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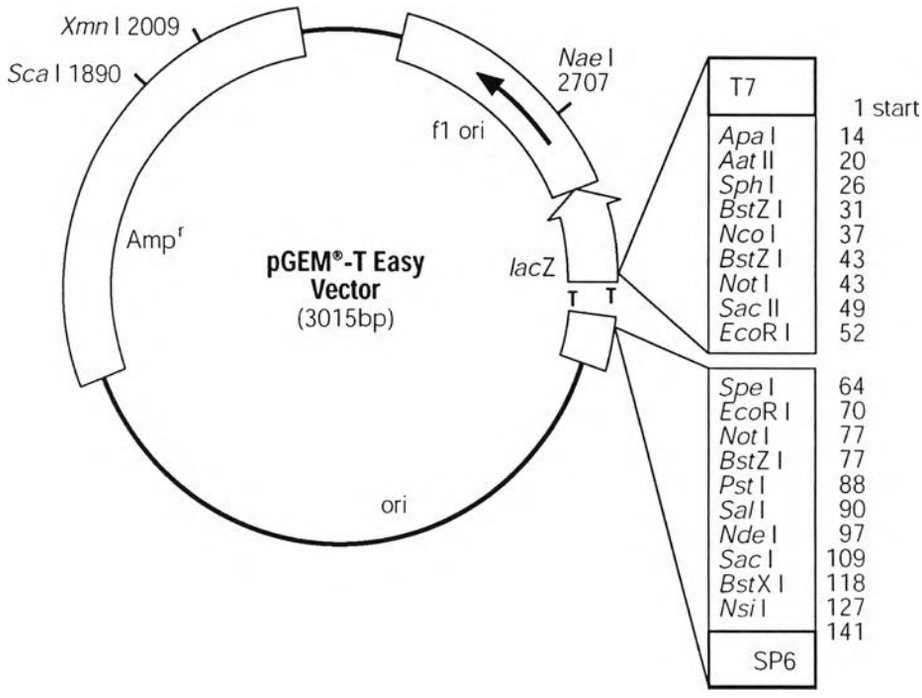
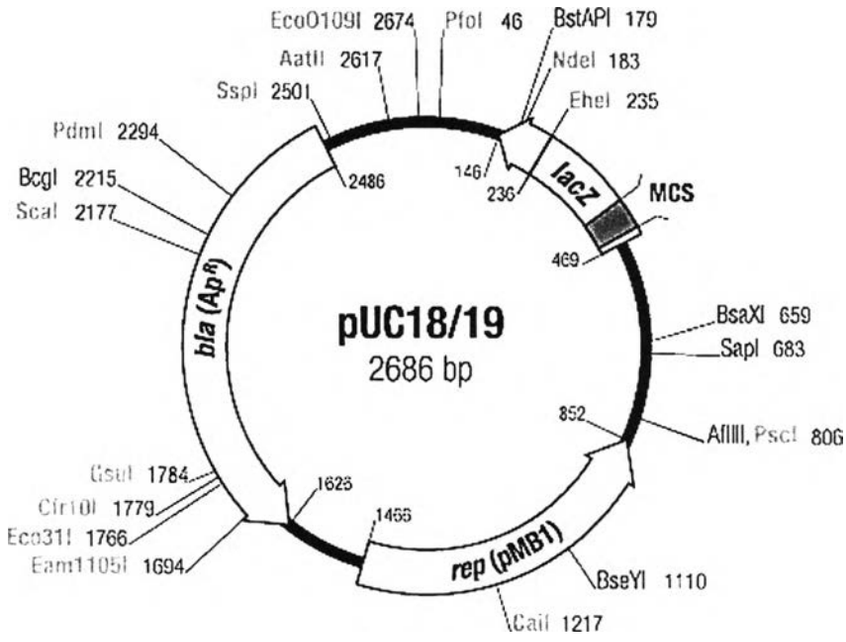
APPENDICES

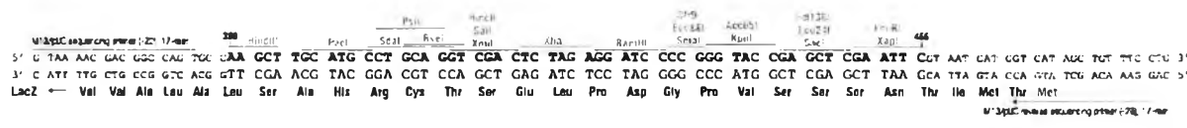
APPENDIX A

Restriction map of pGem®-T Easy vector

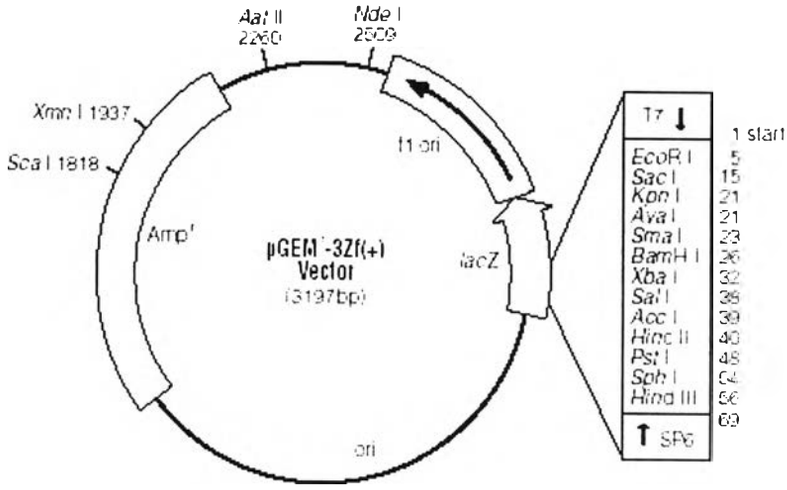


Restriction map of pUC18

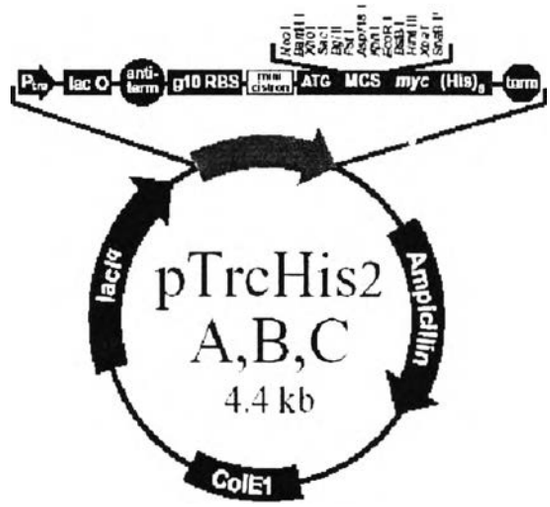




Restriction map of pGem®-3Zf (+) vector



Restriction map of pTrcHis2c



pTrcHis forward priming site

RBS

Mini cistron

RBS

Nco I

```
361  AAAATTAAAG AGGTATATAT TA ATG TAT CGA TTA AAT AAG GAG GAA TAA ACC
                              Met Tyr Arg Leu Asn Lys Glu Glu ***
```

BamH I *Xho* I *Sac* I *Bgl* II *Pst* I *Asp*718 I *Kpn* I *Eco*R I *Bst*B I *Hind* III

```
413  ATG GATCCGAGCT CGAGATCTGC AGCTGGTACC ATATGGGAAT TCGAAGCTTG GGCCC
     Met
```

myc epitope tag

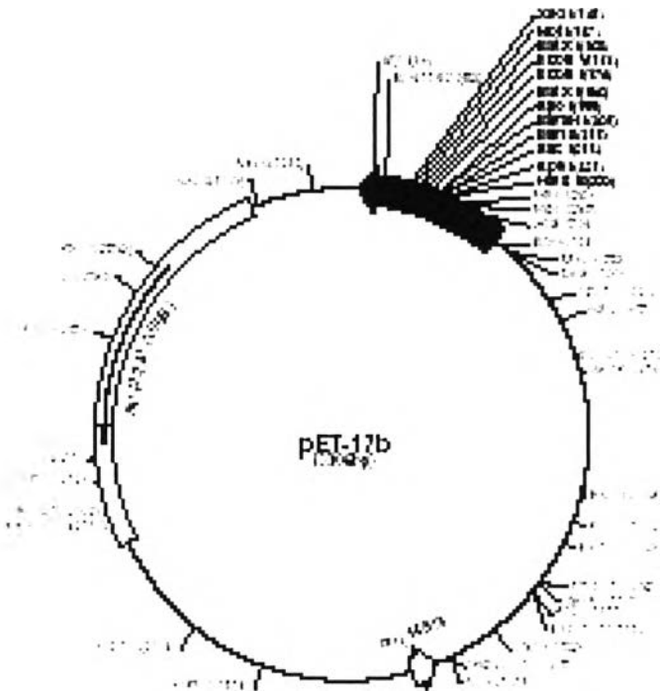
Sal I

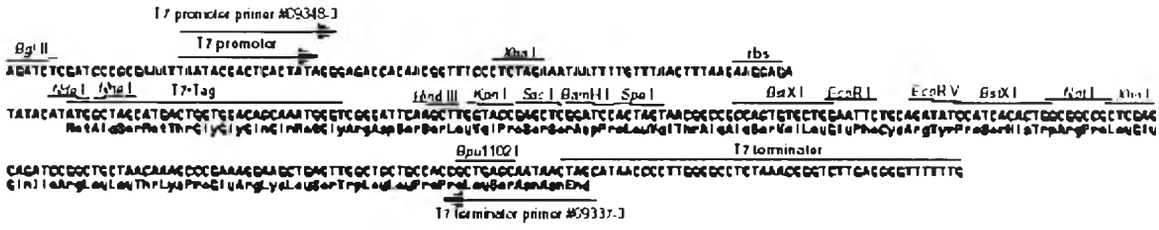
```
461  GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT
     Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His
```

ProBond™ binding domain

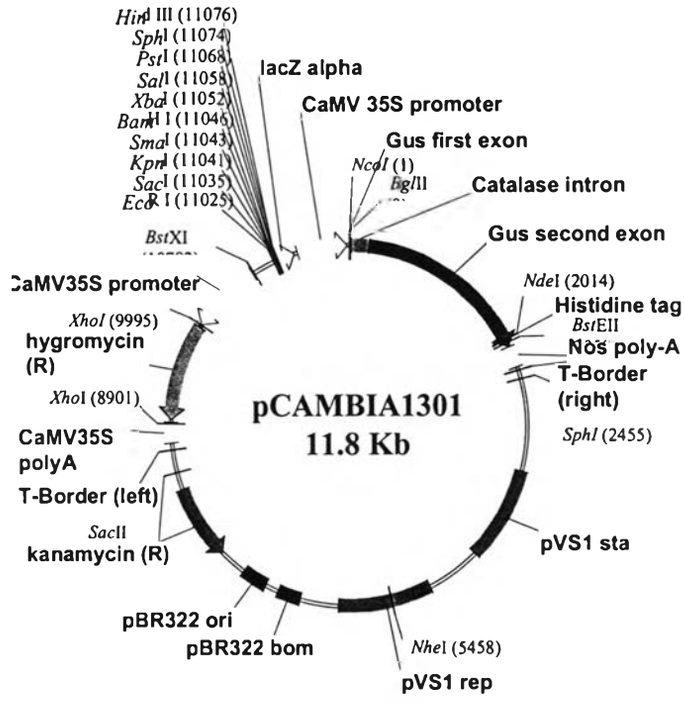
```
510  CAT CAT CAT CAT CAT TGA GTTTA
     His His His His His ***
```

Restriction map of pET17b

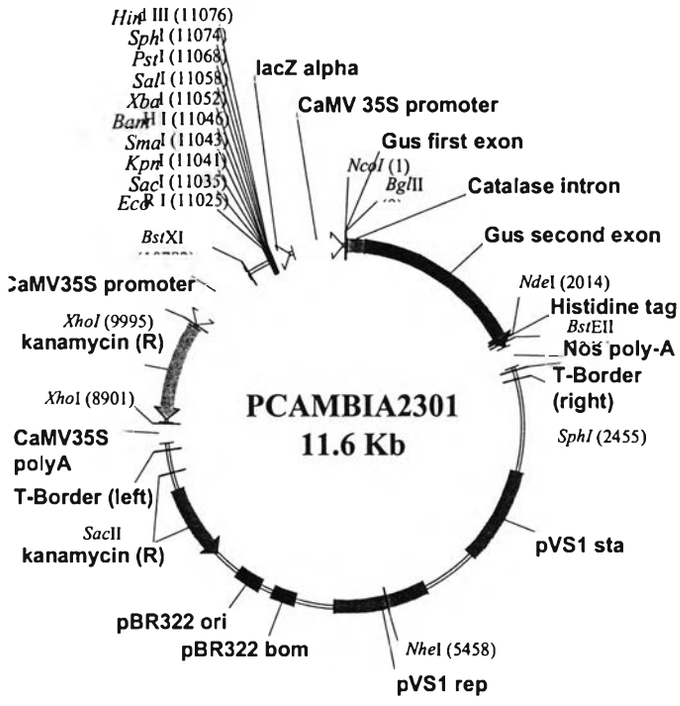




Restriction map of pCAMBIA1301



Restriction map of pCAMBIA2301



APPENDIX B

Table 1 Characterization of transformed plants transformed with pCAMBIA2301-GBSS-AcMRJP1-Nos

Line	Km^R	GUS	AcMRJP gene	AcMRJP mRNA	AcMRJP protein
2301G-A1	+	+	+	ND	ND
2301G-B2	+	+	+	ND	ND
2301G-C3	+	-	+	ND	ND
2301G-D4	+	+	+	+	ND
2301G-E5	+	-	-	-	ND
2301G-H6	+	+	+	+	+
2301G-I7	+	+	+	+	ND
2301G-J8	+	+	+	+	ND
2301G-N9	+	+	+	+	ND

Table 2 Characterization of transformed plants transformed with pCAMBIA2301-B33-AcMRJP1-Nos

Line	Km^R	GUS	AcMRJP gene	AcMRJP mRNA	AcMRJP protein
2301B-A1	+	-	-	ND	ND
2301B-B2	+	-	-	ND	ND
2301B-E3	+	+	+	ND	ND
2301B-F4	+	+	+	ND	ND
2301B-G5	+	+	+	+	ND
2301B-H6	+	+	+	+	+
2301B-I7	+	+	+	+	ND
2301B-L18	+	-	+	+	ND
2301B-M19	+	+	+	+	ND
2301B-N10	+	+	+	+	ND

Table 3 Characterization of transformed plants transformed with pCAMBIA2301-35S-AcMRJP1-Nos

Line	Km^R	GUS	AcMRJP gene	AcMRJP mRNA	AcMRJP protein
2301C-A1	+	-	-	-	ND
2301C-B2	+	+	+	+	+
2301C-C3	+	+	-	-	ND
2301C-D4	+	+	+	+	ND
2301C-E5	+	+	+	ND	ND
2301C-F6	+	+	+	+	ND

Table 4 Characterization of transformed plants transformed with pCAMBIA1301-35S-AcMRJP1-Nos

Line	Hyg^R	GUS	AcMRJP gene	AcMRJP mRNA	AcMRJP protein
1301C-A1	+	+	+	+	ND
1301C-B2	+	+	+	+	ND
1301C-C3	+	+	+	+	+
1301C-D4	+	+	+	+	+
1301C-E5	+	+	+	+	+
1301C-F6	+	+	+	+	ND

ND: Not determined; -: Not detected; +: detected

Km^R : Transformed potato plants were regenerated on medium containing kanamycin.

Hyg^R : Transformed rice plants were regenerated on medium containing hygromycin.

GUS : A fresh leaf lamina of transformed plants was analyzed the β-glucuronidase activity using GUS histochemical staining.

AcMRJP1 : The AcMRJP1 gene integration in transformed plant was detected using PCR analysis.

AcMRJP1 mRNA : The transcription of AcMRJP1 gene in transformed plant was detected using RT-PCR.

AcMRJP1 protein : The AcMRJP1 protein in transformed plant was detected using immunoblot analysis.

APPENDIX C

The composition of rice tissue culture and *Agrobacterium tumefaciens* medium

Table 5. The composition of 2NB medium (callus induction medium) (Li et al. 1993)

Solution	Chemicals	Concentration (mg/l)
N6 Macronutrients	KNO_3	2,830
	$(NH_4)_2SO_4$	463
	$CaCl_2.2H_2O$	166
	$MgSO_4.7H_2O$	185
	KH_2PO_4	460
B5 micronutrients	KI	0.75
	H_3BO_3	3
	$CoCl_2.6H_2O$	0.025
	$MnSO_4.7H_2O$	10
	$ZnSO_4.7H_2O$	2
	$Na_2MoO_4.7H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
FeEDTA	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA.2H_2O$	37.8
B5 vitamins	Myo-inositol	100
	Nicotinic acid	1
	Pyridoxine HCl	1
	Thiamine HCl	10
	2,4-D	2
	Casein hydrolysate	300
	L-Proline	500
	L-Glutamine	500
	Sucrose	30,000
	Agar	8,000

pH 5.8

Table 6. The composition of MS medium (Murashige and Skoog, 1962)

Solution	Chemicals	Concentration (mg/l)
Macronutrients	NH_4NO_3	1,650
	KNO_3	1,900
	$CaCl_2.2H_2O$	440
	KH_2PO_4	170
	$MgSO_4.7H_2O$	370
Micronutrients	KI	0.83
	H_3BO_3	6.2
	$CoCl_2.6H_2O$	0.025
	$MnSO_4.H_2O$	16.9
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
FeEDTA	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA.2H_2O$	37.8
MS vitamins	Myo-inositol	100
	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.1
	Glycine	2
	Sucrose	30,000
	Agar	8,000

pH 5.8

Table 7. The composition of AB medium (Chilton *et al.*, 1974)

Solution	Chemicals	Concentration (mg/l)
AB buffer	K_2HPO_4	1,500
	NaH_2PO_4	200
AB salt	NH_4Cl	1000
	$MgSO_4.7H_2O$	300
	KCl	150
	$CaCl_2.2H_2O$	150
	$FeSO_4.7H_2O$	2.5
	Glucose	5,000
	Agar	15,000

Table 8. The composition of AAM medium (Toriyama and Hinata, 1985)

Solution	Chemicals	Concentration (mg/l)
AA macronutrients	$Na_2HPO_4.2H_2O$	169.6
	$MgSO_4.7H_2O$	500
	KCl	150
	$CaCl_2.2H_2O$	150
AA micronutrients	$MnSO_4.4H_2O$	10
	$Na_2MoO_4.2H_2O$	0.25
	H_3BO_3	3.0
	$ZnSO_4.7H_2O$	2.0
	$CuSO_4.5H_2O$	0.0387
	$CoCl_2.6H_2O$	0.025
	KI	0.75
AA iron	$FeSO_4.7H_2O$	28
MS vitamin	Inositol	100
	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.5
AA amino acid	Glycine	7.5
	Arginine	174
	Glutamine	876
	Casamino acid	500
	Sucrose	68,500
	Glucose	35,000

Acetosyringone 100 μM (add after autoclave)

Journal of Biochemistry and Molecular Biology, Vol. 38, No. 1, January 2005, pp 49-57

JOURNAL OF
Biochemistry
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Cloning, Expression and Genomic Organization of Genes Encoding Major Royal Jelly Protein 1 and 2 of the Honey Bee *(Apis cerana)*

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Received 31 January 2004, Accepted 24 July 2004

Major Royal Jelly Protein cDNAs of *Apis cerana* (AcMRJP) were cloned and characterized. The open reading frames (ORFs) of the AcMRJP1 and AcMRJP2 genes were 1302 and 1392 nucleotides, encoding 433 and 463 amino acid residues, respectively. The sequence divergences between AcMRJP1 and AcMRJP2 and their corresponding protein families in *A. mellifera* were 0.0618 and 0.0934 at the nucleotide level and 0.0912 and 0.1438 at the protein level, respectively. Phylogenetic analysis supports the orthologous similarity between these proteins. The deduced amino acids indicated high essential amino acid contents of AcMRJP1 and AcMRJP2 (47.5 and 44.8%, respectively). The genomic organization of both AcMRJP1 and AcMRJP2 was determined. Both the AcMRJP1 (3663 bp) and AcMRJP2 (3963 bp) genes contained six exons and five introns, where all boundaries conformed to the GT/AG rule. AcMRJP1 and AcMRJP2 cDNAs were cloned into pET17b, and both the recombinant (r) AcMRJP1 (47.9 kDa) and rAcMRJP2 (51.7 kDa) were expressed in the insoluble form. Western blot analysis and *N*-terminal sequencing of the solubilized proteins revealed successful expression of rAcMRJP1 and rAcMRJP2 *in vitro*. The yields of the purified rAcMRJP1 and rAcMRJP2 were approximately 20 and 8 mg protein per liter of the flask culture, respectively.

Keywords: *Apis cerana*, Cloning, Expression, Gene organization, Honeybee, Major royal jelly proteins

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Introduction

Royal jelly (RJ) is secreted from the hypopharyngeal and mandibular glands of 5-15 days old nurse bees and plays an important role in larval development (Lensky and Rakover, 1983; Knecht and Kaatz, 1990; Kubo *et al.*, 1996). Newly emerged bee larvae are fed RJ for 3 days. Only larvae that are developed into queen bees are continually fed throughout their lives (Schmitzova *et al.*, 1998).

The RJ of *A. mellifera* is comprised of 12.7 ± 0.8% proteins, 11.9 ± 0.7% carbohydrates, 6.1 ± 0.4% lipids and 68.3 ± 1.4% moisture contents (Takenaka and Takenaka, 1996). Major royal jelly proteins (MRJPs) represent 82-90% of the total proteins in the RJ of *A. mellifera*. Five families of MRJPs of *A. mellifera* (AmMRJP1-5), with the molecular weights from 49 to 87 kDa were identified based on the *N*-terminal sequences of the purified proteins and cDNA sequences (Klaudiny *et al.*, 1994; Schmitzova *et al.*, 1998; Albert *et al.*, 1999b; Simuth *et al.*, 2001).

Recently, Okamoto *et al.* (2003) reported that AmMRJP3 has potent antiallergic activity by the inhibition of interleukin-4 (IL-4), IL-2 and IFN-γ production. Intraperitoneal administration of AmMRJP3 inhibited the antiOVA IgE and IgG1 levels in the serum of immunized mice, indicating the clinical significance of the potent immunoregulatory effects of AmMRJP3.

The full lengths of AmMRJP3 (RJP57-1) and AmMRJP4 (RJP57-2) cDNA were isolated and characterized from a cDNA library established from heads of *A. mellifera carnica* nurse bees (Klaudiny *et al.*, 1994). Subsequently, complete sequences of abundantly expressed transcripts encoding AmMRJP1 (Judova *et al.*, 1998; Schmitzova *et al.*, 1998) and AmMRJP2 (Bilikova *et al.*, 1999) were characterized and expressed *in vitro*.

In Thailand, an alternative honey bee, *A. cerana*, is indigenously found and widely used for commercial beekeeping, primarily owing to its resistance to the bee mite

(*Varroa jacobsoni*). The composition of *A. cerana* RJ is 16.4 ± 2.5% proteins, 9.4 ± 0.6% carbohydrates, 7.4 ± 0.6% lipids and 65.3 ± 2.5% moisture content, respectively (Takenaka and Takenaka, 1996). Rearing experiments have illustrated that *A. mellifera* queens were not successfully reared with *A. cerana* RJ, and *vice versa* (Pothichot and Wongsiri, 1993). This implied possible differences of the potential compositions of the RJ from *A. mellifera* and *A. cerana*.

Takenaka and Takenaka (1996) analyzed the water soluble proteins in the RJ of *A. mellifera* and *A. cerana* by electrophoresis and fourteen of twenty one protein bands were shared between the RJ proteins of these bees. A highly aggregated protein was found in *A. cerana*, but not in *A. mellifera*. Two major protein bands (bands 10 and 11; 42.7-66.2 kDa) were only found in *A. mellifera* RJ.

Recently, Srisuparbh *et al.* (2003) constructed an expressed sequence tag (EST) library from hypopharyngeal glands of *A. cerana*. Forty-two of sixty-six sequenced ESTs were homologues of AmMRJPs (families 1, 2, 3 and 4). The ORF of AcMRJP1 deduced from 3 separate clones; pCUAC147, pCUAC171 and pCUAC322, was 1302 nucleotides encoding 433 amino acids. In addition, AcMRJP1, 2 and 3 were chromatographically purified using Q-Sepharose and Sephadex G200, and further characterized by *N*-terminal and internal peptide sequencing.

The objectives of this study were to isolate and express AcMRJP1 and AcMRJP2 cDNAs *in vitro* and to examine the organization of these genes in genomic DNA of *A. cerana*. The molecular and physiological properties (e.g. antioxidative, antiinflammary and antihypercholesterolemic activities) of recombinant (r) AcMRJP1 and rAcMRJP2 can be further characterized. In addition, the genomic structure of genes encoding AcMRJP1 and AcMRJP2 provides basic knowledge on the regulatory regions of the AcMRJP genes in *A. cerana*.

Materials and Methods

Biological specimens Nurse bees of *A. cerana* (< 10 days) from a single colony (Bangkok, central Thailand) were collected while feeding their brood. The head of each bee was dissected out, immediately placed in liquid nitrogen and stored at −80°C until needed.

Total RNA and genomic DNA extraction Total RNA was extracted from the heads of *A. cerana* using TRIzol (Invitrogen, San Diego, USA), and further treated with DNase I (Promega, Madison, USA; 2 units/μg of total RNA) at 37°C for 20 min. Genomic DNA was extracted from the thorax of each bee using a phenol-chloroform-SDS method described by Smith and Hagen (1997).

Isolation of AcMRJP1 and AcMRJP2 cDNAs Two micrograms of total RNA were reverse transcribed using an Omniscript RT Kit (Qiagen, Chatsworth, USA). Five microliters of the first strand cDNA were subjected to PCR 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 dNTP and 0.6 μM of FMJ: 5'-TAGGAATTCTAA ATGACAAGGTGGTTGTTCATG3', with the introduced initiation codon ATG and *Eco*RI site and RMJ: 5'-GGGGTACCC(T)$_{15}$A-3' with an introduced *Kpn*I site (Klaudiny *et al.*, 1994; Schmitzova *et al.*, 1998; Albert *et al.*, 1999b) and 1 U of *Pfu* DNA polymerase (Promega, USA). The amplification reaction was composed of denaturation at 92°C for 3 min, followed by 5 cycles of denaturation at 92°C for 1 min, annealing at 50°C for 1.5 min and extension at 68°C for 4 min, with an additional 35 cycles at 94°C for 1 min, 60°C for 1.5 min and 72°C for 4 min. A final extension was carried out at 72°C for 10 min.

The amplification products (1,421 bp and 1,565 bp in size) were treated with proteinase K (50 μg/ml in the presence of 0.5% SDS) at 65°C for 1 h, and purified using a Nucleospin PCR Purification Kit (MACHEREY-NAGEL, Germany), prior to digestion with *Eco*RI and *Kpn*I. DNA fragments were then purified using a QIAquick Gel Extraction Kit (Qiagen), ligated with *Eco*RI/*Kpn*I digested pUC18 and electrotransformed into *E. coli* XL1-Blue. The recombinant clones were selected by a *lac Z'* system (Maniatis *et al.*, 1982). The sizes of the inserts were verified by colony PCR using pUC1: 5'-CCGGCTCGTATGTTGTGTGGA-3' and pUC2: 5'-GTGCTGCAAGGCGATTAAGTTGG-3', as primers. The restriction cleavage sites of the inserts were examined by single and double digestion with *Ssp*I, *Bam*HI, *Eco*RI, *Cla*I and *Pvu*I.

Isolation and characterization of AcMRJP1 and AcMRJP2 genes The AcMRJP1 and AcMRJP2 genes were obtained from amplification of three overlapping regions (Fig. 1 and Table 1). Initially, genomic DNA was amplified using FMRJP and RMRJP primers under the identical conditions described for the RT-PCR. Semi-nested PCR (*n*M1F + RMRJP and *n*M2F + RMRJP) was carried out using the gel-eluted PCR product from the primary amplification. The resulting product was ligated to dephosphorylated/*Sma*I-digested pGEM-3Zf(+), electrotransformed to *E. coli* XL1-Blue and sequenced.

The second overlapping region of MRJP1 and MRJP2 were amplified using 3M1/2F (positions 660th-687th nucleotide) + 3M1R (1,364th-1,387th nt) and 3M1/2F (654th-678th nt) + 3M2R (1,425th-1,449th nt), respectively. The gel-eluted PCR product was then subjected to semi-nested PCR using 3*n*M1F (685th-710th nt) + 3M1R and 3*n*M2F (682nd-706th nt) + 3M2R, respectively. The amplification products were processed as above.

The upstream 5' region of AcMRJP1 and AcMRJP2 were amplified from genomic DNA of *A. cerena* using 5M1F (604 bp upstream region, accession number AF388203) + 5M1R (374th-395th nt of AcMRJP1) and 5M2F (859 bp upstream region, accession number AY078399) + 5M2R (352nd-378th nt of AcMRJP2). Semi-nested PCR was carried out for AcMJRP2 using the original forward primer and 5*n*M2R-1 (133rd-160th nt). The resulting products were cloned and sequenced.

DNA sequencing and data analysis Plasmid DNA was extracted from each recombinant clone and double-strand sequenced using an automated sequencer (Li-Cor, Lincoln, USA). The DNA sequences were further edited with GENETYX (Software Development Inc., Austin, USA) and blasted against data in the GenBank using Blast*N* and Blast*X* (http://www.ncbi.nlm.nih.gov). The putative cleavage

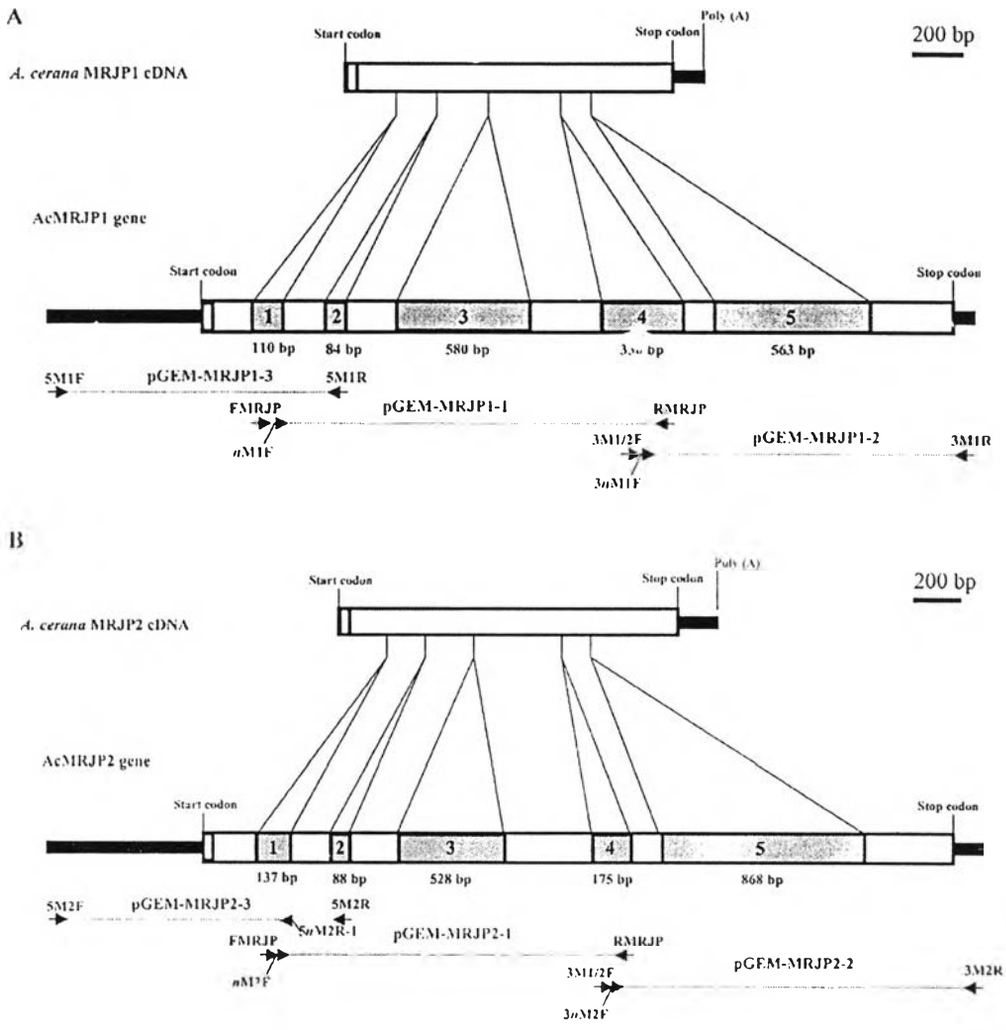

Fig. 1. Schematic diagrams of *A. cerana* MRJP1 (A) and MRJP2 (B) cDNAs and their genes. Complete cDNAs were obtained by RT-PCR, whereas genomic DNA fragments of AcMRJP1 and AcMRJP2 were obtained from the overlapping PCR amplification products. Non-coding regions are represented by solid bars. Introns (with numbers) are gray-shaded. The primers used for amplification of genomic AcMRJP1 and AcMRJP2 and their corresponding clones are illustrated.

site of the signal peptide, the TATA box and the ultraspiracle transcriptional factor (USP-TF) binding sites were predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/), Neural network Promoter Prediction, NNPP2.1 (http://www.fruitfly.org/seq-tools/promoter.html) and Genomatixsuite (http://www.genometix.de), respectively.

Multiple sequence alignments of the nucleotide and translated amino acids were performed using Clustal W (Thompson *et al.*, 1994). The aligned sequences were bootstrapped 1000 times using Seqboot. The 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 Seqboot, Neighbor and Consense. All phylogenetic reconstruction programs were routine in PHYLIP (Felsenstein, 1993). The trees were appropriately illustrated using TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod.html).

Construction of AcMRJP1 and AcMRJP2 expression vectors

Fragments coding mature AcMRJP1 and AcMRJP2 were amplified using primers; Exp1F (5'-CATGCCATGGCTAGC**CATCATCATC ATCATCAT***AGCATTCTTCGAGGAGAAATC*-3') and Exp1R (5'-CG GGGTACC*TTACAGATGTATTGAAATTTTGAAAGG*-3'), and Exp2F (5'-GAAGATCTGGCTAGC**CATCATCATCATCATCAT***GCCAT TATTCGACAAAATTC*-3') and Exp2R (5'-CGGGGTACC*TTAATTG TTAGTATTCTGATTGTTATT*-3'), respectively. A *Nhe*I site (underlined) and six His encoded nucleotides (boldface) and a *Kpn*I site (underlined) were introduced to the forward and reverse primers, respectively.

PCR was carried out as described previously. The amplification product was digested with *Nhe*I and *Kpn*I, ligated to compatible sites of pET17b (Novagen, Madison, USA) and transformed into *E. coli* XL1-Blue. Plasmid DNA was extracted from recombinant clones (pCUAcMRJP1 and pCUAcMRJP2) and subsequently

Table 1. Primers and primer sequences used for amplification of the AcMRJP1 and AcMRJP2 genes

Primer	Sequence (5'-3')
5M1F	ACATCACTATTCTCATTGCATCAGA
5M1R	TTGTCGATCGCAAGTTTTGTGG
FMRJP	TGCCTYGGYATAGYTTGTCAA
RMRJP	TCAYGGGACTRAGWGCMATTC
*n*M1F	AAACTGCAGCTAGCAATTCTTCGAGGAGAATC
3M1/2F	TGATTCYTTCCATCGAWTGACTTCC
3M1R	CGAAAACAATATTTATTTTATACATTCA
3*n*M1F	TCCAAAACTTTCGATTACGATCCTAA
5M2F	TGAGAATGAATTGCAGAATATGGTCGCT
5M2R	GAAAGCGCTCACGATTCCAGAGCAATC
5*n*M2R-1	CAGCTTGTCTTCTTTCTTCGCTACCGAA
*n*M2F	AAACTGCAGCTGCCATTATTCGACAAAATTCTGCAA
3M2R	TAATTTGGTTTATTGATTTTAATGC
3*n*M2F	ACTTTCGATTACGATCCCAGATATG

transformed into *E. coli* Rosetta (DE3)pLysS (Novagen, Madison, USA) for *in vitro* expression.

Expression and purification of rAcMRJP1 and rAcMRJP2 A single colony of recombinant *E. coli* Rosetta (DE3)pLysS carrying either pCUAcMRJP1 or pCUAcMRJP2 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 an OD_{600} of 0.6. After IPTG induction (0.4 mM final concentration), a 1 ml aliquot was taken at various time points (1, 2, 3, 4 and 5 h) and centrifuged at $10000 \times g$ for 10 min at 4°C. The pellet was resuspended in 1 × SDS gel-loading buffer and examined by SDS-PAGE (Laemmli, 1970).

For purification of rAcMRJP1 and rAcMRJP2, 100 ml aliquots of IPTG-induced culture were harvested by centrifugation. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, 1 mM PMSF, pH 7.4), sonicated and centrifuged. The soluble and insoluble fractions were analyzed by SDS-PAGE. The AcMRJPs in the insoluble fraction were purified under denaturing conditions (20 mM sodium phosphate, 500 mM NaCl, 1 mM PMSF, 8 M urea and 250 mM imidazole, pH 7.4) using HiTrap Chelating HP affinity chromatography (Amersham Biosciences, Uppsala, Sweden) and the purified proteins were stored at −20°C.

SDS-PAGE and Western blot analysis Purified rAcMRJP1 and rAcMRJP2 were analyzed in 12% SDS-PAGE. The electrophoresed proteins were transferred to a PVDF membrane (Hybond-P, Amersham Biosciences, Uppsala, Sweden; Towbin, 1979) and incubated with 5% dried skimmed milk-PBS for 1 h at room temperature. The membrane was washed twice in PBS-Tween20 and incubated with diluted Anti-His-HRP Conjugate (1 : 1,000, Penta-His, Qiagen, USA) in 5% dried skimmed milk-PBS for 1 h. The peroxidase activity was detected by adding H_2O_2 and a diaminobenzidine (DAB) chromogenic substrate.

***N*-terminal amino acid sequencing** Purified rAcMRJP1 and rAcMRJP2 were resolved in 12% SDS-PAGE and electroblotted onto a PVDF membrane. The *N*-terminal sequences of these proteins were examined using an ABI 494 protein sequencer (Applied Biosystems, USA at Department of Biological Science, National University of Singapore, Singapore).

Results and Discussion

Isolation, cloning and characterization of AcMRJP1 and AcMRJP2 cDNAs The PCR products (1,421 bp, pRT-AcMRJP1 and 1565 bp, pRT-AcMRJP2) representing the complete ORFs of AcMRJP1 (1,302 nucleotides encoding a polypeptide of 433 amino acid residues, accession number AF525776) and AcMRJP2 (1,392 nucleotides encoding a polypeptide of 463 amino acid residues, AF525777) were successfully cloned and sequenced (Fig. 2 and 3). The putative single (AATAAA) and multiple (AATAAATAAAAT AAA) polyadenylation signals were found at 14 nucleotides upstream from the poly (A) tail of AcMRJP1 and AcMRJP2 cDNAs. The latter also contained a consensus AATAAA at 73 bp upstream from the multiple polyadenylation signal sequence. The sequence and position of overlapping polyadenylation signals in AcMRJP2 were identical with those in AmMRJP3 (Klaudiny *et al.*, 1994).

The similarities of AcMRJP1 and AcMRJP2, and their homologues, in *A. mellifera* were 93 and 92% (nucleotides), and 90 and 86% (deduced amino acids), respectively. The putative cleavage site of the signal peptidase was located between S_{20}-S_{21} and G_{17}-A_{18}. Three (29^{th}, 145^{th} and 178^{th} amino acid residues) and two (145^{th} and 178^{th} amino acid residues) predicted *N*-linked glycosylation sites (NXS/T) were observed in the deduced AcMRJP1 and AcMRJP2 proteins and their homologues in *A. mellifera*. The calculated pI of AcMRJP1 and AcMRJP2 were 5.40 and 7.88, which were concordant with the pI values of 5.2-5.7 and 7.0-8.0 examined from chromatographically purified AcMRJP1 and AcMRJP2, respectively (Srisuparbh *et al.*, 2003).

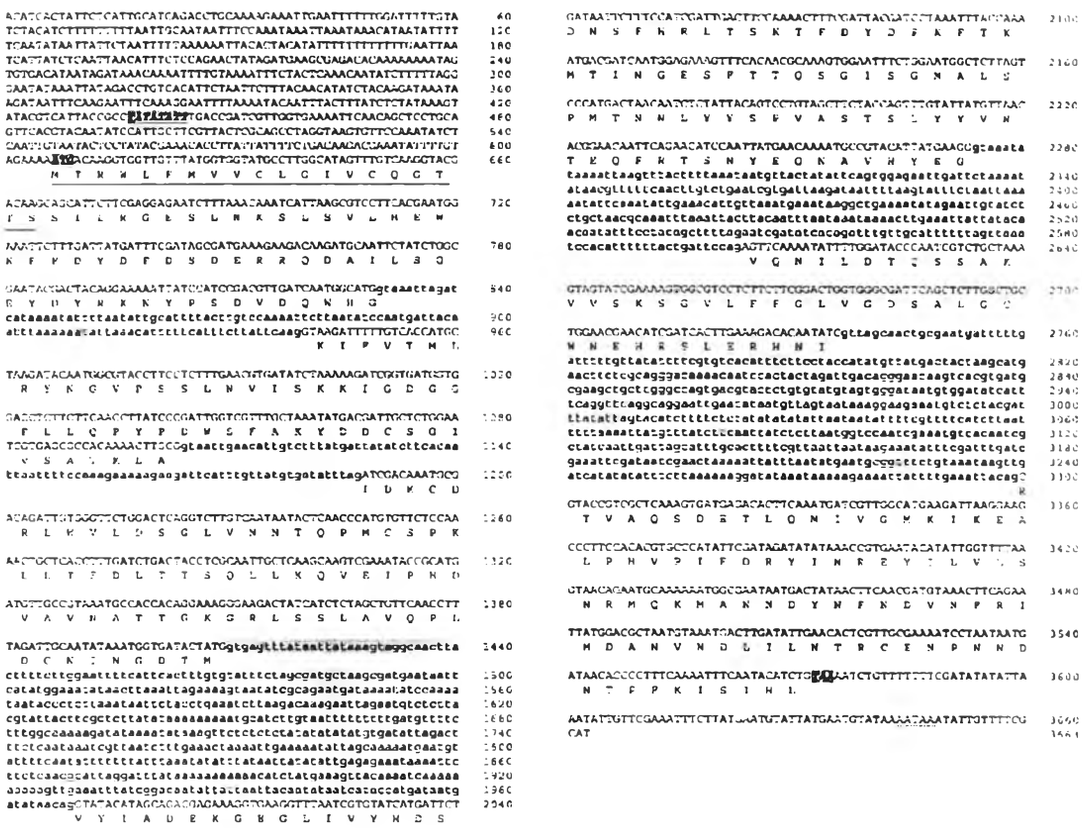

Fig. 2. Organization of the AcMRJP1 gene. Coding nucleotides and deduced amino acids of each exon are capitalized. Introns are shaded, and illustrated with lower letters. The TATA box and start and stop codons are shaded and bold-italicized. The signal peptide sequence and the poly A additional signal site are underlined.

Hanes and Simuth (1998) showed that AmMRJP1 (55 kDa) was separated, by an isoelectricfocusing technique, into eight protein bands with a pI of 4.5-5.0. Sequence comparisons showed 14 mismatches between the AcMRJP1 in the present study and that previously deposited in the GenBank (Srisuparph *et al.*, 2003). Ten of these did not cause amino acid replacement. Nevertheless, non-synonymous mutations were found from P_{106} to Q, A_{112} to S, G_{138} to V and L_{150} to M by substitutions of C_{317}, G_{334}, G_{413} and C_{448} to A, T, T and A, respectively. The internal peptide sequencing of the purified AcMRJP1 in this study supported the existence of Q_{106} and S_{112} for AcMRJP1. Nevertheless, the N_{29} of AcMRJP1 found in both studies was D, as revealed by internal peptide sequencing (Srisuparbh *et al.*, 2003), suggesting possible allelic variants of AcMRJP1. The cDNA and genomic sequences of AcMRJP2 are reported for the first time in this study. No differences were observed between either the genomic DNA sequences of AcMRJP1 and AcMRJP2 or their corresponding cDNA sequences. The essential amino acid contents of the deduced AcMRJP1 and AcMRJP2 were relatively high (48.5 and 45.4%, respectively), which were comparable to those of AmMRJP1 (48%) and AmMRJP2 (47%) (Schmitzova *et al.*, 1998).

Multiple alignments revealed four conserved cysteines typically found across different families of MRJPs (data not shown). No repeated units of amino acids were found in AcMRJP1, AmMRJP1 and AmMRJP4, but repetitive regions with different sequences and localization were found in the remaining protein families. AcMRJP2 contains six complete repeated units; NQKNN encoded by AATCAGAA(A/G)AAT AAC, at the *C*-terminus (423rd to 457th residues). Apparently, pentameric amino acid repeats with Q(N/K)(D/N/T/A/G)(N/ G/D)(I/N/K/R) sequences were found in the *C*-terminus of AcMRJP2, AmMRJP2 and AmMRJP3. In addition DRM and its variants (DRI, DRT and DTM) were found at the C terminus of AmMRJP5 (Schmitzova *et al.*, 1998).

Genes coding for AmMRJPs are present as the single copy gene per haploid genome (Malecova *et al.*, 2003). Bilikova *et al.* (1999) illustrated that the single protein band of purified AmMRJP2, as analyzed by SDS-PAGE, was composed of at least 8 different isoelectric focusing variants of pI 7.5-8.5. Schmitzova *et al.* (1998) examined the nucleotide differences of 4 and 2 isoforms of AmMRJP3 and AmMRJP5, respectively, and indicated that polymorphism of these proteins was related with the length variability of repetitive regions among individual honey bees within the colony.

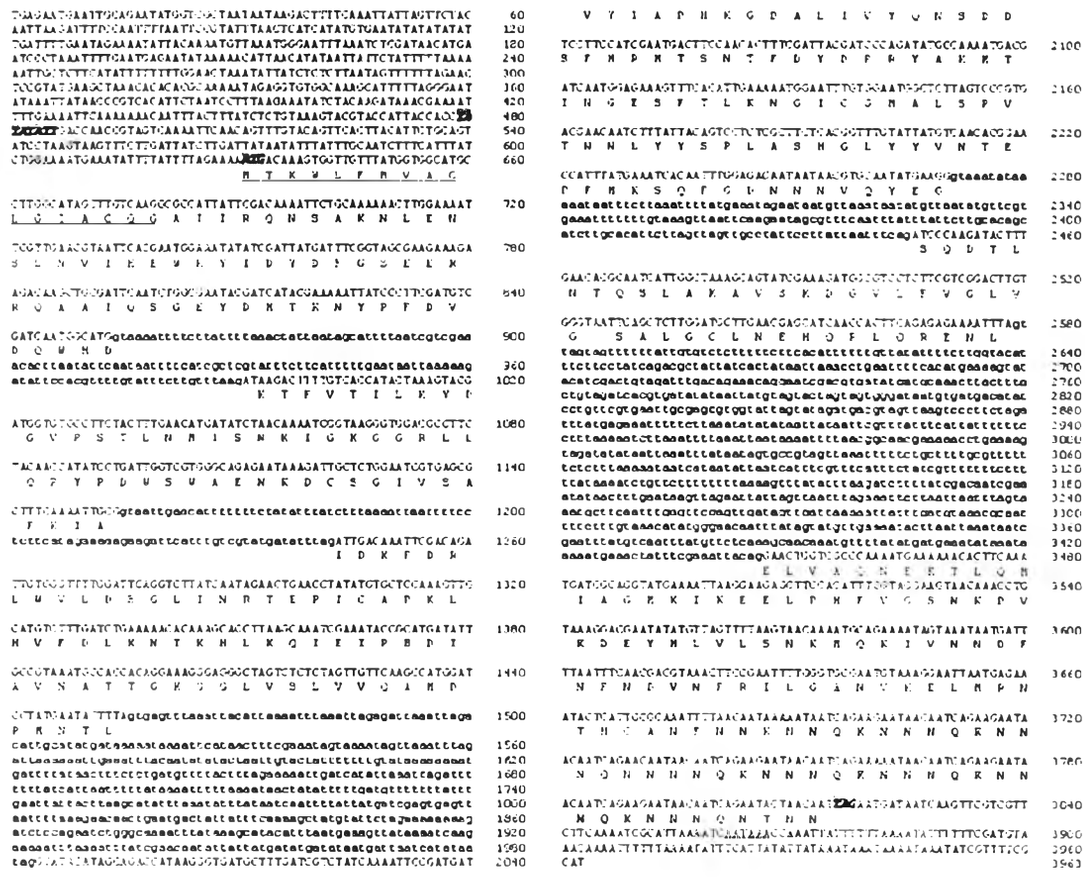

Fig. 3. Organization of the AcMRJP2 gene. Coding nucleotides and deduced amino acids of each exon are capitalized. Introns are shaded, and illustrated with lower letters. The TATA box and start and stop codons are shaded and bold-italicized. The signal peptide sequence and the poly A additional signal site are underlined. Pentameric amino acid repeats (NQKNN) are found at the *C*-terminus of this deduced protein.

Therefore, the intra- and inter-colonial variabilities of an AcMRJP2 gene in different populations of *A. cerana* in Thailand should be further examined.

Gene organization of AcMRJP1 and AcMRJP2 Complete gene sequences of AcMRJP1 and AcMRJP2 were deduced from the nucleotide sequences of the overlapping clones of each protein family. Like AmMRJP1 and AmMRJP2, both AcMRJP1 and AcMRJP2 genes are composed of 6 exons and 5 introns (3,663 bp and 3,963 bp in length; accession numbers AY515688 and AY515689; Fig. 2 and 3). The length of each exon varies from 133 bp (exon 5)-284 bp (exon 4) and 133 bp (exon 5)-372 bp (exon 6) for the AcMRJP1 and AcMRJP2 genes, respectively. The GC content reflects a slightly greater thermal stability in exons (34-42% and 28-42%) than in introns (15-29% and 16-24%) of both AcMRJP1 and AcMRJP2 genes (Table 2).

The exon/intron boundary sites determined by the corresponding cDNA sequences were consistent with the GT/AG rule. Introns 2, 3, and 5 of AcMRJP1 and AcMRJP2 interrupt the ORFs between two codons (type 0 intron), whereas the remaining introns interrupt the ORFs after the 1st or 2nd codon (type 1 intron).

Malecova *et al.* (2003) reported two ultraspiracle transcriptional factor (USP-TF, GGTCA) binding sites in AmMRJP1, but only one binding site in AmMRJP2-5 immediately downstream from the predicted TATA box. The predicted CAAT regulatory box (CCAAT) is located between 69-65 nucleotides downstream from the transcription starting point in AmMRJP1, but is absent from AmMRJP2-AmMRJ5. USP-TF is a member of the ligand-modulated transcription factors that regulate cell homeostasis, reproduction, differentiation and development (Sergaves, 1991). In *Drosophila melanogaster*, USP-TF specifically binds to active juvenile hormones (Jones and Sharp, 1997).

The putative TATA boxes of AcMRJP1 and AcMRJP2 were found at −31 and −32 nt upstream from the transcription initiation sites, respectively. The putative CAAT box was also found in AcMRJP1 (CAAAT) at an identical position to the CCAAT reported in AmMRJP1 (Malecova *et al.*, 2003), but the consensus sequence was not found in AcMRJP2. Both AcMRJP1 and AcMRJP2 contained a single USP-TF binding

Table 2. GC content and length of exons and introns in the AcMRJP1 and AcMRJP2 genes

Exon	Genomic DNA (No. of nucleotides)	GC content (%)	Intron	Genomic DNA (No. of nucleotides)	GC content (%)
AcMRJP1					
1	1-223 (223 bp)	37	1	224-333 (110 bp)	15
2	334-497 (164 bp)	40	2	498-581 (84 bp)	20
3	582-803 (222 bp)	41	3	804-1383 (580 bp)	21
4	1384-1667 (284 bp)	35	4	1668-1997 (330 bp)	20
5	1998-2130 (133 bp)	42	5	2131-2693 (563 bp)	29
6	2694-2969 (276 bp)	34			
AcMRJP2					
1	1-223 (223 bp)	37	1	224-360 (137 bp)	20
2	361-524 (164 bp)	40	2	525-612 (88 bp)	18
3	613-825 (213 bp)	39	3	826-1353 (528 bp)	16
4	1354-1640 (287 bp)	37	4	1641-1815 (175 bp)	20
5	1816-1648 (133 bp)	42	5	1949-2816 (868 bp)	24
6	2817-3188 (372 bp)	28			

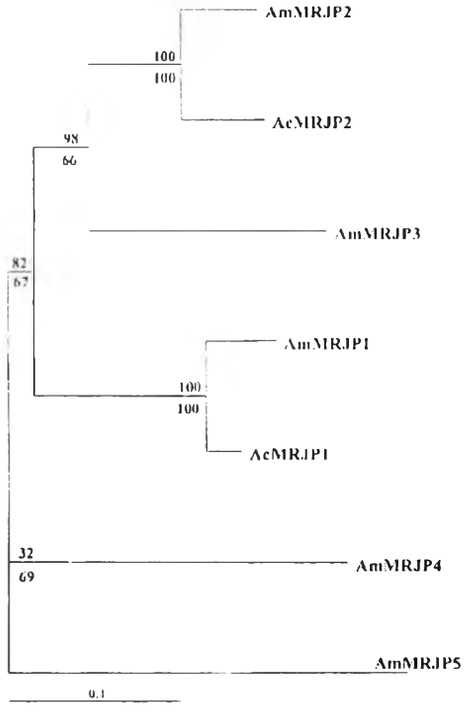

Fig. 4. A bootstrapped neighbor-joining tree illustrating the relationships between different families of AmMRJPs (1-5) and AcMRJP1 and AcMRJP2. Values at the node (nucleotides, above and deduced amino acid, below) indicate the percentage of times that the particular node occurred in 1,000 trees generated by bootstrapping the original nucleotide or deduced protein sequences.

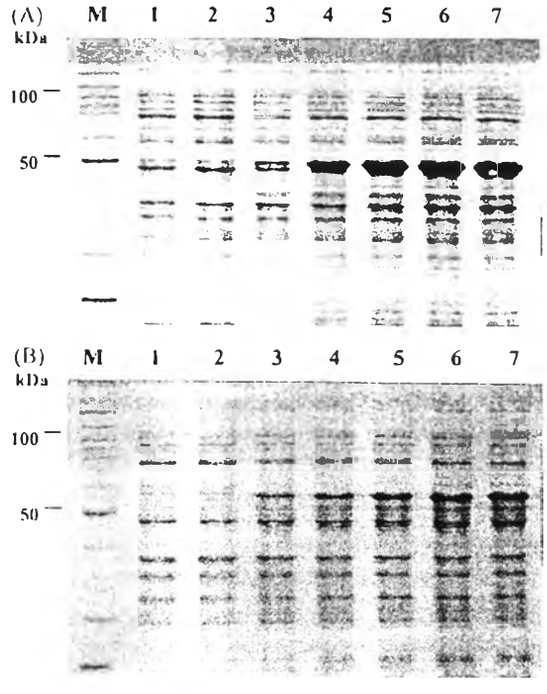

Fig. 5. SDS-PAGE analysis to examine the expressions of pCUAcMRJP1 (A) and pCUAcMRJP2 (B) under non-induced (lanes 1-2, panels A and B) and induced with 0.4 mM IPTG for 1-5 hours (lanes 3-7, A and B) in the crude extracts of *E. coli* Rosetta (DE3)pLysS. Lanes M is the protein standard ladder.

site at the 5' UTR immediately following the TATA box.

Genetic distance and phylogenetic relationships of AcMRJPs

The interspecific sequence divergences between MRJP1 and MRJP2 of *A. cerana* and *A. mellifera* were 0.0618-0.0934 and 0.0912-0.1438, whereas those between different families of MRJPs in *A. mellifera* were 0.2419 (AmMRJP2-AmMRJP3)-0.4490 (AmMRJP3-AmMRJP5) and 0.4252-0.8439 at the nucleotide and deduced protein levels, respectively.

A bootstrapped NJ tree constructed from the sequence divergence of nucleotides and deduced amino acids (Fig. 4) revealed close relationships between AcMRJP1-AmMRJP1

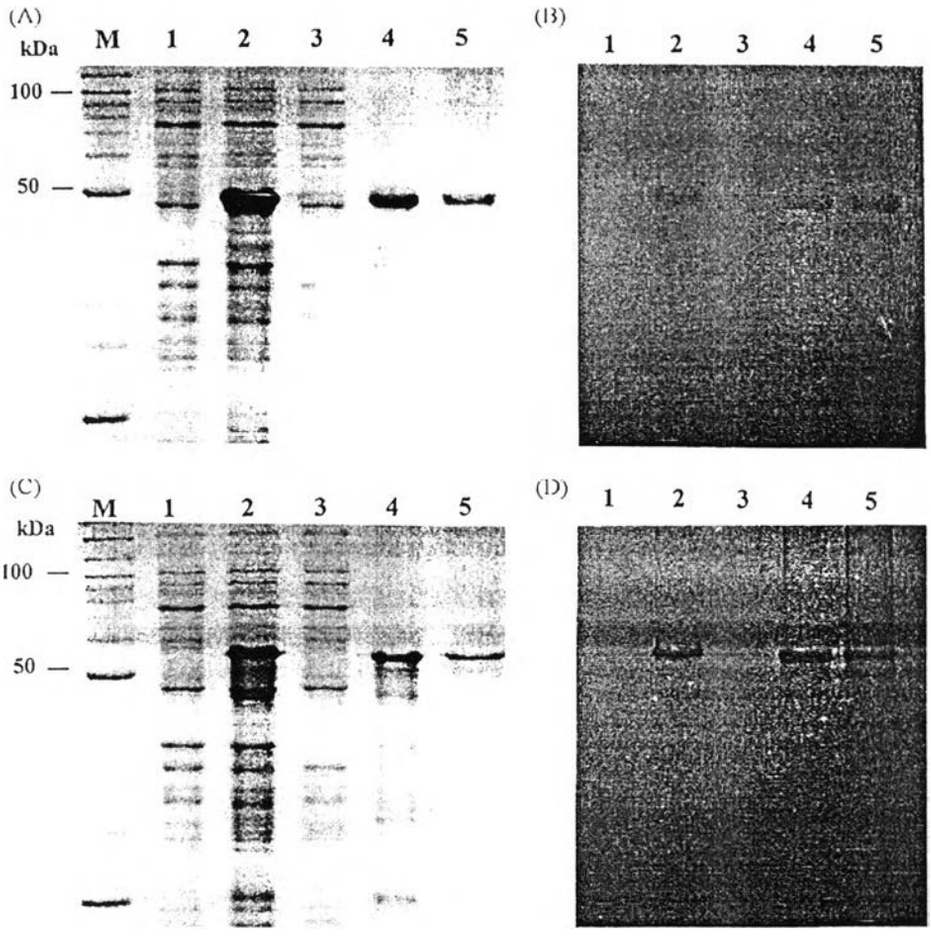

Fig. 6. SDS-PAGE and Western blot analyses to examine the expressions of rAcMRJP1 (A and B) and rAcMRJP2 (C and D) in non-induced (lanes 1, A-D) and IPTG induced (lane 2, A-D) crude extracts, soluble (lane 3, A-D) and insoluble (lanes 4, A-D) fractions and purified rAcMRJP1 (lanes 5 panels A and B) and rAcMRJP2 (lanes 5, panels C and D) of recombinant *E. coli* Rosetta (DE3)pLysS clones. Both recombinant proteins are expressed in the insoluble forms.

and AcMRJP2-AmMRJP2 from different bee species typically found in the genes born from a gene duplication process (Mitsuo *et al.*, 2001). Albert *et al.* (1999a) determined the evolutionary relationships of AmMRJP families, and reported that family variants of MRJP genes resulted from near-simultaneous gene duplication, with MRJP4 possibly being the earliest divergence within these gene families.

Expression, characterization and purification of rAcMRJP1 and rAcMRJP2 proteins Overexpressions of rAcMRJP1 and rAcMRJP2 were induced after the addition of IPTG (0.4 mM final concentration) for 1 h, and reached saturated expression levels after 4 h (Fig. 5). Both proteins were expressed as the insoluble forms and did not degrade during longer incubation periods (data not shown). The sizes of the purified rAcMRJP1 and rAcMRJP2 were 47.9 and 51.7 kDa, as determined by SDS-PAGE, and positively identified by western blot analysis (Fig. 6). *N*-terminal amino acid sequencing revealed that the ASHHHHHHSILRGESLNKSL (rAcMRJP1) and ASHHHHHHAIIRQN(S/N)(S/A)KNL (rAcMRJP2) matched those of the expected sequences, with the exception of a lack of an *N*-terminal methionine (M), which is often removed from expressed proteins in the *E. coli* expression system (Hirel *et al.*, 1989). The yields of the purified rAcMRJP1 and rAcMRJP2 from the 1 liter flask cultures were 20 and 8 mg, respectively.

Judova *et al.*, (1998) and Bilikova *et al.*, (1999) cloned AmMRJP1 and AmMRJP2 cDNAs into pQE32 and pQE30 vectors, and expressed recombinant constructs in *E. coli* M15[pREP4]. The highest productions of recombinant proteins were observed at 1 h (rAmMRJP1) and 5 h (rAmMRJP2) after IPTG induction, respectively. Both proteins were dominantly expressed in the insoluble forms. Only 0.6 mg of purified rAmMRJP1 and a lower amount of rAmMRJP2 were obtained from 1 liter cultures. Unlike rAcMRJP2, rAmMRJP2 was degraded by proteases of the host cells over prolonged culture periods.

Our results indicated the successful isolation and *in vitro*

expressions of AcMRJP1 and AcMRJP2 in the *E. coli* expression system. Relatively high amounts of recombinant proteins were obtained from small scale cultures. Larger quantity of rAcMRJP1 and rAcMRJP2 can be obtained using scaled up batch or continuous culture systems and used for further studies on the antiallergic, antioxidative and/or antitumor activities of these recombinant proteins.

Acknowledgments This work was supported by grants from the Research Affairs, Chulalongkorn University (Ratchadapiseksomphoch) and the TRF/BIOTEC special program for BRT No. R645005, which were awarded to SS. A student grant was supported by the Thai Government Fund for the Academic Staff Development Programme of the Ministry of University Affairs, the Ministry of Education and Office of Commission for Higher Education-CU Graduate Thesis Grant, which were awarded to CI.

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