CHAPTER II

MATERIALS AND METHODS

2.1 Equipments and reagents

2.1.1 Instruments

- Autoclave: LS-2D (Rexall industries Co. Ltd., Taiwan)
- Automatic micropipettes: P2, 10, P20, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
- A -80 °C Freezer (REVCO, BARA LABORATORY CO., LTD.)
- Camera: Pentax K1000 (Asahi Opt. Co., Japan)
- Electronic balance: Alsep EY220A (A&D Co. Ltd., Japan)
- Electrophoresis apparatus (BioRad Laboratories, USA)
- Gel Document: SYNGENE, England
- Gene pulser: (Bio-Rad Laboratories, USA)
- Heating block: Bd 1761G-26 (Sybron Thermermolyne Co., USA)
- Incubator: BM-600 (Memmert GambH, Germany)
- Incubator shaker: (Innova 4080™, New Brunwick Scientific, USA)
- Laminar flow: HT123 (ISSCO, USA)
- Magnetic stirrer and heater (Fisher Scientific, USA)
- Microwave Oven: TRX1500 (Turbora International Co. Ltd., Korea)
- PCR Thermal cycler : model 2400 (Perkin Elmer)
- PCR Workstation : Model # P-036 (Scientific Co., USA)
- pH meter: (Radiometer, Denmark)
- Power supply : Power PAC 300 (Bio-RAD Laboratories, USA)
- Refrigerated microcentrifuge : Kubota 1300 (Kubota, Japan)
- Refrigerated centrifuge : Model J-21C (Beckman Instrument Inc., Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- Ultrasonic homogenizer (SONOPULS, BANDELIN, Germany)
- Vortex: K-550-GE (Scientific Industries, USA)
- White/UV Transilluminator : UVP Image Store 7500 (Mitshubichi Electric Corporation, Japan)

2.1.2 Inventory supplies

- Black and white print film Tmax-400 (Eastman Kodak Company, USA)
- ECL-Nitrocellulose membrane (Amershem Bioscience Inc., USA)
- Filter paper whatman 3MM (Whatman Internation Ltd., England)
- Microcentrifuge tubes 0.5, 1.5 ml (Axygen Hayward, USA)
- Pipette tips 10, 100, 1000 µl (Axygen Hayward, USA)
- PVDF membrane (Amershem Biosciences Inc., USA)
- Thin-wall microcentrifuge tubes 0.2 ml (Axygen Hayward, USA)

2.1.3 Chemicals

- Absolute ethanol (Merck, Germany)
- Acetosyringone (Fluka, Switzerland)
- Acrylamide (Merck, Germany)
- Agarose (FMC BioProducts, USA)
- Ammonium persulfate (Merck, Germany)
- Bacto-agar (Difco, USA)
- Bacto-yeast extract (Difco, USA)
- Bacto-tryptone (Difco, USA)
- 100 Base pair DNA ladder (Promega Corporation Medison, Wisconsin, USA)
- BenchMark™ Protein Ladder (Invitrogen Life Technologies, USA)
- 6-Benzylaminopurine (BAP) (Sigma Chemical Co., USA)
- Beta-mecaptoethanol (Fluka, Switzerland)
- Boric acid (Merck, Germany)
- Bovine serum albumin (Sigma Chemical Co., USA)
- 5-Bromo-4-chloro-3-indolyl-β -D-glucuronic acid (X-Gluc) (CLONTECH Laboratories Inc., USA)
- 5-Bromo-4-chloro-3-indolyl-β -D-galactopyranoside (X-Gal) (Sigma Chemical Co., USA)
- Bromphenol blue (Merck, Germany)
- Casein hydrolysate(Merck, Germany)
- Cetyltrimethylammonium bromide (CTAB) (Sigma Chemical Co., USA)
- Chloroform (Merck, Germany)

- Coomassie brilliant blue R-250 (Sigma Chemical Co., USA)
- 100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin, USA)
- 2,4-dichlorophenoxy acetic acid (2,4-D) (Sigma Chemical Co., USA)
- Diethyl pyrocarbonate sulfoxide (DEPC) (Sigma Chemical Co., USA)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Germany)
- Ficoll type 400 (Sigma Chemical Co., USA)
- Glucose (BDH, England)
- Glycine (Sigma Chemical Co., USA)
- Hydrochloric acid (Merck, Germany)
- Imidazole (Sigma Chemical Co., USA)
- Isoamyl alcohol (Merck, Germany)
- Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., USA)
- Methanol (Merck, Germany)
- Nickel sulphate (Sigma Chemical Co., USA)
- Nicotinic acid (Sigma Chemical Co., USA)
- *N, N'*-methylene-bis-acrylamide (Merck, Germany)
- Phenol, crystal (Fluka, Germany)
- Phenylmethylsulfonyl fluoride: PMSF (USB, USA)
- Phytagel (Sigma Chemical Co., USA)
- Sodium acetate (Merck, Germany)
- Sodium chloride (BDH, England)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- Sucrose (Sigma Chemical Co., USA)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- TRI REAGENT® (Molecular Research Center, Inc., USA)
- Triton X-100 (Merck, Germany)
- Urea (Carlo Erba, Italy)
- Xylene cyanol FF (Sigma Chemical Co., USA)
- Yeast extract (Difco, USA)

2.1.4 Antibiotics

- Ampicilin (Sigma Chemical Co., USA)
- Cefotaxime (Fluka, Switzerland)
- Chloramphenicol (Sigma Chemical Co., USA)
- Hygromycin B (Sigma Chemical Co., USA)
- Kanamycin (Sigma Chemical Co., USA)
- Rifampicin (Sigma Chemical Co., USA)
- Tetracyclin (Sigma Chemical Co., USA)

2.1.5 Kits

- QIAprep Miniprep Kit (Qiagen, USA)
- QIAquick Gel Extraction kit (Qiagen, USA)
- NucleoSpin® PCR Purification Kit (Macherey-Nagel, Germany)
- NucleoSpin® Gel Extraction Kit (Macherey-Nagel, Germany)
- NucleoSpin® Plasmid DNA Purification Kit (Macherey-Nagel, Germany)
- Omniscript RT Kit (Qiagen, USA)
- ImProm-II™ Reverse Transcription System(Promega Corporation Medison, Wisconsin, USA)

2.1.6 Enzymes

- Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA)
- DyNazyme™II DNA polymerase (Finnzymes, Finland)
- *Pfu* DNA polymerase (Promega Corporation Medison, Wisconsin, USA)
- Proteinase K (Sigma Chemical Co., USA)
- Restriction endonucleases (New England BioLabs, USA)
- RNase Free DNase I (Promega Corporation Medison, Wisconsin, USA)
- RNase A (Sigma Chemical Co., USA)
- T4 DNA ligase (Amersham Biosciences, USA)

2.1.7 Oligonucleotides

The oligonucleotides were synthesized at the Bio Basic Inc., USA, ProLigo, USA and BioService Unit, BIOTEC center, National Science and Technolnloy Development Agency (NSTDA), Thailand.

2.1.8 Bacterial strains

Escherichia coli

strain XL1-Blue ($F^{/}$:: Tn*10* *proA*$^{+}$*B*$^{+}$*lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96* (Nal^{r}) *thi hsdR17* (r_k^{-} m_k^{+}) *supE44 relA1 lac*)

strain DH5α$F^{/}$ ($F^{/}$ / *endA1 hsdR17* (r_k^{-} m_k^{+}) *supE44 thi-1 recA1 gyrA* (Nal^{r}) *relA1Δ(lacIZYA-argF)U169 deoR (ϕ80dlacΔ(lacZ)M15)*

strain BL21(DE3)pLysS (F^{-} *ompT hsd*S_B (r_B^{-} m_B^{-}) *gal dcm* (DE3)pLysS (Cm^{R}))

strain BL21 Star (DE3)pLysS (F^{-}*ompT hsd*S_B(r_B^{-} m_B^{-}) *gal dcm rne131*(DE3) pLysS (Cm^{R}))

strain Rosetta (DE3)pLysS (F^{-} *ompT hsd*S_B (r_B^{-} m_B^{-}) *gal dcm lacY1* (DE3) pLysSRARE^{6} (Cm^{R}))

Agrobacterium tumefaciens strain EHA105 (EHA105 (pEHA105); a hypervirulent,

L,L-succinamopine helper strain. (pEHA105 is a T-DNA deletion derivative of pTiBo542, the hypervirulent Ti plasmid of *A. tumefaciens* strain A281)

2.1.9 Vectors

- pUC18
- pGEM®-3Zf(+)
- pGEM®-T easy
- pTrcHis2C
- pET17b
- pCAMBIA1301
- pCAMBIA2301

2.2 Cloning and characterization of Major Royal Jelly Protein 1 and 2 (MRJP1 and MRJP2 cDNAs) from *A. cerana*

2.2.1 Specimens

Nurse worker bees of *A. cerana* were collected from a single colony originating from central area (Bangkok) in Thailand. The head and thorax of nurse bees were dissected out individually and immediately dropped in liquid nitrogen and stored at -80 °C until further needed.

2.2.2 Total RNA extraction

Total RNA was extracted from the head of nurse honeybees using TRI REAGENT® (Molecular Research Center, Inc., USA). Head of nurse honeybee was ground to fine powder in liquid nitrogen. The tissue was immediately transferred to 1.5 ml sterile microcentrifuge tube and homogenized in 1 ml of TRI REAGENT® (Molecular Research Center, Inc., USA). The homogenate was stored at room temperature for 5 minutes followed by addition of 200 µl of chloroform. The mixture was vortexed for 1 minute and then centrifuged at 12,000 rpm for 10 minutes at 4 °C. The aqueous phase was transferred to a new sterile microcentrifuge tube. The total RNA was precipitated by an addition 500 µl of ice-cold isopropanol and incubated at -80 °C for 10 minutes. The precipitated RNA was recovered by centrifugation at 12,000 rpm for 10 minutes and washed with 70% ice-cold ethanol. The supernatant was discarded and the RNA pellet was then briefly air-dried and dissolved in RNase free water. The total RNA was quantified spectrophotometrically and determined for integrity by agarose gel electrophoresis. The concentration of total RNA was determind by measuring the OD at 260 nm and estimated the concentration following equation:

DNA concentration (µg / ml) = OD_{260} x dilution factor x 40

To remove contaminant DNA, aliquots of 1 µg RNA were treated with 2 units of RNase-free DNase (Promega Corporation Medison, Wisconsin, USA) and incubated at 37 °C for 20 minutes. Total RNA was then reextracted by 1 ml of TRI REAGENT®

(Molecular Research Center, Inc., USA) as mention above and finally resuspended in RNase free water and stored at -80 °C.

2.2.3 First stranded cDNA synthesis

First-strand cDNA was synthesized from total RNA extracted from head of nurse honeybee (2.2.2) using the Omniscript RT kit (Qiagen, USA). Two micrograms of total RNA in RNase free water were incubated at 65 °C for 5 minutes and then immediately placed on ice. Subsequently, total RNA was added to the reaction mixture of 20 µl total volume containing 1 µM oligo $(dT)_{12-16}$ primer, 0.5 mM each dNTP, 10 units RNase inhibitor (Promega Corporation Medison, Wisconsin, USA), 1X RT buffer and 4 units of Omniscript reverse transcriptase. The reaction was performed in 20 µl total volume. The mixture was mixed gently and incubated at 37 °C for 1 hour. Subsequently, the mixture was incubated at 70 °C for 10 minutes to terminate the reverse transcription reaction.

2.2.4 Primer designed

The nucleotide and amino acid sequences of MRJPs from *A. mellifera;* AmMRJP1 (GenBank accession number AF000633), AmMRJP2 (AF000632), AmMRJP3 (Z26318), AmMRJP4 (Z26319) and AmMRJP5 (AF004842) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). The computational analysis of the DNA and amino acid sequencing data was carried out using CLUSTAL W sequence alignment program. The oligonucleotide primers were designed based on conserved region of these MRJP genes. The forward primer designated as FMJ and reverse primer designated as RMJ were designed using Primer Premier 5.0 program. The *Eco*R I and *Kpn* I sites were added to the 5' end to facilitate cloning. These primers were further used in RT-PCR analysis. The sequences and length of oligonucleotide primers are shown in Table 2.1.

Table. 2.1 Sequence of oligonucleotide primers used for amplification of AcMRJP1 and cMRJP2 cDNAs. The restriction sites designed for cloning were underlined. *Eco*R I (G/AATTC), *Kpn* I (GGTAC/C)

Primer	Length (bp)	Sequence (5'- 3')
FMJ	33	TAGGAATTCTAA*ATG*ACAAGGTGGTTGTTCATG
RMJ	38	GGGGTACCC$(T)_{28}$A
pUC1	21	CCGGCTCGTATGTTGTGTGGA
PUC2	23	GTGCTGCAAGGCGATTAAGTTGG
1F750	22	TCAGGTCTTGTCAATAATACTC
1R750	20	AATATGGGCACGTGTGGAAG
2F850	21	GATGGTGTGCCTTCTACTTTG
2R850	22	AGCTCTTCCTTAATTTTCATAC

2.2.5 Amplification of the full length AcMRJP1 and AcMRJP2 cDNAs by RT-PCR

The amplification of AcMRJP1 and AcMRJP2 was carried out by using *Pfu* DNA polymerase (Promega Corporation Medison, Wisconsin, USA) or Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA). For *Pfu* DNA polymerase, the amplification reaction was performed in a 25 µl reaction volume containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 mM of each dNTPs, 0.6 µM of FMJ and RMJ primer, 1 units of *Pfu* polymerase (Promega Corporation Medison, Wisconsin, USA) and 5 µl of first stranded cDNA (2.2.3) as template. Amplification by PCR (Perkin-Elmer model 2400) was composed of initial denaturation at 92 °C for 3 minutes followed by 5 cycles of denaturation at 92 °C for 1

minute, annealing at 50 °C for 1.30 minutes and extension at 68 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1.30 minutes and extension at 72 °C for 4 minutes. The final extension was carried out at 72 °C for 10 minutes.

For Ampli *Taq* DNA polymerase, the amplification reactions were performed in a 25 µl reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer and 1.0 unit of Ampli *Taq* DNA polymerase. The reaction was composed of initial denaturation at 92 °C for 3 minutes followed by 5 cycles of denaturation at 92 °C for 1 minute, annealing at 50 °C for 1.30 minutes and extension at 68 °C for 2.30 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1.30 minutes and extension at 72 °C for 2.30 minutes. The final extension was carried out at 72 °C for 10 minutes. The resulting products were electrophoretically analyzed through a 1.2% agarose gel, stained with ethidium bromide and visualized using a UV transilluminator (Maniatis *et al.*, 1982).

2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method used for separation DNA fragments on the basis of their molecular weight and used for rough estimation of DNA on the basis of its direct relationship between the amount of DNA and the intensity of the fluorescence after ethidium bromide staining. The DNA was run on 1.2% agarose gel in 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3). The gel was prepared by adding 0.6 g of agarose to 50 ml of 1X TBE buffer. Agarose was solubilized by heating in a microwave oven and then allowed to cool to 50-60 °C before pouring into a plastic gel former with a preset well-forming comb. Ten microlitres of DNA sample were mixed with 2 µl of the loading dye (0.25% bromophenol blue and 25% Ficoll 400) before loading into the well of gel which was submerged in the 1X TBE buffer. The 100 bp, 200 bp DNA ladders or *λ/Hin*d III were used as standard DNA markers. Electrophoresis was operated at 5 volts/cm until bromophenol blue moved to approximately 0.5 cm from the bottom of the gel. The electrophoresed gel was stained with a 2.5 µg/ml ethidium bromide for 5 minutes and subsequently destained in an appropriate amount of water with gently

shaking for 10 minutes to remove unbound ethidium bromide from agarose gels. The DNA fragments were visualized as fluorescent bands under a UV transilluminator and photographed through a red filter with Kodak Tri-X-Pan 400 film.

2.2.7 DNA fragment elution

The amplification products generated by RT-PCR (2.2.5) were recovered from the agarose gel by using the QIAquick Gel Extraction Kit (Qiagen, USA). After electrophoresis, the desired DNA fragment was excised as gel slice from the 1.2% agarose gel using a scalpel. The gel slice was weighed, and three volumes of buffer QG (supplied by the manufacturer) were added to one volume of the gel and incubated at 50 °C for 10 minutes or until the gel slice was completely dissolved. The gel mixture was vortexed every 2 to 3 minutes during the incubation period. The gel mixture turned to yellow after completely dissolved. The mixture was transferred into a QIAquick column inserted in a provided 2-ml collection tube and centrifuged at 12,000 rpm for 90 seconds. The flow-through solution was discarded and the column replaced in the same collection tube. An another 500 µl of QG buffer was added to the QIAquick column followed by centrifuged at the same speed for 90 seconds. After that, the column was washed with 750 µl of PE buffer and centrifuged for 90 seconds. After discarding the flow through, the QIAquick column was centrifuged for an additional 1 minute to completely removed the residual ethanol from PE buffer and then placed into a sterile 1.5 ml microcentrifuge tube. Finally, the DNA fragment was eluted by an addition of 30 µl of EB buffer (10 mM Tris-HCl, pH 8.5) to the center of the QIAquick membrane and let the column standing for 5 minutes, before centrifuged at 12,000 rpm for 90 seconds. The DNA concentration was determined by agarose gel electrophoresis (2.2.6).

2.2.8 Purification of PCR products using NucleoSpin® PCR Purification Kit (Macherey-Nagel, Germany)

The PCR products were digested with proteinase K (50 µg/ml in the presence of 0.5% SDS) at 65 °C for 1 hour. Subsequently, the PCR products were purified by NucleoSpin® PCR Purification Kit (Macherey-Nagel, Germany). Four volumes of NT2 buffer (supplied by the manufacturer) was added to one volume of reaction

mixture and then loaded into the NucleoSpin column and centrifuged at 10,000 rpm for 1 minute. The flow through was discarded. Then 600 µl of NT3 buffer was added and centrifuged at 10,000 rpm for 1 minute. The flow through was discarded. The column was put back to the collecting tube. Then 200 µl of NT3 buffer was added and centrifuged at 10,000 rpm for 2 minutes. The column was placed into a new microcentrifuge tube and DNA were eluted by addition of 50 µl of the elution NE buffer (5 mM Tris-HCl, pH 8.5) and centrifuged at 10,000 rpm for 1 minute.

2.2.9 Digestion of purified PCR products

The purified PCR products amplified using *Pfu* DNA polymerase were digested with *Eco*R I and *Kpn* I using the condition recommended by the manufacturer. The reaction was carried out in 15 µl containing 5µl of PCR product (approximately 200 ng), 1 units of each restriction enzyme and 1X reaction buffer (supply by the manufacturer). The reaction mixture was incubated at 37 °C overnight. The restricted product was then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The purified DNA fragment in aqueous solution was precipitated by addition of 1/10 volume of 3M sodium acetate, pH 5.5 following by two volumes of absolute ethanol. The mixture was mix throughly and incubated at -80 °C for 30 minutes followed by centrifuged at 12,000 rpm for 20 minutes. The DNA pellet was washed once with 70% ethanol, air-dried and dissolved in 20 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4).

2.2.10 Preparation of vector DNA by digestion of pUC18 with *Eco*R I and *Kpn* I

Five micrograms of pUC18 vector were digested with *Eco*R I (5 unit) and *Kpn* I (5 unit) in the 100 µl reaction mixture using the condition recommended by the manufacturer. The reaction was incubated at 37 °C overnight. The digested product was analyzed by agarose gel electrophoresis and then eluted from agarose gel as described in 2.2.7. The DNA was dissolved in 20 µl of TE buffer.

2.2.11 DNA ligation

DNA ligation was carried out in 20 µl reaction volume using T4 DNA ligase (Amersham Bioscience, USA). Each DNA fragment digested with *Eco*R I and *Kpn* I was ligated to *Eco*R I and *Kpn* I digested pUC18 vector. The ligation reaction containing 300 ng of digested DNA fragment, 100 ng of digested pUC18, 1X T4 DNA ligase buffer (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate and 50 mM potassium acetate), 1 mM of ATP, 5.5 weiss units of T4 DNA ligase. The reaction mixture was gently mixed and incubated at 16 °C overnight.

The pGEM®-T easy vector (Promega Corporation Medison, Wisconsin, USA) was used for cloning of DNA fragment amplified from Ampli *Taq* DNA polymerase. The ligation reaction was performed in the total volume of 10 µl containing 300 ng of DNA fragment, 50 ng pGEM®-T easy vector, 5 µl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2mM ATP and 10 % PEG 8000), 3 units of T4 DNA ligase. The ligation mixture was mixed and incubated at 4 °C overnight. The ligation product was subsequently electrotransformed to *E. coli* XL1-Blue.

2.2.12 Transformation of ligated products to *E. coli* host cells by electroporation (Dower *et al.*, 1988)

2.2.12.1 Preparation of competent *E. coli* cells

The glycerol stock of *E. coli* was streaked onto LB agar plate (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl and 1.5% Bacto agar) and incubated at 37 °C overnight. A single colony of bacterial cells was innoculated into 10 ml of LB-broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.2) and incubated with vigorous shaking at 37 °C overnight. The starting culture was inoculated into 1 litre of LB-broth and continued culture at 37 °C with vigorous shaking to the OD_{600} of 0.5 to 0.8. The cells were chilled briefly on ice for 15 to 30 minutes, and harvested by centrifugation in a prechilled rotor at 5,000 rpm for 15 minutes at 4 °C. The cell pellet were washed by resuspending in 1 litre of cold sterile distilled water and centrifuged as above. The supernatant was discarded. The cell

pellet were resuspended in 500 ml of cold sterile distilled water and recentrifuged as above. Subsequently, the pellet were resuspended in 20 ml of 10% glycerol and recentrifuged as above. The supernatant was removed. Finally, the cell pellet were resuspended in 2 to 3 ml of 10% glycerol. This concentrated cell suspension was devided to 45 µl aliquots. These cells could be used immediately or stored at -80 °C for later used.

2.2.12.2 Electrotransformation of recombinant DNA to *E. coli* host cell

The competent cells were thawed on ice for 5 minutes. One or two microlitres of the ligation mixture (2.2.11) was added and gently mixed by pipetting. The mixture was left on ice for at least 1 minute. The mixture was transferred to a prechilled 0.2 cm cuvette and electroporated by using a Gene pulser (Bio-Rad) with the setting parameters of 25 µF, 200 Ω and 2.5 KV. After electroporation, the mixture were immediately removed from the cuvette and added to a new tube containing 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) or LB broth. The cell suspension was incubated with shaking at 37 °C for 1 hour. Approximately 10-50 µl of this were spreaded on a selective LB agar plates containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG and 20 µg/ml of X-Gal and further incubated at 37 °C overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.2.13 Characterization of the insert DNA fragment of recombinant clones

Usually, identification of bacterial colonies that carry putative recombinant plasmids can be done by α-complementation insertional inactivation, restriction analyis of plasmid DNA and screening by colony PCR. The recombinant plasmids that exhibited α-complementation with the compatible host cells were first selected by selection of the white colonies and these extracted plasmids by minipreparation were then verified by restriction analysis. In the case that the recombinant plasmid could not be selected by α-complementation, the putative recombinants were identified by colony PCR followed by restriction analyis.

2.2.13.1 Restriction enzyme analysis of recombinant plasmids

2.2.13.1.1 Isolation of recombinant plasmid DNA by alkaline lysis method

Plasmid DNA was isolated using a modification of the alkaline lysis DNA method (Li *et al.*, 1997). A single colony of *E. coli* harboring plasmid was selected and grown overnight with shaking at 37 °C in 3 ml of LB broth supplemented with appropriate antibiotic. The cells were harvested by centrifugation at 10,000 rpm for 30 seconds in a microcentrifuge tube and resuspended in 100 µl of solution I (50 mM glucose, 10 mM EDTA, pH 8.0, 25 mM Tris-HCl, pH 8.0). After addition of 200 µl of freshly prepared solution II (0.2 N NaOH and 1% SDS) followed by gentle mixing by inversion of the tube and incubated on ice for 5 minutes. The lysate was subsequently neutralized by addition of 150 µl of solution III (3 M sodium acetate, pH 4.8). After gentle mixing, the tube was centrifuged at 10,000 rpm for 15 minutes to pellet the cell debris. The supernatant was transferred into a new microcentrifuge tube. The plasmid DNA in the supernatant was precipitated by addition of equal volume of ice-cold absolute ethanol followed by centrifugation at 12,000 rpm for 10 minutes. The pellet was washed twice with 70% ethanol followed by air-dried *in vacuo* for 5 minutes. The pellet was dissolved in 50 µl of TE buffer. RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA. The reaction mixture was incubated at 37 °C for 30-60 minutes. Plasmid DNA was stored at -20 °C.

2.2.13.1.2 Restriction enzyme analysis

The recombinant plasmids isolated by alkaline lysis method (2.2.13.1.1) were analyzed for the presence of the interested cloned fragments by digestion with appropriate restriction endonucleases. The existence of the insert DNA fragment was examined by digestion of recombinant DNA with *Eco*R I and *Kpn* I. The reaction was carried out in a 20 µl standard mixture at 37 °C overnight. At the end of digestion, the resulting product was electrophoretically analyzed by 1% agarose gel electrophoresis. The size of DNA insert is investigated by comparing with that of a λ / *Hin*d III and 200 bp DNA ladder.

2.2.13.2 Identification of recombinant clones by colony PCR

Recombinant clone was identified by colony PCR using pUC1 and pUC2 primers (Table 2.1). The colony was picked up and resuspended in a 25 µl amplification reaction containing 10mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 100 µM of each dNTP, 0.4 µM of pUC1 and pUC2 primers and 1 unit of DyNazyme^{TM}II DNA polymerase. The reaction was predenatured at 94 °C for 2 minutes following by 30 cycles of denaturing at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 1.30 minutes. The final extension was performed at 72 °C for 10 minutes. After amplification, 5 µl of reaction product was electrophoretically analyzed on 0.7% agarose gel.

The amplification products from colony PCR were then digested with restriction enzymes *Ssp* I, *Bam*H I, *Eco*R I, *Cla* I, *Pvu* I. The reaction was carried out in 15 µl containing 5µl of PCR product, 5 units of each restriction enzyme and 1X reaction buffer (supply by the manufacturer). The reaction mixture was incubated at 37 °C overnight. The digested product was electrophoretically analyzed through 0.7% agarose gel. The restriction patterns of restricted products were compared with restriction map of AmMRJP to identify the recombinant plasmid. Subsequently, the recombinant plasmids that had restriction map correlated with desired MRJP were selected for nucleotide sequencing.

2.2.14 Sequence analysis of MRJP1 and MRJP2 cDNA of *A. cerana*

2.2.14.1 Isolation of recombinant plasmid DNA by QIAprep Miniprep kit (Qiagen)

A single colony was inoculated into a sterile tube containing 2 ml of LB broth supplemented with appropriate antibiotic and incubated with shaking at 37 °C for 16 hours. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 30 seconds. The supernatant was carefully decanted. The pellet was resuspended in 250 µl of P1 Buffer (supplied by the manufacturer). Then, 250 µl of P2buffer was added and gently mixed by inversion of the tube. A volume of 350 µl of N3 buffer was added and immediately followed by inverting the

tube for 4-6 times before centrifugation at 10,000 rpm for 15 minutes to pellet the cell debris. The supernatant was transferred into a QIAprep Miniprep column inserted in a 2 ml collection tube. After centrifuged for 1 minute, the flow-through solution was discarded. After that, the column was washed twice with 750 µl of PB buffer and then with 500 µl of PE buffer, respectively followed by centrifugation for 1 minute at each wash. After this step, the flow through solution was discarded. The QIAprep Miniprep column was centrifuged to remove a trace amount of the washing solution. Finally, the plasmid DNA was eluted into a sterile 1.5 ml microfuge tube by addition of 30 µl of EB buffer (10mM Tris-HCl, pH 8.5) and centrifuged at 12,000 rpm for 90 seconds. Plasmid DNA was stored at -20 °C.

2.2.14.2 DNA sequencing and sequence analysis

Plasmid DNA extracted from each recombinant clones were sequenced for both directions using automatic sequencer (Li-Cor, Lincoln, USA). The universal M13 forward and reverse primers were initially used for sequencing. Internal sequencing primers were subsequently designed by Oligo program, and used for sequencing along the entire length of the insert (Table 2.1).

DNA sequences data derived from both strands were assembled and analyzed with GENETYX (Software Development Inc.) and blasted against data in the GenBank using BlastN and BlastX (http://www.ncbi.nlm.nih.gov). The putative cleavage site of the signal peptide was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignments of nucleotide and translated amino acids were performed using Clustal W (Thompson *et al.*, 1994). Aligned sequences were bootstrapped 1000 times using Seqboot. Sequence divergence between different families of MRJPs was calculated based on the two parameter method (Kimura, 1980) using Dnadist. Boostrapped neighbor-joining trees were constructed using Neighbor and Consense. All phylogenetic reconstruction programs are routine in PHYLIP (Felsenstein, 1993). Trees are appropriately illustrated using TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod.html).

2.3 Cloning and characterization of MRJP1 and MRJP2 genes from *A. cerana*

2.3.1 Specimens

Nurse worker bees of *A. cerana* were collected as described in 2.2.1.

2.3.2 Genomic DNA extraction

Genomic DNA was extracted from the thorax of honeybee using modified phenol-chloroform-SDS method of Smith and Hagen (1997). Briefly, the tissue was placed in a prechilled microcentrifuge tube containing 600 µl of extraction buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 200 mM sucrose, 50 mM EDTA, pH 8.0) and homogenized with a pestle. Then the solution of 1.0% (w/v) SDS and 10 mg/ml of RNase A solution were added to a final concentration of 1.0% (w/v) and 100 µ g/ml, respectively. The resulting mixture was incubated at 37 °C for 1 hour. Subsequently, the solution of 20 mg/ml proteinase K was added to a final concentration of 300 µg / ml and further incubated at 65 °C for at least 3 hours. Then, the mixture was extracted by a standard phenol-chloroform method. The extraction was carried out twice by the addition of an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and gently mixed for 15 minutes. The solution was centrifuged at 12,000 rpm for 10 minutes at 4 °C. The upper aqueous phase was transferred to a new sterile microcentifuge tube and further extracted once with an equal volume of chloroform-isoamyl alcohol (24:1). The upper aqueous phase was transferred to a new microcentrifuge tube. One-tenth volume of 3 M sodium acetate, pH 5.5 was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80 °C for 30 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 15 minutes and briefly washed twice with 70% ethanol followed by centrifugation at 12,000 rpm for 5 minutes. The supernatant was discarded. The DNA pellet was air-dried and resuspended in 100 µl of TE buffer. The DNA solution was incubated at 37 °C for 1-2 hours for complete redissolved and then stored at 4 °C until further needed. The genomic DNA were quantified spectrophotometrically and determined for integrity by agarose gel electrophoresis.

2.3.3 Measurement of DNA concentrations

The concentration of extracted DNA was spectrophotometrically quantitated by measuring the optical density of 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml double stranded DNA. Therefore, the DNA concentration was calculated from the following equation:

DNA concentration (μg/ml) = OD_{260} x dilution factor x 50

The purity of DNA samples can be obtained by calculating a ratio of OD_{260} / OD_{280}. The ratio of 1.8 to 2.0 indicates pure prepared DNA whereas much higher and lower values of this ratio indicate RNA or protein contamination of isolated DNA samples, respectively (Maniatis *et al.*, 1982).

2.3.4 Designation of oligonucleotide primers

The oligonucleotide primers used in amplification of AcMRJP1 and AcMRJP2 genes from genomic DNA (2.3.2) were designed based on the nucleotide sequence of AcMRJP1 and AcMRJP2 cDNAs obtained from RT-PCR experiment. The sequence of the 5′ upstream region of AmMRJP1 (AY078399) and AmMRJP2 (AF388203) genes were retrived from GenBank (http://www.ncbi.nlm.nih.gov/) and used to designed the primers. The primers were designed using Primer Premier 5.0 program. The nucleotide sequences and length of primers were shown in Table 2.2.

2.3.5 Amplification of the AcMRJP1 and AcMRJP2 genes by PCR

The AcMRJP1 and AcMRJP2 genes were isolated by using PCR approch. AcMRJP1 and AcMRJP2 genes were obtained from amplification of three overlapping regions. The nucleotide sequences of the gene specific and degenerate primers used in genomic amplification of AcMRJP1 and AcMRJP2 were shown in Table 2.2. Initially, genomic DNA was amplified by degenerate FMRJP and RMRJP primers (designed from conserved cDNA sequence at nucleotideposition of 767^{th}-787^{th} nucleotides in AcMRJP1 cDNA and 758^{th}-778^{th} in AcMRJP2 cDNA).

Table. 2.2 Sequence of oligonucleotides primers used for amplification of AcMRJP1 and cMRJP2 genes from extracted *A. cerana* genomic DNA.

Primer	Length (bp)	Sequence (5'- 3')
5M1F	25	ACATCACTATTCTCATTGCATCAGA
5M1R	22	TTGTCGATCGCAAGTTTTGTGG
FMRJP	21	TGCCTYGGYATAGYTTGTCAA
RMRJP	21	TCAYGGGACTRAGWGCMATTC
*n*M1F	32	AAACTGCAGCTAGCAATTCTTCGAGGAGAATC
3M1/2F	25	TGATTCYTTCCATCGAWTGACTTCC
3M1R	28	CGAAAACAATATTTATTTTATACATTCA
3*n*M1F	26	TCCAAAACTTTCGATTACGATCCTAA
5M2F	28	TGAGAATGAATTGCAGAATATGGTCGCT
5M2R	27	GAAAGCGCTCACGATTCCAGAGCAATC
5*n*M2R-1	28	CAGCTTGTCTTCTTTCTTCGCTACCGAA
*n*M2F	36	AAACTGCAGCTGCCATTATTCGACAAAATTCTG CAA
3M2R	25	TAATTTGGTTTATTGATTTTAATGC
3*n*M2F	25	ACTTTCGATTACGATCCCAGATATG

The amplification reaction was performed in a 25 µl reaction volume containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 mM of each dNTPs, 0.6 µM of each primer, 1 units of *Pfu* polymerase (Promega Corporation Medison, Wisconsin, USA) and 50 ng of genomic DNA (2.3.2). The PCR condition composed of denaturation at 93 °C for 3 minutes followed by 5 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and extension at 72 °C for 4 minutes for 25 cycles. The final extension was carried out at 72 °C for 10 minutes. The resulting products were electrophoretically analyzed through a 1.2% agarose gel, stained with ethidium bromide and visualized using a UV transilluminator (Maniatis *et al.*, 1982). The PCR products were then extracted from the agraose gel using NucleoSpin® gel Extraction Kit as described in 2.3.6.

In order to increase the specificity of the primary PCR product, nested primer specific for AcMRJP1 and AcMRJP2 were designed; (*n*M1F; designed from cDNA sequence at position 61^{st}-80^{th} in AcMRJP1 whereas *n*M2F; designed from cDNA sequence at position 52^{nd}-76^{th} for AcMRJP2). The semi-nested PCR (*n*M1F + RMRJP and *n*M2F + RMRJP) was carried out with the same PCR condition using the gel-eluted PCR product (2.3.6) as template.

The resulting PCR product was analyzed by agarose gel electrophoresis and recovered from the agarose gel. The expected DNA fragment was phosphorelated with T4 polynucleotide kinase and ligated to dephosphorylated / *Sma* I - digested pGEM®-3Zf(+) and subsequently electrotransformed to *E. coli* XL1-Blue. The transformants were selected on LB agar plates containing 50 µg/ml of ampicillin, 25 µ g/ml of IPTG and 20 µg/ml of X-Gal and further incubated at 37 °C overnight. The recombinant clones were identified by colony PCR as described in 2.2.13.2.

The recombinant plasmids were extracted and sequenced. Three clones were selected for sequencing in both directions by M13 forward and reverse primers using an automated sequencer (Li-Cor, Lincoln, USA).

In the second overlapping region, the specific primer designed from cDNA sequence of AcMRJP1 (3M1R; designed from cDNA sequence at nucleotide position of 1364^{th} - 1387^{th}) and specific primer for AcMRJP2, (3M2R; designed from cDNA

sequence at nucloetide position of 1425th - 1449th) were used to amplified genomic DNA with the degenerated primer (3M1/2F; designed from conserved cDNA sequence at nucleotide position of 660th - 687th of AcMRJP1 cDNA and nucleotide at 654th - 678th of AcMRJP2 cDNA) using PCR condition as described above. The obtained PCR products were eluted from agarose gel (2.3.6) and used as template for the second semi-nested PCR using 3*n*M1F (designed from cDNA sequence at nucleotide position of 685th - 710th) + 3M1R and 3*n*M2F (designed from cDNA sequence at nucleotide position of 682nd - 706th) + 3M2R, respectively. The amplification products were eluted, cloned and sequenced as described above.

In the third region, the upstream region of both genes, were performed using primer in the 5' upstream region of the AmMRJP1 and AmMRJP2 genes which deposited in GenBank (accession number AF388203 and AY078399, respectively). The upstream 5' region of AcMRJP1 and AcMRJP2 genes were amplified from genomic DNA of *A. cerena* using 5M1F (604 bp upstream region, accession number AF388203) + 5M1R (designed from AcMRJP1cDNA sequence at position 374th - 395th) and 5M2F (859 bp upstream region, accession number AY078399) + 5M2R (designed from AcMRJP2 cDNA sequence at position 352nd - 378th). Semi-nested PCR was carried out for AcMJRP2 gene using the original forward primer and 5*n*M2R-1 (designed from cDNA sequence at position 133rd - 160th). The resulting products were cloned and sequenced. The sequences obtained from these three regions were assembled and compared to cDNA sequence to determine the genomic organization.

2.3.6 DNA fragment elution using NucleoSpin®Gel Extraction Kit (Macherey-Nagel, Germany)

The DNA fragments were fractionated by agarose gel electrophoresis and excised from the gel using a scalpel. Three volumes of NT1 binding buffer (supplied by the manufacturer) were added and incubated at 50 °C until the gel slice has completely dissolved. The mixture was applied into a NucleoSpin®Extract column inserted in a 2 ml collecting tube and centrifuged at 12,000 rpm for 1 minute. The flow-through solution was discarded. Then 600 µl of NT3 washing buffer was added to the NucleoSpin® Extract column and recentrifuged for 1 minute. An another 200 µl

of NT3 buffer was added to NucleoSpin® Extract column and centrifuged for 2 minutes to remove a trace amount of the washing solution. The flow through solution was discarded. The NucleoSpin® Extract column was placed into a clean 1.5 ml microcentrifuge tube. DNA was eluted by an addition of 50 µl of prewarmed (70 °C) NE elution buffer (10 mM Tris-HCl, pH 8.5) and let the column standing for 5 minutes, before centrifuged at 12,000 rpm for 1 minute.

2.3.7 Preparation of dephosphorylated / *Sma* I - digested pGEM®-3Zf(+) vector

Ten micrograms of pGEM®-3Zf(+) were digested with *Sma* I in the 100 µl reaction mixture using the condition recommended by the manufacturer. The reaction was incubated at 37°C overnight. The digested product was analyzed by agarose gel electrophoresis and then eluted from agarose gel as described in 2.3.6. The DNA was dissolved in 20 µl of TE buffer. The linearized pGEM®-3Zf(+) was dephosphorylated with bacterial alkaline phosphatase (GIBCO BRL) using the condition recommended by the manufacturer. The reaction was incubated at 37 °C for 1 hour and then extracted once with phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was recoverred by precipitate with equal volume of absolute ethanol and dissolved in 20 µl of sterile disstilled water.

2.3.8 Ligation of DNA fragment to dephosphorylated / *Sma* I - digested pGEM®-3Zf(+) vector

Each DNA fragment was ligated to dephosphorylated / *Sma* I - digested pGEM®-3Zf(+) vector. The ligation reaction was performed in 20 µl reaction mixture containing 300 ng of eluted DNA fragment, 100 ng of dephosphorylated / *Sma* I - digested pGEM®-3Zf(+) vector, 1X T4 DNA ligase buffer (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate and 50 mM potassium acetate), 1 mM of ATP, 5.5 weiss units of T4 DNA ligase (Amersham Biosciences, USA). The reaction was gently mixed by briefly centrifuged to collect the contents to the bottom. The reaction mixture was incubated at 4 °C overnight. The ligation product was electrotransformed to *E. coli* XL1-Blue and the white colonies of the recombinant clones were selected as described in 2.2.12.

2.3.9 Sequence analysis of MRJP1 and MRJP2 gene

2.3.9.1 Isolation of recombinant plasmid DNA by NucleoSpin® Plasmid DNA Purification Kit (Macherey-Nagel, Germany)

A single colony of recombinant clone was inoculated into 2 ml of LB medium supplemented with appropriate antibiotic and incubated with shaking at 37 °C for 16 hours. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 30 seconds. The supernatant was carefully decanted. Then, 250 µl of A1 buffer (supplied by the manufacturer) was added to the cell pellet and resuspended by vortexing. Two hundred and fifty microlitres of A2 buffer was added and gently mixed by inversion of the tube and incubated at room termperature for 5 minutes. Three hundred microlitres of A3 buffer was added and mix by trapping of the tube. The tube was centrifuged at 10,000 rpm for 15 minutes to pellet the cell debris. The supernatant was transferred into a NucleoSpin® Plasmid column inserted in a 2 ml collecting tube and centrifuged at 12,000 rpm for 1 minute. The flow-through solution was discarded. An another 500 µl of prewarmed (50 °C) AW buffer was added to the column and recentrifuged for 1 minute. After this step, a 600 µl of A4 buffer was added to the column and centrifuged. The flow through solution was discarded. The NucleoSpin® Plasmid column was centrifuged to remove a trace amount of the washing solution. The NucleoSpin® Plasmid column was placed into a sterile 1.5 ml microfuge tube. Plasmid DNA was eluted by an addition of 50 µl of prewarmed AE buffer (10 mM Tris-HCl, pH 8.5) and let the column standing for 5 minutes, before centrifuged at 12,000 rpm for 1 minute. Plasmid DNA was stored at -20 °C.

2.3.9.2 DNA sequencing and sequence analysis

The recombinant plasmids were extracted and sequenced for both directions. The sequencing data from all selected clones were assembled and analyzed with GENETYX (Software Development Inc.) and blasted against the sequence data in the GenBank using BlastN and BlastX (http://www.ncbi.nlm.nih.gov). The transcription start site was predicted using the Neural network Promoter Prediction, NNPP2.1 (http://www.fruitfly.org/seq-tools/promoter.html). Identification of transcription

factor binding sites (TATA box and the ultraspiracle transcriptional factor (USP-TF) binding sites) were performed using Genomatixsuite (http://www.genometix.de). Multiple sequence alignments of nucleotide and translated amino acids were performed using Clustal W (Thompson *et al.*, 1994). Aligned sequences were bootstrapped 1000 times using Seqboot. Sequence divergence between different families of MRJPs was calculated based on the two parameter method (Kimura, 1980) using Dnadist. Boostrapped neighbor-joining trees were constructed using Neighbor and Consense. All phylogenetic reconstruction programs are routine in PHYLIP (Felsenstein, 1993). Trees are appropriately illustrated using TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod.html).

2.4 Expression of AcMRJP1 and AcMRJP2 in *E. coli* expression system

2.4.1 Construction of AcMRJP1 and AcMRJP2 expression vector under control of Lac promoter of pGEM®-3Zf(+) vector

2.4.1.1 Primer designation

The oligonucleotide primers were designed from AcMRJP1 and AcMRJP2 cDNA sequences. AcMRJP1 coding sequence was amplified using the primers 1500Fexpression and 1500Rexpression whereas the AcMRJP2 coding region was amplified with the 1600Fexpression and 1600Rexpression (Table 2.3). The *Pst* I and *Kpn* I restriction sites were introduced to facilitate cloning of PCR product.

2.4.1.2 PCR amplification

The fragment of AcMRJP1 and AcMRJP2 cDNAs were amplified from the plasmid pRT-AcMRJP1-Taq and pRT-AcMRJP2. The amplification reactions were performed in a 25 µl reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer and 1.0 unit of Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA). PCR amplification composed of initial denaturation at 95 °C for 3 minutes, 25 cycles of denaturation at 95 °C for 1 minute, annealing at 65 °C for 30 seconds and extension at 72 °C for 4 minutes. Post amplification was performed at 72 °C for 10 minutes. The PCR-amplified fragments were purified as described in 2.2.8.

Table. 2.3 Sequence of oligonucleotides primers used for amplification of AcMRJP1 and cMRJP2 cDNAs in *E. coli* expression experiment. The restriction sites designed for cloning were underlined. Hexa-histidine coding sequence shown in boldface. *Pst* I (CTGCA/G), *Kpn* I (GGTAC/C), *Nhe* I (G/CTAGC)

Primer	Length (bp)	Sequence (5'- 3')
1500Fexpression	31	AAACTGCAGCTAGCATTCTTCGAGGAGAATC
1500Rexpression	33	CGGGGTACCCAGATGTATTGAAATTTTGAAA GG
1600Fexpression	36	AAACTGCAGCTGCCATTATTCGACAAAATTC TGCAA
1600Rexpression	33	GGGGTACCATTGTTAGTATTCTGATTGTTATT
Exp1F	53	CATGCCATGGCTAGC**CATCATCATCATCATC ATA**GCATTCTTCGAGGAGAATC
Exp1R	36	CGGGGTACCTTACAGATGTATTGAAATTTTG AAAGG
Exp2F	53	GAAGATCTGGCTAGC**CATCATCATCATCAT CAT**GCCATTATTCGACAAAATTC
Exp2R	36	CGGGGTACCTTAATTGTTAGTATTCTGATTGT TATT
T7	20	TAATACGACTCACTATAGGG

2.4.1.3 Digestion of purified PCR products

The purified cDNA fragments were digested with *Pst* I and *Kpn* I using the condition recommended by the manufacturer. The reaction was incubated at 37 °C overnight. The restricted product were analyzed on an agarose gel and extracted from the agraose gel. The DNA fragment was dissolved in 20 µl of TE buffer.

2.4.1.4 Digestion of pTrcHis2c vector with *Pst* I and *Kpn* I

Five micrograms of pTrcHis2c was digested with *Pst* I and *Kpn* I in the 100 µl reaction mixture using the condition recommended by the manufacturer. The reaction was incubated at 37 °C overnight. The restricted product was then extracted from agarose gel and dissolved in 20 µl of TE buffer.

2.4.1.5 Ligation of AcMRJP cDNAs to *Pst* I and *Kpn* I-digested pTrcHis2c vector

The ligation reaction was performed in the total volume of 10 µl containing 250 ng of purified DNA fragment (2.4.1.3), 50 ng *Pst* I and *Kpn* I - digested pTrcHis2c vector (2.4.1.4), 1X T4 DNA ligase buffer (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate and 50 mM potassium acetate), 1 mM of ATP, 5.5 weiss units of T4 DNA ligase. The reaction mixture was gently mixed and incubated at 16 °C overnight. The ligation product was electrotransformed to *E. coli* XL1-Blue and the recombinant clones were selected as described in 2.2.12. After transformation, white colonies were random selected. The recombinant plasmids were extracted and analyzed by *Pst* I and *Kpn* I digestion. The restricted products were analyzed on agarose gel electrophoresis.

2.4.1.6 Subcloned of AcMRJP1 and AcMRJP2 cDNA fragments into pGEM®-3Zf(+) expression vector

The recombinant plasmids were digested with *Xho* I and *Kpn* I and then analyzed on agarose gel electrophoresis. The DNA fragment of ~ 1.5 Kb was eluted from agarose gel (2.3.6). The *Xho* I and *Kpn* I digested cDNA fragments were ligated to the *Sal* I and *Kpn* I digested pGEM®-3Zf(+). The ligated products were subsequently transformed into *E. coli* DH5α. Transformed cells were spreaded on the

LB agar plate containing 50 μg/ml ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-Gal and then incubated at 37 °C overnight. Recombinant plasmid contained AcMRJP1 and AcMRJP2 cDNA was extracted. Three white recombinant colonies were selected and the plasmids were purified and analyzed by restriction digestion with *Hin*d III and *Eco*R I followed by agarose gel electrophoresis (2.2.13.1). The recombinant plasmid was designated as pGEM®-3Zf(+)-AcMRJP1 and pGEM®-3Zf (+)-AcMRJP2.

2.4.1.7 Expression of AcMRJP1 cDNA and AcMRJP2 cDNAs in *E. coli*

A single colony of *E. coli* DH5α carrying recombinant plasmid pGEM®-3Zf (+)-AcMRJP1 or pGEM®-3Zf(+)-AcMRJP2 as well as *E. coli* DH5αharbouring pGEM®-3Zf(+) which served as negative control of the expression was inoculated into 2 ml of LB medium containing 50 μg/ml ampicillin at 37 °C. The overnight culture was then added to 50 ml of LB medium and further incubated with shaking to the OD_{600} of 0.6. Expression of AcMRJP1 or AcMRJP2 was induced by the addition of 1 mM IPTG and cells were for another 3 hours. To monitor protein expression, 1 ml of bacterial culture was taken at 1-hour intervals and collected by centrifugation and store at -80 °C. The protein extract prepared from collected cells was analyzed by 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970).

2.4.1.8 Protein detection by SDS-PAGE

2.4.1.8.1 Protein concentration determination

The concentration of each protein sample was determined according to Bradford's method (Bradford,1976). Protein samples were diluted with experiment buffer and 10 μl of each dilution was mixed with 90 μl of distilled water to make a total volume of 100 μl. Subsequently, 1 ml of Bradford working buffer [0.1% (w/v) Serva Blue G, 10% (v/v) of 85% phosphoric acid and 5% (v/v) of 95% ethanol] was added and vortexed. After 5 minutes, the absorbance of the blue color generated was measured at 595 nm using BSA as the standard.

2.4.1.8.2 Protein sample preparation

Cell pellet collected at various time intervals of induction was mixed with 100 µl of 2X sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% Bromophenol blue and 20% glycerol and 100 mM of 2-mercaptoethanol). After the cells were completely suspended, the suspensions were boiled for 5 minutes. Subsequently, the cell debris were eliminated by centrifugation at 10,000 rpm for 5 minutes and 5 µl of clear homogenate was applied to the SDS-polyacrylamide gel.

2.4.1.8.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The expression of AcMRJP1 or AcMRJP2 in *E. coli* was detected by 12% SDS-PAGE according to the method of Laemmli (1970). A discontinuous slap gel contained a lower separating gel with 12% or 10% (w/v) acrylamide and 0.1% (w/v) SDS in 0.375 mM Tris-HCl, pH 8.8 and an upper stacking gel with 5% acrylamide and 0.1% SDS in 0.125 M Tris-HCl, pH 6.8. The stacking gel and separating gel were prepared to the desired concentration from a stock 30% (w/v) acrylamide solution with acrylamide to *N*, *N*′-methylene-bis-acrylamide with the ratio of 29:1. Tris-glycine buffer [25 mM Tris, 192 mM glycine; pH 8.3 and 0.1% (w/v) SDS] was used as electrode buffer. Five microliters of protein sample (2.4.1.8.2) was loaded to the gel. The electrophoresis was carried out at 20 mA constant current per slab gel at room temperature. The electrophoresis was conducted until the bromophenol blue dye marker reached the bottom of the gel. A 10-200 kDa Protein ladder was used as the standard molecular weight marker.

The protein bands on the gel were visualized by staining with Coomassie brilliant blue R-250 [0.1% (w/v) in 10% (v/v) acetic acid and 45% (v/v) methanol] for at least 20 minutes. Destaining was performed by immersing the gel in destaining solution [10% (v/v) acetic acid and 10% (v/v) methanol] followed by several change of destaining solution until a clear gel background was obtained. To estimate the amount of recombinant AcMRJP1 and AcMRJP2 in crude extract, the Coomassie Brilliant Blue stained polyacrylamide gel were photographed. The photographs were

scanned and analyzed by comparison with known concentration of the protein standards, using Gel Document (BioRad, USA).

2.4.2 Construction of AcMRJP1 and AcMRJP2 as fusion protein with His-Tag under control of Trc promoter of pTrcHis2C vector

To facilitate purification, AcMRJP1 or AcMRJP2 gene was cloned into pTrcHis2c expression vector in order to express the recombinant AcMRJP1 or AcMRJP2 as fusion protein with His-Tag at the C-terminus. The insert gene was put between *Pst* I and *Kpn* I site and under the control of Trc promoter.

2.4.2.1 PCR amplification

A set of specific oligonucleotide PCR primers were designed to amplified the mature AcMRJP1 and AcMRJP2 cDNA from the plasmid pRT-AcMRJP1-Pfu and pRT-AcMRJP2-Pfu. AcMRJP1 coding sequence was amplified using the primers 1500Fexpression and 1500Rexpression whereas the AcMRJP2 coding region was amplified with the 1600Fexpression and 1600Rexpression (Table 2.3). The *Pst* I and *Kpn* I restriction sites were introduced into the primer sequences to facilitate cloning of PCR product into the corresponding sites of pTrcHis2c vector. For *Pfu* DNA polymerase (Promega Corporation Medison, Wisconsin, USA), 25 µl of the amplification reaction contained 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 mM of each dNTPs, 0.6 µM of each primer, 1 unit *Pfu* polymerase. Amplification by PCR (Perkin-Elmer model 2400) was composed of initial denaturation at 92 °C for 3 minutes followed by 25 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1.30 minutes and extension at 72 °C for 4 minutes. The final extension was carried out at 72 °C for 10 minutes. The PCR-amplified fragments were purified as described in 2.2.8.

2.4.2.2 Subcloned into pTrcHis2c expression vector

The vector pTrcHis2c was linearized with *Pst* I and *Kpn* I restriction endonucleases. The digested product was electrophoretically analyzed and eluted from agarose gel. The purified AcMRJP1 and AcMRJP2 cDNA fragment from 2.4.2.1 was digested with both *Pst* I and *Kpn* I. Finally, the digested product was

eletrophoretically analyzed and eluted from agarose gel. The *Pst* I and *Kpn* I digested cDNA fragments were ligated to the corresponding sites of pTrcHis2c expression vector (Invitrogen, USA). The ligated product was used to transform *E. coli* DH5α or XL1- blue competent cells and the ampicillin-resistant transformant were selected.

2.4.2.3 Identification of recombinant clones by restriction analyis

After transformation, whites colonies were selected. The recombinant plasmid DNA was extracted by alkaline lysis method and then analyzed for the presence of the insert fragments by digestion with *Pst* I and *Kpn* I. At the end of digestion period, the resulting product was electrophoretically analyzed by 1% agarose gel. The size of DNA insert was calculated by using λ / *Hin*d III and 100 bp DNA ladder as the standard DNA. These recombinant plasmids were designated as pTrcHis2c-AcMRJP1 and pTrcHis2c-AcMRJP2, respectively.

2.4.2.4 Transformation of recombinant plasmid to expression host

Fifty nanograms of recombinant plasmid pTrcHis2c-AcMRJP1 or pTrcHis2c-AcMRJP2 was subsequently electrotransformed to a competent *E. coli* strain DH5α host cells. Transformed cells were spreaded on the LB agar plate containing 50 μg/ml ampicillin 25 μg/ml of IPTG and 20 μg/ml of X-Gal and then incubated at 37 °C overnight. Recombinant clones were identified by colony PCR and analyzed on agarose gel electrophoresis.

2.4.2.5 Expression of AcMRJP1 cDNA and AcMRJP2 cDNA in *E. coli*

A single colony of *E. coli* carrying recombinant plasmid or pTrcHis2c vector was inoculated into 2 ml of LB medium containing 50 μg/ml ampicillin at 37 °C. The overnight culture was then transferred to 50 ml of LB medium and further incubated to the OD_{600} of 0.6. Expression of AcMRJP1 or AcMRJP2 was induced by the addition of 0.4 mM IPTG and cells were grown for another 5 hours. To monitor protein expression, 1 ml of bacterial culture was taken at 1-hour intervals and collected by centrifugation and store at -80 °C. The crude protein extract from collected cells was analyzed by 12% SDS-PAGE the same as those in 2.4.1.9.

2.4.2.6 Purification of recombinant AcMRJP1 and AcMRJP2 proteins

2.4.2.6.1 Preparation of recombinant AcMRJP1 and AcMRJP2 proteins from bacterial cell lysate

For purification of recombinant AcMRJP1 (rAcMRJP1) and rAcMRJP2, aliquots of 100 ml of IPTG-induced culture were harvested by centrifugation at 10,000 rpm for 10 minutes at 4 °C. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 5 mM imidazole and 1 mM PMSF). The cell suspension was disrupted by sonication (Sonopuls,Germany). The crude extract was centrifuged at 4 °C for 10 minutes and the supernatant was used as the soluble fraction whereas the pellet was used as the insoluble fraction. Each of the above fractions and the whole-cell proteins were analyzed for solubility of the recombinant protein by SDS-PAGE, followed by visualization with Coomassie brilliant blue. The fraction showing recombinant AcMRJP1 and AcMRJP2 proteins band in SDS-PAGE was used for further purification.

2.4.2.6.2 Purification of recombinant AcMRJP1 and AcMRJP2 proteins by affinity chromatography

The MRJPs were purified under denaturing conditions by using HiTrap Chelating HP affinity chromatography (Amersham Biosciences,USA) according to manufacturer's instructions. The pellet (insoluble fraction) was washed three times with inclusion body washing buffer (20 mM sodium phosphate, pH 7.4 and 1% Triton X-100) and collected by centrifugation at 10,000 rpm for 10 minutes. The inclusion body was dissolved in solubilizing buffer (20 mM sodium phosphate, pH 7.4, 5 mM imidazole, 500 mM NaCl and 8 M urea) and stirred for 1 hour at room temperature. The samples were centrifuged at 10,000 rpm for 20 minutes at 4 °C to pellet any insoluble cell debris. After centrifugation, the supernatant was collected and applied to the HiTrap Chelating HP affinity columns. The column was charged with 0.5 ml of 0.1 M $NiSO_4$ followed by washing the column twice with 5 ml distilled water. The column was equilibrated with 10 ml of equilibrate buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 5 mM imidazole and 8 M urea). The urea solubilized sample was applied to the column. The column was washed with wash buffer (20 mM sodium

phosphate, pH 7.4, 500 mM NaCl, 10 mM imidazole and 8 M urea). The elution of His-tagged fusion protein could be achieved by using step gradient starting with 5 ml of elution buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 250 mM imidazole and 8 M urea). Purified protein was stored at -20 °C. Fractions of about 1 ml were collected and analyzed by SDS-PAGE. The protein fraction was dialyzed against 20 mM sodium phosphate, pH 7.4 and 50 mM NaCl at 4 °C and changed every 4 hours for 24 hours to remove urea. After dialysis, the protein sample were centrifugation at 10,000 rpm for 10 minutes and protein concentration was determined as described in section 2.4.1.9.1.

2.4.2.7 Western blot analysis of recombinant AcMRJP1 and AcMRJP2 proteins

2.4.2.7.1 Transfer of recombinant AcMRJP1 and AcMRJP2 proteins to PVDF membrane

Purified rAcMRJP1 and rAcMRJP2 were analyzed in 12% SDS-PAGE. Electrophoresed proteins were transferred to a PVDF membrane (Hybond-P, Amersham Biosciences, USA) by the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad Laboratories, USA). The gel was soaked in the ice-cold electroblotting buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.3, and 20 % methanol) recommended by Towbin (1979) for 30 minutes. A piece of PVDF membrane was cut to the dimensions of the gel and pre-wetted with methanol followed by equilibrated in the electroblotting buffer for 15 minutes. The blotted sandwich was assemble in a large shallow tray contaning cold electroblotting buffer in the following order: two pieces of Whatman No.1 saturated with electroblotting buffer, electrophoretic gel, PVDF membrane and covered with two layers of saturated filter papers. The air bubbles between layers of the component were removed by rolling a clean glass rod across the surface of each component. Finally, the electroblotting of proteins on the gel to the PVDF membrane was carried out at 80 V, 4 °C for 1 hour. When the transfer was completed, the gel was stained with Coomassie brilliant blue to check the efficiency of the transfer.

2.4.2.7.2 Western blot analysis of recombinant AcMRJP1 and AcMRJP2 proteins

Purified rAcMRJP1 and rAcMRJP2 were resolved in 12% SDS-PAGE and electroblotted onto a PVDF membrane as described in 2.4.7.1. The membrane was washed twice for 10 minutes each time with TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) at room temperature. Subsequently, the membrane was incubated in blocking buffer (3% BSA in TBS, 0.05% (v/v) Tween 20) for 1 hour at room temperature. Then the membrane was washed twice for 10 minutes each time with TBS-Tween/Triton buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween 20, 0.2% (v/v) Triton X-100) followed by TBS buffer for 10 minutes at room temperature. The membrane was incubated with diluted Anti-His-HRP Conjugate (1:1000, Penta-His, Qiagen, USA) in blocking buffer for 1 hour with gently shaking. Then the membrane was washed twice in TBS-Tween/Triton buffer followed by TBS buffer for 10 minutes at room temperature. The peroxidase activity was detected by adding H_2O_2 into staining solution containing diaminobenzidine (DAB) chromogenic substrate (6 mg/ml DAB in 0.9% (w/v) NaCl, 100 mM Tris-HCl, pH 8.0). The reactive areas would turn brown color within 1-15 minutes. The reaction was terminated by washing the membrane several times in distilled water.

2.4.3 Construction of AcMRJP1 and AcMRJP2 as fusion protein with His-tag of pET system under control of T7 promoter of pET17b vector

In order to improve the level of expression of the fusion protein, AcMRJP1 or AcMRJP2 cDNA was cloned into pET17b vector for ecpression under the control of T7 promoter.

2.4.3.1 Primer designation

Oligonucleotide PCR primers were designed to amplified the coding sequences of MRJPs from AcMRJP1 or AcMRJP2 cDNA. The AcMRJP1 coding region was amplified with the Exp1F and Exp1R primers whereas the AcMRJP2 coding region was amplified with the Exp2F and Exp2R primers (Table 2.3). The forward oligonucleotides contained an *Nhe*I restriction site followed by nucleotide

sequences encoding six histidine residuces. The reverse primers contain *Kpn* I recognition site to facilitate the cloning.

2.4.3.2 PCR amplification

The amplification reaction of 25 µl contained 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 mM of each dNTPs, 0.6 µM of each primer, 1 unit *Pfu* polymerase (Promega Corporation Medison, Wisconsin, USA). Amplification by PCR (Perkin-Elmer model 2400) was composed of initial denaturation at 92 °C for 3 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1.30 minutes and extension at 72 °C for 4 minutes. The final extension was carried out at 72 °C for 10 minutes. The resulting products were electrophoretically analyzed through a 1.2% agarose gel.

2.4.3.3 Subcloned into pET17b expression vector

The vector pET17b was linearized with *Nhe* I and *Kpn* I restriction endonucleases. The digested product was electrophoretically analyzed and eluted from agarose gel. The purified AcMRJP1 and AcMRJP2 DNA fragment was digested with *Nhe* I and *Kpn* I and then eluted from agarose gel. The *Nhe* I and *Kpn* I digested DNA fragments were ligated to the corresponding sites of pET17b expression vector (Novagen, Madison, USA). The ligated product was used to transform *E. coli* DH5α or XL1-blue competent cells and the ampicillin-resistant transformants were selected.

2.4.3.4 Identification of recombinant clones by colony PCR

Identification of recombinant clones harbouring the recombinant plasmids was performed by colony PCR screening using T7 primer as forward primer and Exp1R or Exp2R primer as reverse primer. The protocol was the same as described in 2.2.13.2. After amplification, 5 µl of reaction product was electrophoretically analyzed using 0.7% agarose gel. The transformant identified as the positive clones were picked for plasmid extraction and double digested with *Nhe* I and *Kpn* I. The recombinant plasmid was sent to Bioservice Unit for sequencing using T7 primer. A desired plasmid was designated as pET17b-AcMRJP1 and pET17b-AcMRJP2.

2.4.3.5 Transformation of recombinant plasmid to Expression host

The recombinant plasmids were subsequently transformed into *E. coli* BL21 (DE3)pLysS, BL21 Star (DE3)pLysS and Rosetta (DE3)pLysS for expression. The recombinant plasmids and pET17b vector were electrotransformed to a competent *E.coli* cells. The preparation of *E. coli* BL21(DE3)pLysS, BL21 Star (DE3)pLysS and Rosetta (DE3)pLysS competent cells and the electrotransformation method were the same as described in 2.2.12.1. Transformed cells were spreaded on the LB agar plate containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and then incubated at 37 °C overnight.

2.4.3.6 Expression of AcMRJP1 cDNA and AcMRJP2 cDNA in *E. coli*

A single colony of each *E. coli* host cells carrying recombinant plasmid (pET17b-AcMRJP1 or pET17b-AcMRJP2) was inoculated into 2 ml of LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. The overnight culture was then transferred to 50 ml of LB medium and further incubated to the OD_{600} of 0.6. Expression of AcMRJP1 and AcMRJP2 were induced by the addition of 0.4 mM IPTG and cells were grown for another 5 hours. To monitor protein expression, 1 ml bacterial culture was taken at 1-hour intervals and collected by centrifugation and stored at -80 °C. The protein extract prepared from cell pellets (2.4.1.9.2) was analyzed by 12% SDS-PAGE. The protein was purifed (2.4.6) and analyzed by Western blot analysis (2.4.7).

2.4.3.7 *N*-terminal amino acid sequencing

Purified rAcMRJP1 or rAcMRJP2 was resolved in 12% SDS-PAGE and electroblotted onto a PVDF membrane. After blotting, the membrane was removed and rinse with large amount of distilled water prior to staining. The membrane was stained with 0.1% (w/v) Coomassei Brilliant Blue R-250 in 40% (v/v) methanol and 1 % (v/v) acetic for 1-5 minutes. Subsequently, the membrane was destained in 50 % methanol, rinsed with deionized water and air-dried. The protein band was excised from PVDF membrane and further analyzed by ABI 494 automated protein sequencer (Department of Biological Science, National University of Singapore).

2.5 Expression of AcMRJP1 in plant expression system

2.5.1 Construction of plant expression vector

2.5.1.1 Construction of potato expression vector under control of GBSS gene promoter

2.5.1.1.1 Preparation of GBSS gene promoter fragment

The pPGB121 containing fragment of GBSS gene promoter (kindly obtained from Dr. Richard Visser) was digested with *Hin*d III and *Sal* I to release ~800 bp of GBSS gene promoter. This fragment was eluted from agarose gel and ligated to the corresponding site of pGEM®-3Zf(+) vector. This plasmid was called pGEM®-GBSS.

2.5.1.1.2 Preparation of AcMRJP1 coding fragment

DNA fragments coding for mature AcMRJP1 were amplified by PCR using F1500SalKosak and R1500Exp primers (Table 2.4). The KDEL signal at the C-terminus was included. The restriction site of *Sal* I and *Kpn* I was introduced to the 5' end of the primers to facilitate cloning. The PCR reaction contained 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 mM of each dNTPs, 0.6 μM of each primer, 1 unit *Pfu* polymerase (Promega Corporation Medison, Wisconsin, USA). The PCR profile was composed of initial denaturation at 92 °C for 3 minutes followed by 25 cycles of denaturation at 94 °C, 1 minute, annealing at 60 °C for 1.30 minutes and extension at 72 °C for 4 minutes. The final extension was carried out at 72 °C for 10 minutes. The PCR-amplified fragment of AcMRJP1 was purified and digested with *Sal* I and *Kpn* I and ligated into pGEM®-GBSS containing GBSS promoter gene to generate pGEM®-GBSS-AcMRJP1.

Table. 2.4 Sequence of oligonucleotide primers used for amplification of AcMRJP1 cDNA in plant expression experiment. The restriction sites designed for cloning were underlined. *Sal* I (G/TCGAC), *Kpn* I (GGTAC/C), *Eco*R I (G/AATTC)

Primer	Length (bp)	Sequence (5'- 3')
F1500Sal Kosak	39	ACGCGTCGACACC*ATG*GCGAGCATTCTTCGAGG AGAATC
R1500Exp	33	CGGGGTACCCAGATGTATTGAAATTTTGAAAGG
FNosHis	51	GCCGGTACCCACCACCACCACCACCACAAGGA TGAATTGTGAATTACAGGT
RNosHis	30	CCGAATTCCCGATCTAGTAACATAGATGAC
FGBSS	25	TGCTATAAACGTGGGGTTGATCGTG
RGBSS	25	CAAAACTTTAGGTGCCTCTAGGGCT
Fpatatin	25	ACCTTGGAGAAACTCGTGTGCATCA
Rpatatin	25	TAACATCCATCGTAGAGGACCCCAT
F18S	20	CTGCCCGTTGCTGCGATGAT
R18S	20	GGAATTACCGCGGCTGCTGG
Factin	20	TGATGCGCCCAGGGCTGTCT
Ractin	20	CGATTGGCCTTGGGGTTGAG

2.5.1.1.3 Preparation of Nopaline synthase (Nos) terminator fragment

The Nos terminator was amplified by PCR using FNosHis and RNosHis primers (Table 2.4). The restriction site of *Kpn* I and *Eco*R I was added to the 5' end of primers. The Nos terminator was amplified from plasmid pCAMBIA2301. The amplification reaction contained 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 mM of each dNTPs, 0.6 μM of each primer, 1 unit *Pfu* polymerase (Promega Corporation Medison, Wisconsin, USA). The PCR profile composed of initial denaturation at 92 °C for 3 minutes followed by 25 cycles of denaturation at 94 °C, 1 minute, annealing at 60 °C for 1.30 minutes and extension at 72 °C for 1 minute. The final extension was carried out at 72 °C for 10 minutes. The ~300 bp of PCR-amplified fragment of Nos was purified and digested with *Kpn* I and *Eco*R I and ligated into pGEM®-GBSS-AcMRJP1 (containing GBSS gene promoter and coding sequence of AcMRJP1) to generate pGEM®-GBSS-AcMRJP1-Nos.

2.5.1.1.4 Preparation of expression vector

Finally, the expression cassette containing the GBSS gene promoter, AcMRJP1 and the nos terminator from pGEM®-GBSS-AcMRJP1-Nos and cloned into *Hin*d III and *Eco*R I site of pCAMBIA2301 vector to generate pCAMBIA2301-GBSS-AcMRJP1-Nos

2.5.1.2 Construction of potato expression vector under control of B33 patatin gene promoter

2.5.1.2.1 Preparation of patatin gene promoter fragment

The pART7 containing fragment of patatin gene promoter (kindly obtained from Dr. Eva Farre) was digested with *Sac* I and *Xho* I to release 1,400 bp of patatin gene promoter. This fragment was eluted from agarose gel and ligated to the corresponding site of pET17b vector to generate the *Hin*d III and *Kpn* I recognition sequences. This plasmid was called pET-B33.

2.5.1.2.2 Preparation of Nos terminator fragment

The Nos terminator was amplified by PCR using the PCR primers and condition as described in 2.6.1.1.3. The PCR product was purified and digested with *Hin*d III and *Eco*R I. The fragment was eluted from agarose gel and ligated to the corresponding site of pGEM®-3Zf(+) to generated pGEM®-Nos.

2.5.1.2.3 Preparation of AcMRJP1 coding fragment

DNA fragments coding for mature AcMRJP1 was amplified by PCR using F1500SalKosak and R1500Exp primers (Table 2.4). The PCR reaction mixture and condition were the same as described in 2.6.1.1.2 The PCR-amplified fragment was purified and digested with *Sal* I and *Kpn* I and ligated into the compatible site of pGEM®-Nos. The construct recombinant plasmid was called pGEM®-Nos-AcMRJP1.

2.5.1.2.4 Preparation of expression vector

The B33 patatin gene promoter cassette was isolated by *Hin*d III / *Xho* I from plasmid pET-B33 and cloned into *Hin*d III / *Sal* I of plasmid pGEM®-Nos-AcMRJP1 containing the AcMRJP1 coding sequence and Nos cassette giving rise to plasmid pGEM®-Nos-AcMRJP1-B33. Finally, the expression cassette of the patatin B33 gene promoter, AcMRJP1 coding sequence and the Nos terminator was excised by *Hin*d III and *Eco*R I and joined into *Hin*d III and *Eco*R I site of pCAMBIA2301 vector. Resulting recombinant plasmid was called pCAMBIA2301-B33-AcMRJP1-Nos.

2.5.1.3 Construction of potato expression vector under control of 35S promoter

The AcMRJP1-Nos cassette was excised from plasmid pGEM®-Nos-AcMRJP1 by *Sal* I and *Eco*R I digestion and ligated with compatible site of pCMABIA2301 to generate pCAMBIA-AcMRJP1-Nos. Finally, the 35S fragment was isolated from plasmid pCAMBIA2301 by digestion with *Hin*d III and *Xho* I and cloned into *Hin*d III and *Sal* I of plasmid pCAMBIA2301-AcMRJP1-Nos. The resulting recombinant plasmid wass called pCAMBIA2301-35S-AcMRJP1-Nos which contained the expression cassette of the 35S promoter, AcMRJP1 coding sequence and the Nos terminator.

2.5.1.4 Construction of rice expression vector under control of 35S promoter

For rice transformation, pCAMBIA1301 was employed. The AcMRJP1-Nos cassette was excised from plasmid pGEM®-Nos-AcMRJP1 by *Sal* I and *Eco*R I digestion and ligated with compatible site of pCMABIA1301 to generate pCAMBIA-AcMRJP1-Nos. Finally, the 35S fragment was isolated from pCAMBIA2301 vector by *Hin*d III and *Xho* I digestion and cloned into *Hin*d III and *Sal* I of plasmid pCAMBIA1301-AcMRJP1-Nos. The resulting recombinant plasmid was called pCAMBIA1301-35S-AcMRJP1-Nos which contained the expression cassette of the 35S promoter, AcMRJP1 coding sequence and the nos terminator.

2.5.2 Plant Tissue Culture

2.5.2.1 Potato tissue culture

The tuber of potato (*Solanum tuberosum*) cultivar Atlantic was obtained from the Agricultural Research Center, Phang, Chiang Mai. The tubers were incubated at room temperature for 2 months until potato tuber sprouted. The sprouting shoot were detached from the potato tubers and rinse in tap water for 10 minutes. The sprouting shoot were disinfected in 10% Clorox with 1 drop of Tween 20 for 20 minutes with agitation. The shoots were further rinsed 3 times with sterile water. Bleached tissues were trimmed away using a sterilize razor blade and a forcepts. Subsequenctly, the shoot tip explants were transferred to MS basal medium (Murashige and Skoog, 1962). The cultures were incubated at 25 °C under a 16/8 hours light/dark photoperiod for 3 weeks. Whole plants were regenerated from shoot tip. Micropropagation technique was done by subculture. The intact shoots were removed from stock cultures and placed in sterile petridish. Subsequenctly, they were subdivided into single node cuttings using a sterile scalpel. The cultures were transferred to fresh medium and incubated at 25 °C under a 16/8 hours light/dark photoperiod for 3 weeks. *In vitro* grown leaves were used as starting material in transformation experiments.

2.5.2.1.1 Regeneration of potato

Leaf disk of potato was transferred to SIM (MS basal medium supplemented with 2 mg/l zeatin riboside, 0.02 mg/l NAA, 0.02 mg/l GA3) and incubated at 25 °C under a 16/8 hours light/dark photoperiod. Regenerated shoots were rooted on MS medium.

2.5.2.1.2 *In vitro* tuberization

Microtubers were induced from nodal segments of *in vitro* grown plants on 8MS medium (MS basal medium supplemented with 8% (w/v) sucrose). The cultures were incubated at 20-22 °C in the dark for 3 weeks.

2.5.2.2 Rice tissue culture

The *indica* rice cultivar (*Oryza sativa* L.) KDML 105 was obtained from the Bangkhen rice research center. Mature seeds were dehulled and first sterilized with 70% ethanol for 1 minute and then with 15% Clorox with 1 drop of Tween 20 for 30 minutes with shaking. The seeds were further rinsed 3 times with sterile water. These were cultured on 2NB medium [Li *et al.*, 1993; NB basal medium supplemented with 2 mg/l of 2,4-dinitrophenoxy acetic acid (2,4-D)] for callus induction. The cultures were incubated in the dark at 28 °C for 6 weeks. The calli observed as being compact, yellowish and granular (Peterson and Smith, 1991) were separated with sterile scalpel and subcultured on fresh medium. Actively growing calli (1-2 mm in diameter) were used for plant regeneration and transformation experiments. For regeneration, calli were transferred to regeneration medium (NB basal medium supplemented with 4 mg/l BAP). The cultures were incubated at 28 °C under a 16/8 hours light/dark photoperiod.

2.5.3 Transformation of pCAMBIA2301-GBSS-AcMRJP1-Nos, pCAMBIA2301-B33-AcMRJP1-Nos, pCAMBIA2301-35S-AcMRJP1-Nos and pCAMBIA1301-35S-AcMRJP1-Nos to *A. tumefaciens* EHA105 host cells by electroporation

2.5.3.1 Preparation of competent *A. tumefaciens* EHA105 cells (Gelvin and Schilperoort, 1994)

A. tumefaciens EHA105 was streaked on solid LB medium supplemented with 25 μg/ml rifampicin and incubated at 28 °C for 2 days. A well-separated colony was innoculated in 10 ml of LB-broth supplemented with 25 μg/ml rifampicin and incubated with vigorous shaking at 28 °C for 8 hours. The starting culture was inoculated into 1 litre of L-broth and continued culture at 28 °C with vigorous shaking to the OD_{600} of 1.0 to 1.5. The cells were harvested by centrifugation in a cold rotor at 5,000 rpm for 15 minutes. The pellet were washed by resuspending in 1 litre of cold sterile distilled water and centrifuged as above. The supernatant was carefully poured off. The cell pellet was resuspended in 500 ml of cold sterile distilled water and centrifuge as above. Subsequently, the pellet was resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and supernatant was removed. Finally, the cell pellet was resuspended in 1 ml of 10% glycerol. Usually, cells can be resuspended in the 10% glycerol that remains in the centrifuge bottle. This concentrated cell suspension was devided to 45 μl aliquots. These cells could be used immediately or kept at -80 °C for 6 months.

2.5.3.2 Electrotransformation of binary vector to *A. tumefaciens* EHA105

The recombinant vector, pCAMBIA2301-GBSS-AcMRJP1-Nos, pCAMBIA2301-B33-AcMRJP1-Nos, pCAMBIA2301-35S-AcMRJP1-Nos or pCAMBIA1301-35S-AcMRJP1-Nos was transformed into *A. tumefaciens* EHA105 by electrotransformation using condition as described above in section 2.2.12.2. The electroporated cells were immediately removed from the cuvette and added to a new tube containing 1 ml of SOC medium. The cell suspension was incubated at 28 °C with shaking for 2 hours. Approximately 10-50 μl of the cell suspension was spreaded on the selective LB agar plate containing 50 μg/ml of kanamycin, 25 μg/ml rifampicin and incubated at 28 °C for 2 days.

2.5.4 Identification of *A. tumefaciens* transformant by colony PCR

The transformants were screened by colony PCR using F1500SalKosak and R1500Exp primers. The transformant colony was picked up and resuspended in a 25 µl amplification reaction (2.2.13.2). Amplification by PCR was composed of initial denaturation at 94 °C for 4 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was carried out at 72 °C for 10 minutes. After amplification, 5 µl of reaction product was electrophoretically analyzed on agarose gel.

2.5.5 Transformation of binary vectors by co-cultivation with *Agrobacterium*

2.5.5.1 Co-cultivation of rice calli

A. tumefaciens strain EHA105 harbouring pCAMBIA13005.1-35S-AcMRJP1-Nos was streaked on solid AB medium (Appendix A) supplemented with 50 µg/ml hygromycin and 50 µg/ml kanamycin. The bacteria were incubated at 28 °C for 2 days and collected by scraping from plates with platinum loop. The bacteria were resuspended in AAM medium (Appendix A) containing 100 µM of acetosyringone with vigorous shaking. The optical density at 600 nm of the bacterial suspension was adjusted to 0.01 by diluting with AAM medium. The calli from section 2.6.2.1 were subcultured to fresh medium and incubated in the same condition for 4 days before using in co-cultivation. The four-day incubated calli were immersed in bacterial suspension for 10 minutes with occasional shaking. The excess of bacteria was removed by decanting the liquid and calli were blotted dry on sterile filter papers. The calli were then transferred to the co-cultivation medium (2NB-AS; 2NB supplemented with 100 µM of acetosyringone) and incubated in the dark at 25 °C for three days.

2.5.5.2 Selection and regeneration of transformed calli

After the co-cultivation, the calli were removed from the co-cultivation medium and bloted dry on sterile filter papers whereas the *Agrobacterium* overgrowing calli were discarded. The co-cultivated calli were transferred to selection medium (2NB-CH; 2NB supplemented with 500 µg/ml cefotaxime and 50 µg/ml hygromycin) and incubated at 28 °C for 4 weeks. The hygromycin resistant calli

obtained after first round of selection were subcultured for two cycles onto fresh 2NB-CH medium every two weeks. The hygromycin resistant calli were then transferred to regeneration medium without any antibiotics [NB4-RE; NB-RE containing 4 mg/l 6-benzylaminopurine (BAP)] and incubated at 28 °C under 16 hours light photoperiod for 3-4 weeks. Green buds / shoots were observed after 4 weeks. When the transformed shoots became 1-1.5 cm in length, they were transferred to hormone-free NB medium for stimulation of rooting and stem elongation for 4 weeks. One-month old transformed plants were transferred to sterile soil under the natural condition.

2.5.5.3 Co-cultivation of potato leaf disk

A. tumefaciens strain EHA105 habouring pCAMBIA2301-35S-AcMRJP1-Nos, pCAMBIA2301-GBSS-AcMRJP1-Nos or pCAMBIA2301-B33-AcMRJP1-Nos was separately streaked on solid YEB medium (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% saccharose, 0.49 g/l $MgSO_4.7H_2O$) supplemented with 50 μg/ml kanamycin. The bacteria were incubated at 28 °C for 2 days. A single colony was inoculated to 25 ml of YEB medium and incubated at 28 °C, 130 rpm overnight. The leaves of potato form 2.6.2.1 were detached from *in vitro* plantlets and placed on a petri dish. The leaves were cut off the base and made 1-2 mm cuts across the midrib in two places separated by 4-5 mm. These leaves were placed upside down (adaxial-side down) in 2MS medium. The 50 μl of *Agrobacterium* suspension was added to the medium. The plate was shaked gently by hand and incubated at 22 °C in the dark for 2 days.

2.5.5.4 Selection and regeneration of transformed potato

After 2 days, these leaves were transferred onto callus induction medium; CIM medium (MS basal medium supplemented with 1.6% glucose, 5 mg/l NAA, 0.1 mg/l BAP, 250 mg/l cefotaxime and 50 mg/l kanamycin) and incubated at 22 °C in the dark for 7 days. Then these leaves were transferred to shoot induction medium, SIM medium incubated at 22 °C for 7 days under 16 hours light photoperiod and transferred to fresh medium every 2 weeks for 8 weeks. The regenerated shoot of 1-

1.5 cm were removed from leaf explants and transferred to RIM or root induction medium (MS basal medium supplemented with 250 mg/l cefotaxime) for 2 weeks.

2.5.6 Characerization of transformed plants

2.5.6.1 Histochemical analysis for β -Glucuronidase (GUS) activity

GUS activity was assayed histochemically by a procedure based on that described by Jefferson, 1987. Histochemical GUS assays were made after co-cultivation of calli leaves and roots. The tissues were placed in microcentrifuge tube and 100 µl of histochemical (X-Gluc) staining solution composed of 0.5 mg/ml X-Gluc, 50 mM $NaPO_4$, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na_2EDTA, 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100 and 0.1% sodium N-laurylsarcosine was added. The tissues were vacuum infiltration for one minute and incubated at 37°C overnight. To facilitate detection of the blue color, 95% of ethanol was added to remove the chlorophyll and to detect the formation of blue color.

2.5.7 Detection of AcMRJP1 gene in transformed plant

2.5.7.1 Analysis of AcMRJP1 in genomic DNA of transformed plant

2.5.7.1.1 Plant DNA extraction using modified CTAB method (Weising *et al.*, 1995)

Genomic DNA was extracted from control and putative transformed plants using the modified CTAB method. The CTAB extraction buffer (2% (w/v) CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 0.2 M EDTA and 0.1 M Tris-HCl, pH 8.0) was preheated at 60 °C. Then 100-200 mg of fresh plant tissue was harvested and grinded to a fine powder in liquid nitrogen with mortar and pestle. The forzen powder was immediately transferred to a 1.5 ml microcentrifuge tube. The 500 µl of preheated CTAB extraction buffer was added to the tissue powder and mix throughly. The mixture was incubated at 65 °C for 30 minutes with regularly swirling to ensure efficient extraction. The plant cell debris was removed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was transferred to a new tube and then extracted once with an equal volume of chloroform-isoamyl alcohol (24:1) by mixed gently for

15 minutes followed by centrifuged at 10,000 rpm for 10 minutes to separate phases. The upper aqueous phase was transferred to a new tube and equal volume of ice-cold isopropanol was added. The mixture was incubated at -80 °C for 10 minutes. The precipitated DNA pellet was recovered by centrifugation at 12,000 rpm for 15 minutes and briefly washed once with 70% ethanol. The DNA pellet was air-dried and dissolved in 100 µl of TE buffer (10 mM Tris-HCl, pH7.4 and 1 mM EDTA). RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA. The mixture was incubated at 37 °C for 30 minutes. The DNA was then extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) by mixed gently for 15 minutes and centrifuged at 12,000 rpm for 10 minutes to separate phases. The upper aqueous phase was transferred to a new tube and further extracted once with an equal volume of chloroform-isoamyl alcohol. One-tenth volume of 3 M sodium acetate, pH 5.5 was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80 °C for 15 minutes. The precipitated DNA pellet was recovered by centrifugation at 12,000 rpm for 15 minutes and washed once with 70% ethanol. The pellet was air-dried and resuspended in 20 µl of TE buffer. The DNA was analyzed by spectrophotometry and agarose gel electrophoresis.

2.5.7.1.2 Verification of AcMRJP1 gene in transformed plant by PCR

The presence of the AcMRJP1 gene in putative transformed plants was assesed by PCR. The transformants were screened using F1500SalKosak and R1500Exp primers (Table 2.4). The amplification reactions were performed in a 25 µl reaction volume containing 10mM Tris-HCl pH 8.8, 50mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 100 µM of each dNTP, 0.4 µM of each primers, 1 unit of DyNazyme^{TM}II DNA polymerase and 50 ng of genomic DNA template. The reaction was predenatured at 94°C for 2 minutes following by 30 cycles of denaturing at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 1.30 minutes. The final extension was performed at 72°C for 10 minutes. After amplification, 5 µl of reaction product was eletrophoretically analyzed on agarose gel.

2.5.8 Expression analysis of AcMRJP1 mRNA in transformed plants by RT-PCR

The transcription of AcMRJP1 gene in transformed plant was detectd using RT-PCR method.

2.5.8.1 Total RNA extraction

Total RNA was extracted from 50-100 mg of leaves of transformed rice and tuber of transformed potato using TRI REAGENT® (Molecular Research Center, Inc., USA). Plant tissue was homogenized with mortar and pestle in the presence of liquid nitrogen and then transferred to mirocentifuge tube immediately. One ml of TRI REAGENT® (Molecular Research Center, Inc., USA) was added to the tissue powder. The mixture was homogenized and incubated at room temperatue for 5 minutes. The plant cell debris was removed by centrifuge at 12,000 rpm for 10 minutes at 4 °C. The supernatant was transferred to a new microcentrifuge tube and then 200 µl of chloroform was added. The homogenate was vortexed for 2 minutes and left at room temperature for 10 minutes. The mixture was centrifuged at 12,000 rpm for 10 minute at 4 °C. The upper aqueous phse was transferred to an new microcentrifuge tube. The total RNA was recovered by addition of 500 µl of ice-cold isopropanol and mixed throughly. The mixture was incubated at -80 °C for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with ice-cold 70% ethanol and centifuged at 12000 rpm for 5 minute. The RNA pellet was air-dried and dissolved in RNase free water by incubating at 55-60 °C for 10 minute. Contaminant DNA was eliminated by incubation of the RNA with 1 unit of DNase I for 15 minutes at 37 °C followed by reextracted with TRI REAGENT® (Molecular Research Center, Inc., USA). The concentration of total RNA was then determined by spectrophometry at the wavelength of 260 nm and calculated by following the equation:

RNA concentration (µg/ml) = A_{260} x dilution factor x 40

2.5.8.2 First stranded cDNA synthesis

First-strand cDNA was synthesized from total RNA using the ImProm-II™ Reverse Transcription system (Promega Corporation Medison, Wisconsin, USA) according to the manufacturer recommendations. Approximately 1 μg of total RNA in RNase free water was combined with 0.5 μg of oligo $(dT)_{12-16}$ primer followed by incubated at 70 °C for 5 minutes and immediately chilled on ice for at least 5 minutes. Subsequently, total RNA was added to the reaction containing 0.5 mM of each dNTP, 20 units of RNase inhibitor (Promega Corporation Medison, Wisconsin, USA), 1X RT buffer and 1 μl of ImProm-II™ Reverse Transcriptase. The reaction was carried out at 25 °C for 5 minutes followed by 42 °C for 90 minutes and then the reaction was terminated by heating at 70 °C for 15 minutes.

2.5.8.3 RT-PCR amplification

The presence of the AcMRJP1 mRNA in putative transformed plants was assesed by RT-PCR. The first-strand cDNA (2.5.8.2) of various transformants were screened using F1500SalKosak and R1500Exp primers. The sequence of oligonucleotide primers were shown in Table 2.4. The amplification reaction contained 10mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 100 μM of each dNTP, 0.4 μM of pUC1 and pUC2 primers and 1 unit of DyNazyme™II DNA polymerase. The PCR profile was composed of initial denaturation at 92 °C for 3 minutes followed by 30 cycles of denaturation at 94 °C, 1 minute, annealing at 60 °C for 1 minute and extension at 72 °C for 1.30 minutes. The final extension was carried out at 72 °C for 10 minutes. After amplification, 5 μl of reaction product was eletrophoretically analyzed on agarose gel.

2.5.9 Analysis of AcMRJP1 protein in transformed plants

2.5.9.1 Preparation of protein sample

Microtubers were homogenized in liquid nitrogen in an equal volume of buffer containing 625 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol. The homogenate was centrifuged at 12,000 rpm for 15 minutes at 4 °C and the

supernatant was used for protein concentraion determination by method of Bradford's (Bradford, 1976).

2.5.9.2 Western blot analysis of recombinant AcMRJP1 proteins using ECL-immunodetection

The homogenate from transformed microtubers was resolved in 7.5% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Amersham Biosciences, USA) by the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad Laboratories, USA). A piece of nitrocellulose membrane was cut to the dimensions of the gel and pre-wetted with distilled water followed by equilibration in the electroblotting buffer for 15 minutes. The electroblotting was carried out as described in 2.4.7.1. The blotted membrane was blocked in blocking buffer in TBS containing 1% Tween 20 for 1 hour at room termperature. The membrane was then incubated in a new blocking solution containing the primary antibody solution (anti-His-HRP conjugate) for 1 hour. The membrane was washed three times with TBS containing 0.1% (v/v) Tween 20 for 10 minutes. In the detection step, the membrane was incubated in the detection solution (equal volume of Detection solution A and B) for 5 minutes at room temperature with out agitation. The excess detection reagent was drained off and the membraane was wrapped in Saranwrap without air bubbles. Then the membrane was exposed to autoradiography film (Kodak, USA) for 3-30 minutes and the film was developed by using developing reagent (Kodak, USA).

By using polyclonal rabbit antiserum against WSPs of RJ of *A. mellifera* as primary antibody, the membrane was incubated in a new blocking solution containing polyclonal rabbit antiserum against WSPs of RJ of *A. mellifera* at a dilution of 1:2,000 for 1 hour. After three times washing with TBS containing 0.1% Tween 20 for 10 minutes, the membrane was subsequently incubated with the secondary antibody (anti-rabbit IgG, horseradish peroxidase-linked antibody) at the dilution of 1:1,000 for 1 hour at room temperature with gentle agitation on a platform shaker. The membrane was washed three times with TBS contining 0.1% (v/v) Tween 20 for 10 minutes and then detected as described above.