M were detected at a similar level to that of the control. Interestingly, the expression level of *PaGAMYBBP* mRNA in leaf treated with 10^{-4} M GA_3 was significantly decreased as compared to the control (Fig. 7). The expression level of *PaGAMYBBP* mRNA was inhibited by GA_3 at the concentrations of 10^{-4} M.

3.2 Heat Treatment

The transcription level of *PaGAMYBBP* in leaves was analyzed after heat (37℃) treatment for 0 hr, 1 hr, 4 hr, 6 hr, 12 hr, 24 hr, 36 hr and 48 hr. Northern blot was analyzed by hybridization to the gene specific probe as previously described. Compare the expression level of *PaGAMYBBP* mRNA in heat treated leaves, slightly increased as compared to that in the control (Fig. 8).

3.3 Cold Treatment

The transcription level of *PaGAMYBBP* in leaves was analyzed after cold (4℃) treatment leaves for 0 hr, 1 hr, 6 hr, 12 hr, and 24 hr. Northern blot was analyzed by the gene specific probe as previously described RNA gel blot was hybridized

with gene specific DIG labeling probe as former described. The expression pattern showed no difference after cold treatment (Fig. 9).

According to the information from the level of *PaGAMYBBP* mRNA, *PaGAMYBBP* is slightly induced by high temperature stress but not affected by cold stress. The expression level of *PaGAMYBBP* mRNA was also inhibited at 10^{-4} M GA_3. The expression level of *PaGAMYBBP* mRNA in leaf was similar to that in floral bud.

Discussion

The GAMYB-binding protein was first observed in barley. Four GAMYB-binding protein unigenes in barley aleurone layer or anthers were found in NCBI uni-gene database, including a novel WD40 protein (AY167560), a homologue of the human nuclear co-activator SKIP (AY167563), KGM (Kinase associated GAMYB-binding protein; AY167561) (Woodger *et al*., 2003) and GMPOZ (GAMYB associated BTB/POZ protein; AY587552) (Woodger *et al*., 2004). The functions of KGM and GMPOZ had been characterized (Woodger *et al*., 2003; 2004).

Transient expression of KGM repressed promoter activities of α-amylase which is a GA-responsive hydrolase under the control of GAMYB (Woodger *et al*., 2003). The orthologue of KGM in Arabidopsis is At4g19110 which is annotated with functions of ser/thr kinase and ATP binding activity. *GMPOZ* has been identified in cereals, tomato (*Lycospericon esculentum*), soy bean (*Glycine max*) and cotton (*Gossypium hirsutum*) but there is no characterized relative of *GMPOZ* in the gene databases (Woodger *et al*., 2004). BTB/POZ domain is a homo- and heterodimers forming about 120 amino acids, which is found at the N terminus found in variety of transcriptional regulators, cytoskeleton and chromatin (Collins *et al*., 2001). In barley, GMPOZ is a nuclear factor functions as an activator of α-amylase (Woodger, *et al*., 2004). Orthologue of GMPOZ in Arabidoposis is At3g61660 which is a hypothetic protein with unknown function.

The full length of *PaGAMYBBP* cDNA is 2552 bp, from 253 to 2124 bp is the coding region. According to the results of blastx (Table 1.) and alignment of amino acid sequence (Fig. 5), this gene is similar to the rice putative

GAMYB-binding protein (XP_467904) and barley GAMYB-binding protein (AAO25542). *Phalaenopsis*, *Oryza Sativa*, and *Hordeum vulgare* are all monocotyledons and their GAMYB-binding protein orthologues all contain SKIP/SNW domain.

SKIP was originally discovered as Ski interacting protein. Using v-Ski sequence as bait, the human gene skip was identified as encoding a protein which interacts with both the cellular and viral forms of Ski in the two-hybrid system (Dahl *et al*., 1998). SKIP/SNW domain exists in chromatin protein and nuclear skip protein. Skip interacts with ski protein through its conserved SNW domain (Prathapam *et al*., 2001). Skip has a role in regulation of transcription. Bx42 is the homologue of human Skip in *Drosophilia melanogaster* which is found to be associated with chromatin in transcriptionally active puffs of salivary glands, suggesting a role in regulation of transcription (Prathapam *et al*., 2001). Skip also involves in activation of Notch signaling. NotchIC is a constitutively activated Notch receptor which translocates to nucleus where targets to CBF1. The CBF1 is a member of the CSL DNA-binding protein family, CSL stands for CBF1, Su (H), and Lag-1, and CBF1 functions as a transcriptional repressor of Notch signaling (Ronchini and Capobianco, 2001). Skip was shown to interact with NotchIC and CBF1 to facilitate the function in the activation of downstream target genes of Notch signaling (Zhou *et al*., 2000). These downstream genes affect the gene expression that in turn to determine cell fate during development (Lodish *et al*., 2003).

According to these informations, we hypothesize that *PaGAMYBBP* affects the expression of GA-responsive gene through protein-protein interaction level. PaGAMYBBP might interact with GAMYB through its SKIP/SNW domain to regulate GA-responsive hydrolase gene expression at transcription level. In barley, KGM repressed α-amylase activity at GAMYB level which might avoid precocious α-amylase synthesis and premature hydrolysis of starchy endosperm reserves in non-GA treated aleurone cell (Woodger *et al*., 2003). The mRNA expression level of *PaGAMYBBP* decreased by GA_3 treatment at 10^{-4} M indicating that *PaGAMYBBP* expressed under conditions of insufficient GA concentration condition to inhibit GA-responsive hydrolase expression.

The EST was first isolated from our PCR-select subtraction library based on heat subtraction, and the expression level of *PaGAMYBBP* mRNA was slightly

induced by heat (37°C) treatment. ABA induction can be an important component of thermo tolerance, and ABA is a GA antagonist. The effect of gibberellin on high temperature tolerance is the reverse of ABA (Maestri *et al*., 2002). An inherently heat-resistant dwarf mutant of barley impaired in the synthesis of GA was rendered to heat-sensitive by application of gibberellin (Vettakkorumakankav *et al*., 1999). In barley, when the aleurone layer was exposed to heat shock treatment, the synthesis of α-amylase was suppressed while heat shock proteins were induced (Belanger *et al*., 1986). Comparison the heat-shock responses of barley aleurone layers with or without GA treatment found that heat shock (40°C) blocked the synthesis and secretion of secretory protein, like α-amylase, from GA treated layers but not untreated layers (Grindstaff *et al*., 1996). According to these findings, we hypothesize that at 37°C, the amount of PaGAMYBBP increases and functions as a repressor of GAMYB to block the expression of GA responsive hydrolase gene.

In our experimental sets, the mRNA expression pattern of *PaGAMYBBP* showed no difference between cold treatment and the control, but GA signal is related to low temperature. A subset of genes involve in GA biosynthesis were up-regulated in response to low temperature, including *AtGA3ox1*and *AtGA3ox3* (Yamauchi *et. al*., 2004). Induction of seed germination or flowering (vernalization) by exposure to low temperature is processes in which GA have been implicated (Hedden and Kamiya, 1997). Although cold treatment increases the biosynthesis of endogenous GA however, cold treatment did not inhibit the expression of *PaGAMYBBP* in our experiment.

GAMYB also functions in tissues outside the aleurone. In barley, an increase in GAMYB level caused a progressive decrease in anther size, particularly a decrease in anther length, and anther also became increasingly lighter in color (Murray *et al*., 2003). In Arabidopsis, there were three genes with GAMYB-like activities identified, *AtMYB33, AtMYB65, AtMYB101,* which can substitute for barley GAMYB in trans-activation of barley α-amylase promoter (Gocal *et al*., 2001). *AtMYB101* expressed in sub-apical tissues which maybe involved in stem elongation. *AtMYB33* may mediate a GA signaling in flowering was supported by its ability to bind to the promoter of the *LEAFY* (*LFY*) which functions to establish floral meristem-identity. *AtMYB65* and *AtMYB33* may constitute a pair of redundant gene with common functions in the plant (Gocal *et al*., 2001; Taiz and

Zeiger, 2002). Although *AtMYB33* and GAMYB in barely also involve in flower development, but the expression level of *PaGAMYBBP* mRNA in leaf was similar to that in floral bud.

The promoter region of Arabidopsis At1g77180 was analyzed (Appendix 2). Motifs of GA signaling, drought, and light response were found indicating that At1g77180 might be regulated by light. Light is involved in GA signaling to influence plant development. Far red light long day exposure can increase the activity of key group of biosynthetic enzymes, the 20-oxidases (Hisamatsu *et al*., 2005). Since the plants were treated under a long day condition, *PaGAMYBBP* expression may be directly affected by light signal or the endogenous GA was induced by light. To investigate the mRNA expression pattern of *PaGAMYBBP* under temperature stress or hormone (GA and ABA) treatment, it might be necessary to eliminate the effects light.

(a)

(b)

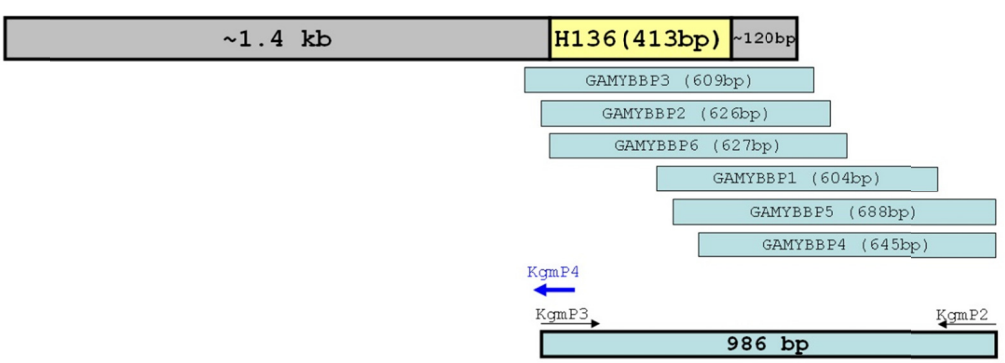


Fig. 1. Strategy for obtaining full length *PaGAMYBBP* cDNA. (a) Yellow box indicates the partial cDNA of 413 bp from subtraction library and grey boxes indicate the lacked part at 5'-end and 3'-end. (b) Six cloned were screened from cDNA library screening could be aligned and a pairs of primers KgmP2 and KgmP3 were designed for RT-PCR obtaining a fragment about 1kb. Primer Kgm4 was designed for 5'-RACE.

(a) (b)

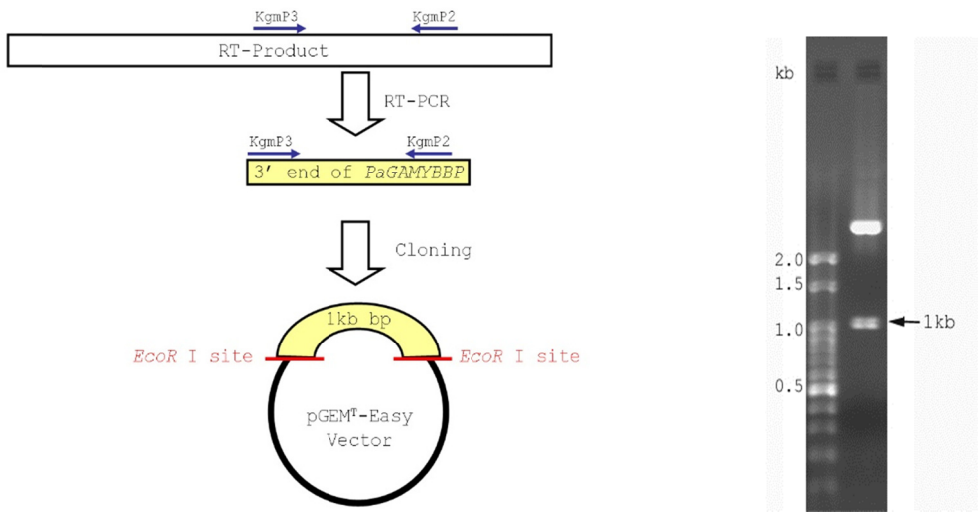


Fig. 2. To obtain 3' end of *PaGAMYBBP* cDNA through cDNA library screening and RT-PCR. (a) Strategy used for obtaining the 3' end of *PaGAMYBBP* cDNA. The primers were used for RT-PCR amplification corresponding to the known sequence from cDNA library screening. (b) The plasmid was digested with *EcoR* I and electrophoresis on 1% agarose gel. The arrow indicates the 3' partial cDNA fragment of about 1000 bp. Molecular size marker was on the left and indicated by kb.

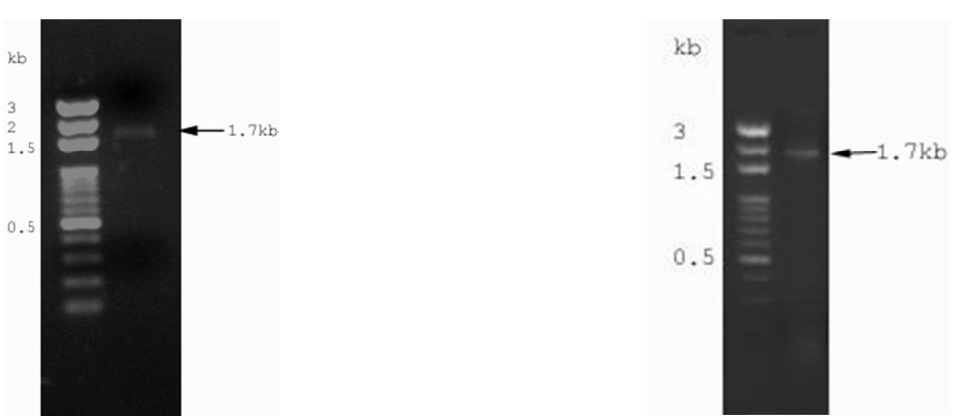
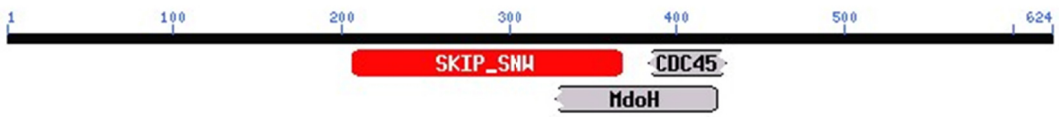


Fig. 3. The 5' region of *PaGAMYBBP* was obtained with 5'-RACE. (a) The arrow indicates the primary 5'-RACE PCR product of ~1.7 kb. (b) 5'-RCAE PCR

product was cloned into pGEM®-T Easy vector and digested with *EcoR* Ⅰ to confirm the size of insert. The arrow indicates the desired fragment of~1.7 kb. Molecular marker was on the left lane and indicates by kb.

(a)



(b)

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Query:  205  SKYIKYKPSQQSAAFISGAKERIIRMVEMPSDPLEPPS--SAQKVPRAFGSPPFPVMHSP  262
Sbjct:  1    AQYIRYTPSNQNGANNNSANQRIIRMVEKQQDPLEPPKFKIHKKVPRAPNSPPAPVLHSP  60

Query:  263  PRPVTVKDQQDWKIPPCISNWKNPKGYTIPPDKRLAADGRGLQEVQINDNFAKLSEALYV  322
Sbjct:  61   PRKVTVKDQNDWKIPPCVSNWKNPKGYTIPLDKRLAADGRGLQDVEINDNFAKLSEALYI  120

Query:  323  AEQKAREAVAMRSKVQRELMLKEKERKEQELRALAQKARSERTGIA  368
Sbjct:  121  AEKKAREEVRARAELERKLAQKEKEEKEDKLKELAQKAREERGGAR  166
```

Fig. 4. Prediction of SKIP/SNW Domain in PaGAMYBBP. The conserved domain in the deduced PaGAMYBBP amino acid (624 amino acids) was found with CDART (Conserved Domain Architecture Retrieval Tool) program on the NCBI website. (a) Red box indicates the SKIP/SNW domain in PaGAMYBBP. Gray boxes indicate the putative conserved domain with very low scores. (b) The SKIP/SNW domain from amino acid sequence 205 to 368 was found in PaGAMYBBP. The red words indicate the identical amino acids, the blue ones indicate the amino acids with similar characteristics and the black ones indicate the different amino acids. The length of SKIP/SNW domain in PaGAMYBBP is 164 residues, and 98.8 % are aligned. The score is 221 bits and E-value is 2e-58.

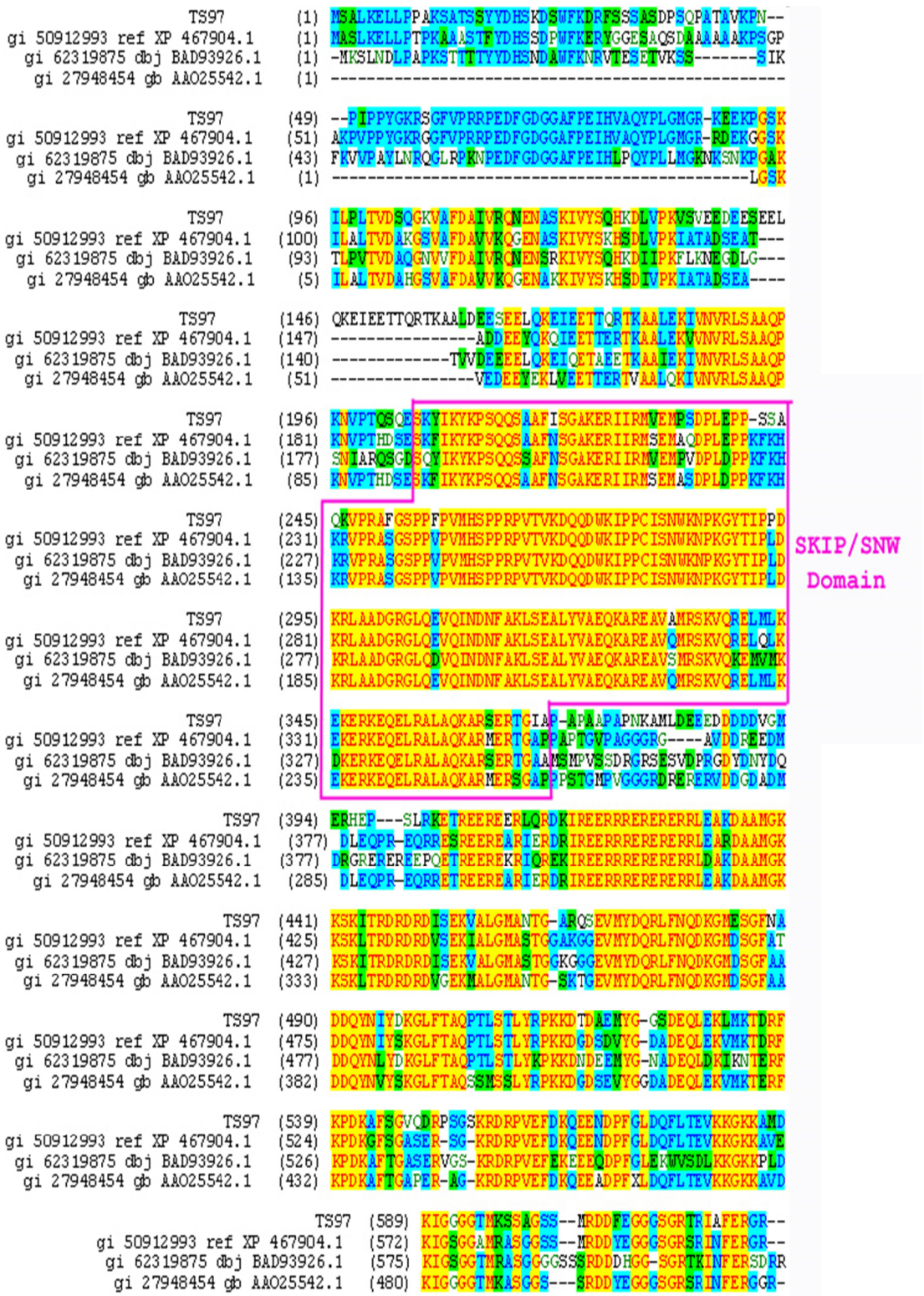

SKIP/SNW
Domain

Fig. 5. Alignment of four GAMYB-binding protein amino acid sequence. The SKIP/SNW domain from amino acid 205 to 368 is indicated within a pink frame.

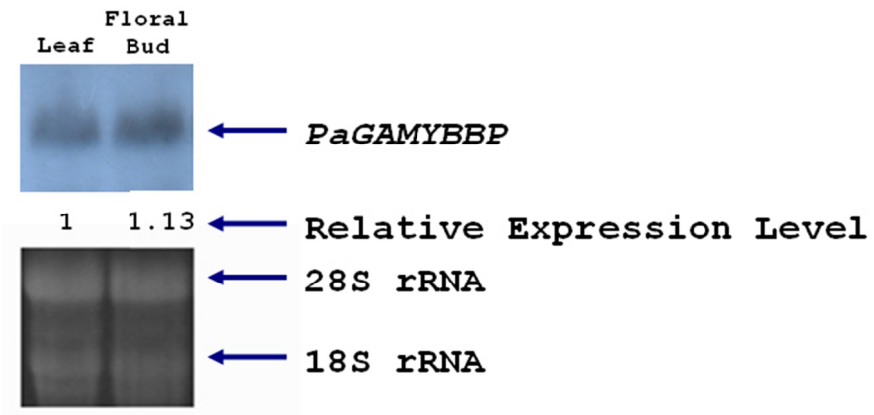
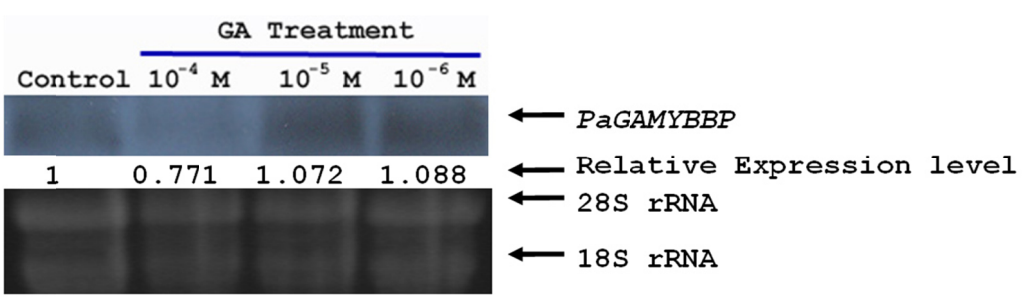


Fig. 6. Analysis of *PaGAMYBBP* transcriptional level in leaf and floral bud. Extract total RNA from leaves and flower buds of *P. amabilis* and analyzed on a formaldehyde denatured agarose gel. The membrane was hybridized to a DIG-labeled DNA probe prepared from a DNA fragment containing nucleotides 1 to 1644 of the full length *PaGAMYBBP* cDNA. Use total lab software to quantify the relative mRNA expression level.

(a)



(b)

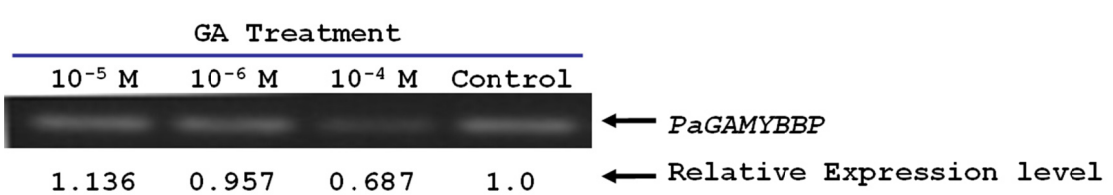


Fig. 7. Analysis of the *PaGAMYBBP* transcriptional level in leaves under different GA_3 concentration. (a) Total RNA (25 μg) extracted from leaves of *P. amabilis* treated with GA_3 for 6 hours at concentrations of 1 x 10^{-6} M, 1 x 10^{-5} M, or 1 x 10^{-4} M, and analyzed on a formaldehyde denatured agarose gel. The membrane was hybridized to a DIG-labeled DNA probe prepared from a DNA fragment containing nucleotides 1 to 1644 of the full length *PaGAMYBBP* cDNA. (b) RT-PCR products was electrophoresis on 2% agarose gel. The total lab software was used to quantify the relative mRNA expression level. The intensity of the control was set as 1 and the intensity of each sample was a relative amount of the control.

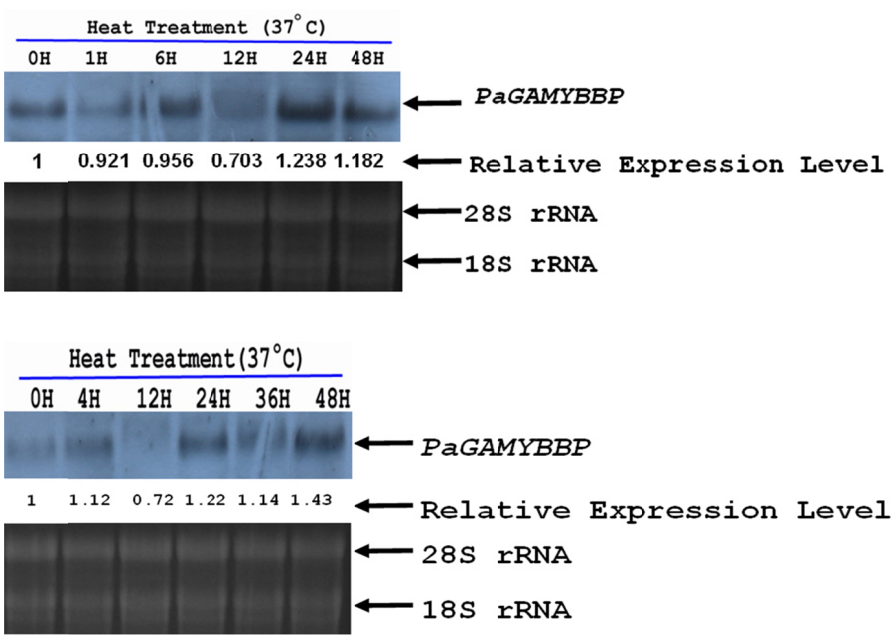
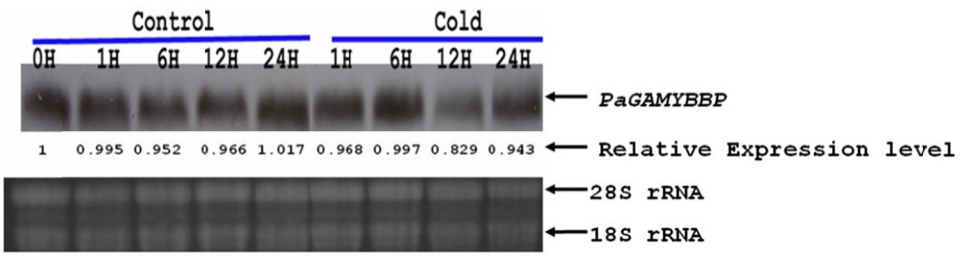


Fig. 8. Analysis of the *PaGAMYBBP* mRNA expression level in leaves under heat (37°C) treatment. Extract total RNA from leaves of *P. amabilis* treated with 37°C for the indicated time and analyzed on a formaldehyde denatured agarose gel. The membrane was hybridized to a DIG-labeled DNA probe prepared from a DNA fragment containing nucleotides 1 to 1644 of the full length *PaGAMYBBP* cDNA. The total lab software was used to quantify the relative mRNA expression level. The intensity of the control was set as 1 and the intensity of each sample was a relative amount of the control (0 hr).

(a)



(b)

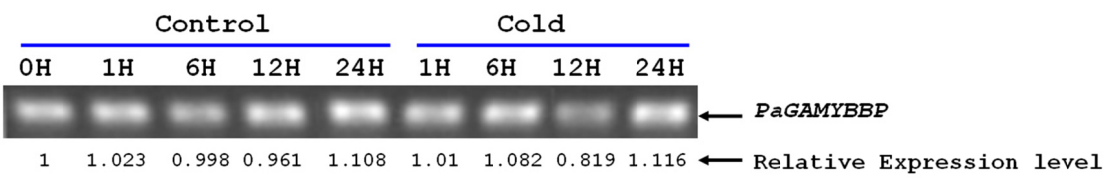


Fig. 9. Analysis of the *PaGAMYBBP* mRNA expression level in leaves under cold (4°C) treatment. (a) Extract total RNA from leaves of *P. amabilis* treated with 4°C for the indicated time and analyzed on a formaldehyde denatured agarose gel. The membrane was hybridized to a DIG-labeled DNA probe prepared from a DNA fragment containing nucleotides 1 to 1644 of the full length *PaGAMYBBP* cDNA. (b) RT-PCR products was electrophoresis on 2% agarose gel. Use total lab software to quantify the relative mRNA expression level.. The intensity of the control was set as 1 and the intensity of each sample was a relative amount of the control (0 hr).

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