CHAPTER III

RESULTS

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

3.1.1 Primer designation for isolation of AcMRJP1 and AcMRJP2

The AcMRJP1 and AcMRJP2 cDNAs were isolatated by RT-PCR using total RNA extracted from head of *A. cerana* nurse honeybee. The MRJPs were regarded as member of MRJP family resulted in high homology among this group. The AcMRJP1 and AcMRJP2 gene specific primers were designed on the basis of the conserved nucleotide and amino acid sequences of the *N*-terminal. The nucleotide and amino acid sequences of AmMRJP1-5 from *A. mellifera* were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/) and aligned together (Figure 3.1). The gene-specific oligonucleotide primers which spanned the putative first ATG codon were designed and used for amplification of its homologue in *A. cerana*.

The forward primer, designated as FMJ, was 5'-TAG*GAATTC*TAAATGACAAGGTGGTTGTTCATG-3'. The initiation codon ATG and *Eco*R I site were underlined. The sequence of reverse primer, designated as RMJ, was:5'-GGGGTACCC$(T)_{28}$A-3'. The *Kpn* I site added to the 5' end to facilitate cloning were underlined. The expected full length of AcMRJP1 and AcMRJP2 cDNAs were approximately 1,500 bp and 1,600 bp as estimated from their corresponding size of AmMRJPs cDNA.

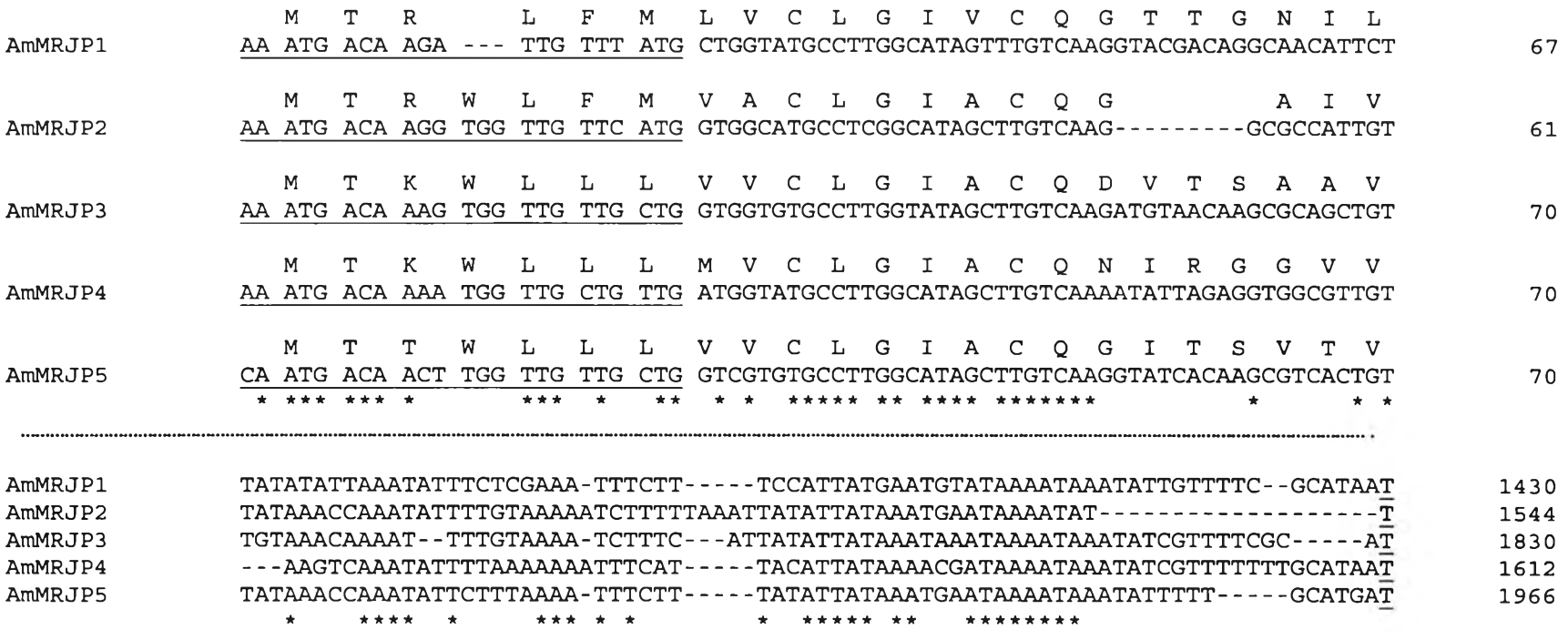

Figure 3.1 Multiple alignment of the cDNA sequence of AmMRJP1-5. The nucleotide sequences of MRJPs from *A. mellifera;* AmMRJP1 (GenBank accession number AF000633), AmMRJP2 (AF000632), AmMRJP3 (Z26318), AmMRJP4 (Z26319) and AmMRJP5 (AF004842). Conserve sequences used for designation of primers were underlined.

3.1.2 Total RNA extraction

The total RNA was extracted from head of individual nurse honeybee (*A. cerana)* using TRI REAGENT® (Molecular Research Center, Inc., USA). The concentration of total RNA was determined by measuring the optical density at 260 nm. An amount of extracted RNA was approximately 3-5 μg per honeybee individual. An OD_{260} / OD_{280} ratio in the range of 1.9 to 2.0 indicated pure prepared RNA.

An ethidium bromide stained 1.0% agarose gel of total RNA revealed predominant bands; 28S and 18S ribosomal RNAs and low-molecular-weight RNAs along with smeared RNA with molecular sizes up to approximately 8-12 kb (Figure 3.2)

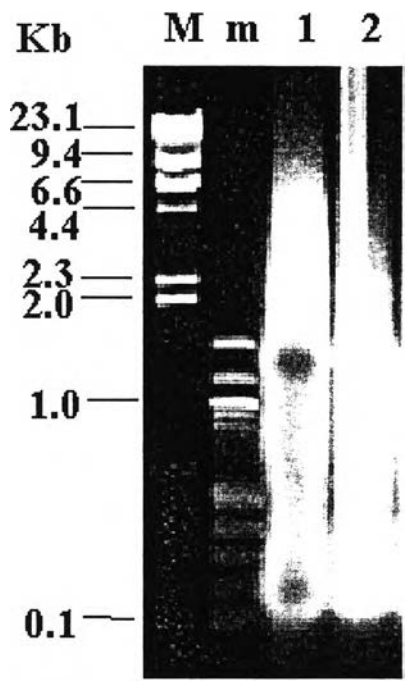

Figure 3.2 A 1.0% agarose gel electrophoresis of total RNA extracted from head of *A. cerana.*

Lane M = λ-*Hind* III DNA marker

Lane m = 100 bp DNA marker

Lane 1 = extracted total RNA from individual 1

Lane 2 = extracted total RNA from individual 2

3.1.3 RT-PCR analysis

The first strand cDNA synthesized from total RNA extracted from head of nurse honeybee was used as template in RT-PCR reaction. The expected full length of AcMRJP1 and AcMRJP2 cDNA were about 1,500 bp and 1,600 bp as estimated from their corresponding size of AmMRJP1 and AmMRJP2 cDNA, respectively. The amplification product was analyzed on 1.2% agarose gel electrophoresis.

Two bands of PCR product at expected sizes approximately 1,500 and 1,600 bp were obtained from amplification with specific primers using Ampli *Taq* DNA polymerase (Figure 3.3) and *Pfu* DNA polymerase (Figure 3.4). These PCR products were selected and purified from agarose gel by using QIAquick Gel Extraction kit.

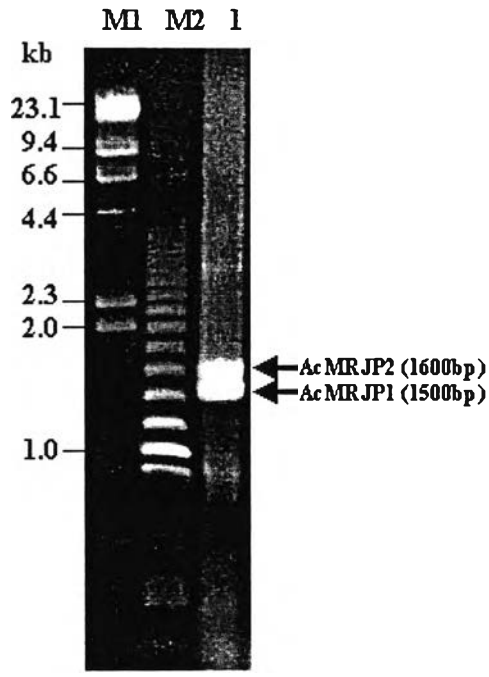

Figure 3.3 Amplification products of AcMRJP1 and AcMRJP2 cDNAs using Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA) analyzed on 1.2% agarose gel.

Lane M = λ / *Hin*d III standard marker

Lane m = 200 bp DNA ladder

Lane 1 = RT-PCR amplification product

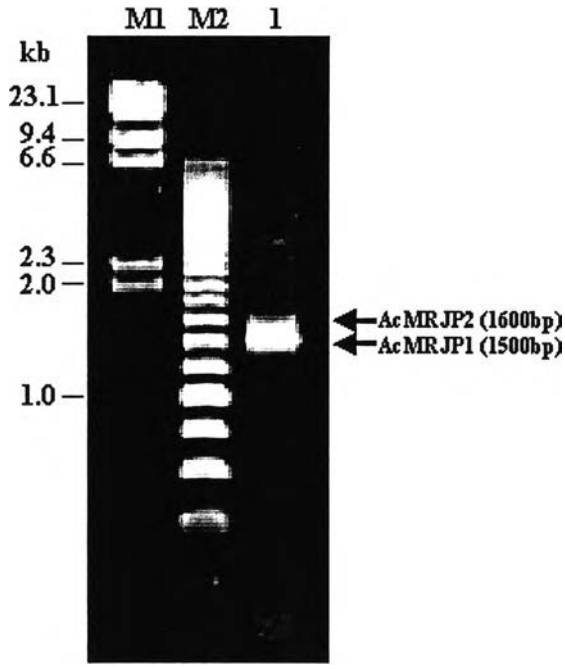

Figure 3.4 Amplification products of AcMRJP1 and AcMRJP2 cDNAs using *Pfu* DNA polymerase (Promega Corporation Medison, Wisconsin, USA) analyzed on 1.2% agarose gel

Lane M = λ / *Hin*d III standard marker

Lane m = 200 bp DNA ladder

Lane 1 = RT-PCR amplification product

3.1.4 Cloning and restriction analysis

The DNA fragments amplified from Ampli *Taq* DNA polymerase were purified from agarose gel and ligated to the pGEM®-T easy vector. The ligation products were used to transform *E. coli* XL1-Blue. The transformants were selected by ampicillin resistance and blue/white selection. Twelve of white colonies from each experiment were randomly picked for recombinant plasmid extraction. The restriction endonuclease analyses using several enzymes were exploited as a means to characterize the transformed clones. The AmMRJPs sequences were mapped by analyzed on NEBcutter V2.0 on the www Server (http://tools.neb.com/NEBcutter2/index.php). The DNA insert fragment from each recombinant clones were mapped by digestion with restriction endonucleases on the basis that MRJP cDNA sequence should have the restriction map related to restriction map predicted from *A. mellifera* MRJPs cDNA. From sequence analysis, all AmMRJPs cDNA sequences contain *Ssp* I recognition sites at differnt location. In addition, the recognition sites of *Bam*H I was present in AcMRJP2 cDNA but not in AmMRJP1 cDNA. Therefore, these enzymes can be used to differentiate the family of MRJP cDNAs before being verified by their nucleotide sequence. Restriction fragment sizes of MRJP1 and MRJP2 cDNAs after digestion with *Ssp* I and *Bam*H I were shown in Table 3.1.

The 1,500 bp insert DNA fragment was digested with restriction endonuclease *Ssp* I (Figure 3.5, lane 2). Two excised fragments of 901 and 427 bp were obtained whereas the restriction enzymes *Bam*H I can not cut within the cDNA insert (Figure 3.5 lane 5). These digested products sizes were similar to those obtained from AmMRJP1 cDNA (Table 3.1). It showed that 1,500 bp cDNA insert was most likely be AcMRJP1.

The 1,600 bp insert DNA fragment was digested with restriction enzymes *Ssp* I (Figure 3.5 lane 3). The digested product size of 1,458 bp was detected. When digested with *Bam*H I the digested product sizes of 582, 304 and 679 bp were obtained (Figure 3.5, lane 6). The sizes of digested product were compared with those of AmMRJP2 cDNA (Table 3.1). The result showed similar digested product with the addition of one recognition site of *Bam*H I in AcMRJP2 cDNA sequence. Therefore, the 1,600 bp cDNA insert might be AcMRJP2. These recombinant plasmids

designated as pRT-AcMRJP1-Taq and pRT-AcMRJP2-Taq were subjected to nucleotide sequencing.

The DNA fragments amplified from *Pfu* DNA polymerase (Promega Corporation Medison, Wisconsin, USA) were digested with *Eco*R I and *Kpn* I and cloned into corresponding site of pUC18 vector. The ligation products were used to transform *E. coli* XL1-Blue. The transformants were selected by ampicillin resistance and blue/white selection. Six white colonies of each transformant were randomly selected and screened by colony PCR approach. All recombinant clones were found to carry approximately 1,500 or 1,600 bp DNA fragment insert (Figure 3.6). The amplification product from colony PCR was singly digested with restriction enzymes (*Ssp* I, *Bam*H I). The fragment sizes of restricted products were compared with those of AmMRJP. Then the recombinant plasmids were designated as pRT-AcMRJP1-Pfu and pRT-AcMRJP2-Pfu according to the restriction digested results. Furhter confirmation of those two recombinant plasmids was performed by DNA sequencing.

Table 3.1 Restriction fragments of AmMRJP1-AmMRJP2 and AcMRJP1-AcMRJP2 cDNA digested with restriction enzyme *Ssp* I (AAT/ATT) and *Bam*H I (GG/ATCC)

Family	Length (bp)	Digested fragment size (bp)		Reference
		Ssp I	*Bam*H I	
AmMRJP1	1444	898, 427, 42, 77	1444	Schmitzova *et al.*, 1998
AcMRJP1	**1421**	**901, 427, 47, 46**	**1421**	This study
AmMRJP2	1579	1424, 22, 44, 89	883, 696	Schmitzova *et al.*, 1998
AcMRJP2	**1565**	**1458, 32, 75**	**582, 304, 679**	This study

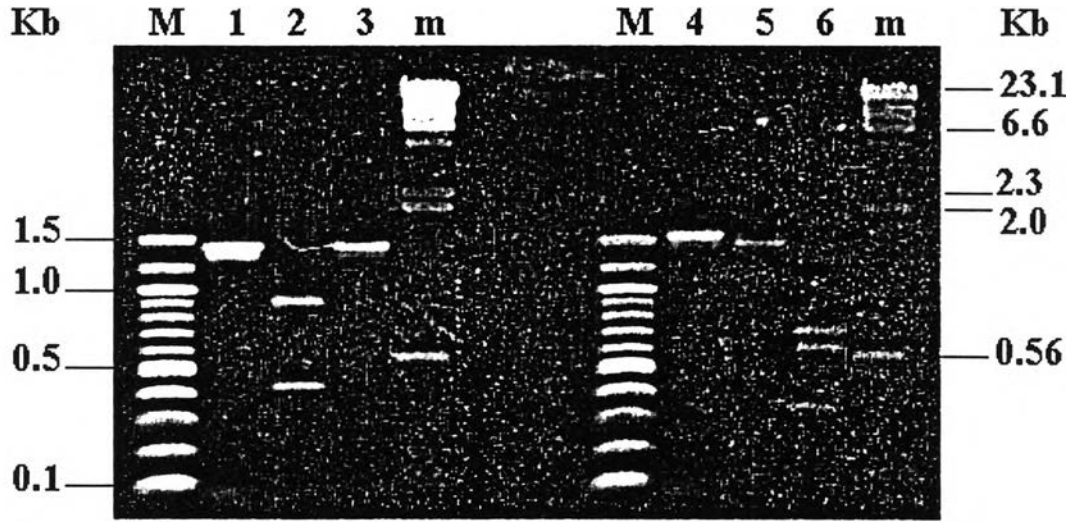

Figure 3.5 Restriction analysis of recombinant plasmid pRT-AcMRJP1 and pRT-AcMRJP2 analyzed on 1.2% agarose gel

Lane M = 100 bp DNA ladder

Lane 1 = Gel-eluted 1,500 bp DNA fragment insert

Lane 2 = Gel-eluted 1,500 bp DNA fragment insert digested with *Ssp* I

Lane 3 = Gel-eluted 1,600 bp DNA fragment insert digested with *Ssp* I

Lane 4 = Gel-eluted 1,600 bp DNA insert fragment

Lane 5 = Gel-eluted 1,500 bp DNA fragment insert digested with *Bam*H I

Lane 6 = Gel-eluted 1,600 bp DNA fragment insert digested with *Bam*H I

Lane m = λ / *Hin*d III standard marker

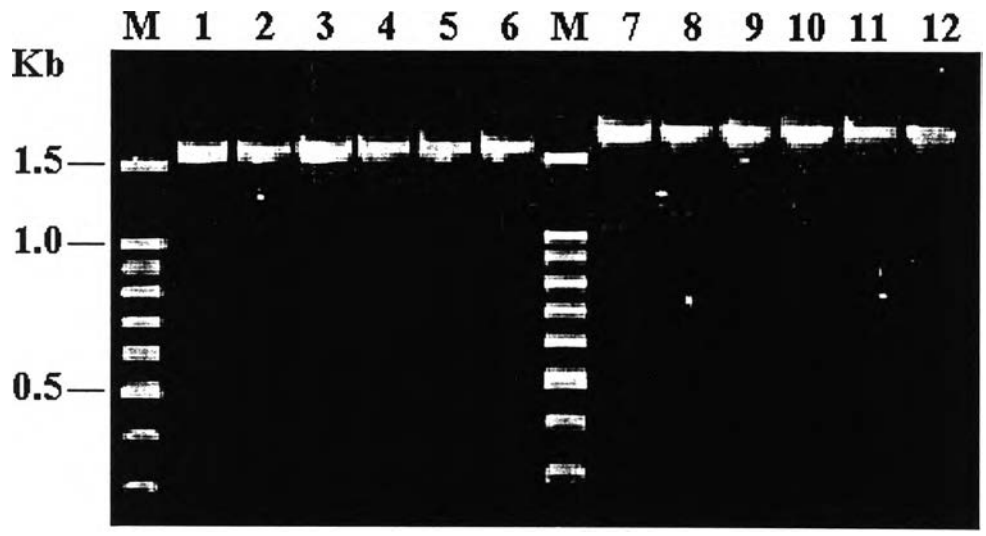

Figure 3.6 Colony PCR analysis analyzed on 1.2% agarose gel

Lane M = 100 bp DNA ladder

Lane 1-6 = 1,500 bp insert

Lane 7-12 = 1,600 bp insert

3.1.5 Characterization of cDNA sequences of AcMRJP1

The recombinant plasmids containing 1,500 bp cDNA fragment, pRT-AcMRJP1-Taq and pRT-AcMRJP1-Pfu which were expected to be AcMRJP1 cDNA from *Ssp* I and *Bam*H I digestion analysis, was initially sequenced using M13 forward and M13 reverse primers. Internal sequencing primers (1F750 and 1R750) of this gene were then designed and used for sequencing along the entire length. The nucleotide sequences derived from both strands were assembled and blasted against data in the GenBank using Blast*N* and Blast*X* (http://www.ncbi.nlm.nih.gov).

The nucleotide sequences of pRT-AcMRJP1-Taq and pRT-AcMRJP1-Pfu showed an insert of 1,420 bp and 1,421 bp, respectively. The sequence comparisons showed a single deletion at $A_{1,015}$ in pRT-AcMRJP1-Taq. This deletion giving rise to a frameshift that introduces fifteen alternative amino acids followed by a termination codon representing the open reading frame (ORF) of 1,062 nucleotides encoding 353 amino acid residues.

In pRT-AcMRJP1-Pfu, nucleotide sequences obtained revealed an insert of 1,421 bp representing the complete open reading frame (ORF) of 1,302 nucleotides encoding 433 amino acid residues (GenBank accession number AF525776). The putative polyadenylation signal, AATAAA, is present 14 nucleotide upstream of the poly (A) tail (Figure 3.7). The nucleotide and deduced amino acid sequence comparison showed significant similarity of this sequence to the homologues *A. mellifera* MRJP1 (designated as AmMRJP1). Sequence analysis showed 93% nucleotide identity and 90% deduced amino acid similarity to AmMRJP1. The alignment of nucleotide sequences and deduced amino acid residues of MRJP1 was shown in Figure 3.8 and 3.9. This sequence was most likely to be AcMRJP1 cDNA. The sequence analysis of deduced AcMRJP1 revealed putative cleavage site of signal peptidase located between S_{20} - S_{21}. The deduced amino acid (without putative signal peptide) composition of AcMRJP1 contained 43.3% hydrophobic, 28.1% neutral and 28.6% hydrophilic amino acid residues. The essential amino acid content was 47.4%. The estimated molecular weight was 46.7 kDa. The pI-value of AcMRJP1 was estimated to be 5.3. Three putative *N*-link glycosylation sites (NXS/T) were predicted at 29th, 145th and 178th amino acid residues using GENETYX (Software Development Inc.) (Figure 3.7).

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ATGACAAGGTGGTTGTTCATGGTGGTATGCCTTGGCATAGTTTGTCAAGGTACGACAAGC     60
M  T  R  W  L  F  M  V  V  C  L  G  I  V  C  Q  G  T  T  S

AGCATTCTTCGAGGAGAATCTTTAAACAAATCATTAAGCGTCCTTCACGAATGGAAATTC    120
S  I  L  R  G  E  S  L  N  K  S  L  S  V  L  H  E  W  K  F

TTTGATTATGATTTCGATAGCGATGAAAGAAGACAAGATGCAATTCTATCTGGCGAATAC    180
F  D  Y  D  F  D  S  D  E  R  R  Q  D  A  I  L  S  G  E  Y

GACTACAGGAAAAATTATCCATCCGACGTTGATCAATGGCATGGTAAGATTTTTGTCACC    240
D  Y  R  K  N  Y  P  S  D  V  D  Q  W  H  G  K  I  F  V  T

ATGCTAAGATACAATGGCGTACCTTCCTCTTTGAACGTGATATCTAAAAAGATCGGTGAT    300
M  L  R  Y  N  G  V  P  S  S  L  N  V  I  S  K  K  I  G  D

GGTGGACCTCTTCTTCAACCTTATCCCGATTGGTCGTTTGCTAAATATGACGATTGCTCT    360
G  G  P  L  L  Q  P  Y  P  D  W  S  F  A  K  Y  D  D  C  S

GGAATCGTGAGCGCCACAAAACTTGCGATCGACAAATGCGACAGATTGTGGGTTCTGGAC    420
G  I  V  S  A  T  K  L  A  I  D  K  C  D  R  L  W  V  L  D

TCAGGTCTTGTCAATAATACTCAACCCATGTGTTCTCCAAAACTGCTCACCTTTGATCTG    480
S  G  L  V  N  N  T  Q  P  M  C  S  P  K  L  L  T  F  D  L

ACTACCTCGCAATTGCTCAAGCAAGTCGAAATACCGCATGATGTTGCCGTAAATGCCACC    540
T  T  S  Q  L  L  K  Q  V  E  I  P  H  D  V  A  V  N  A  T

ACAGGAAAGGGAAGACTATCATCTCTAGCTGTTCAACCTTTAGATTGCAATATAAATGGT    600
T  G  K  G  R  L  S  S  L  A  V  Q  P  L  D  C  N  I  N  G

GATACTATGGTATACATAGCAGACGAGAAAGGTGAAGGTTTAATCGTGTATCATGATTCT    660
D  T  M  V  Y  I  A  D  E  K  G  E  G  L  I  V  Y  H  D  S

GATAATTCTTTCCATCGATTGACTTCCAAAACTTTCGATTACGATCCTAAATTTACCAAA    720
D  N  S  F  H  R  L  T  S  K  T  F  D  Y  D  P  K  F  T  K

ATGACGATCAATGGAGAAAGTTTCACAACGCAAAGTGGAATTTCTGGAATGGCTCTTAGT    780
M  T  I  N  G  E  S  F  T  T  Q  S  G  I  S  G  M  A  L  S

CCCATGACTAACAATCTCTATTACAGTCCTGTAGCTTCTACCAGTTTGTATTATGTTAAC    840
P  M  T  N  N  L  Y  Y  S  P  V  A  S  T  S  L  Y  Y  V  N

ACGGAACAATTCAGAACATCCAATTATGAACAAAATGCCGTACATTATGAAGGAGTTCAA    900
T  E  Q  F  R  T  S  N  Y  E  Q  N  A  V  H  Y  E  G  V  Q

AATATTTTGGATACCCAATCGTCTGCTAAAGTAGTATCGAAAAGTGGCGTCCTCTTCTTC    960
N  I  L  D  T  Q  S  S  A  K  V  V  S  K  S  G  V  L  F  F

GGACTGGTGGGCGATTCAGCTCTTGGCTGCTGGAACGAACATCGATCACTTGAAAGACAC   1020
G  L  V  G  D  S  A  L  G  C  W  N  E  H  R  S  L  E  R  H
```

Figure 3.7

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ATATCCGTACCGTCGCTCAAAGTGATGAGACACTTCAAATGATCGTTGGCATGAAGATT  1080
N  I  R  T  V  A  Q  S  D  E  T  L  Q  M  I  V  G  M  K  I

AAGGAAGCCCTTCCACACGTGCCCATATTCGATAGATATATAAACCGTGAATACATATTG  1140
K  E  A  L  P  H  V  P  I  F  D  R  Y  I  N  R  E  Y  I  L

GTTTTAAGTAACAGAATGCAAAAAATGGCGAATAATGACTATAACTTCAACGATGTAAAC  1200
V  L  S  N  R  M  Q  K  M  A  N  N  D  Y  N  F  N  D  V  N
TTCAGAATTATGGACGCTAATGTAAATGACTTGATATTGAACACTCGTTGCGAAAATCCT  1260
F  R  I  M  D  A  N  V  N  D  L  I  L  N  T  R  C  E  N  P

AATAATGATAACACCCCTTTCAAAATTTCAATACATCTGTAAAATCTGTTTTTTTCGATA  1320
N  N  D  N  T  P  F  K  I  S  I  H  L  *

TATATTAAATATTGTTCGAAATTTCTTATGAATGTATTATGAATGTATAAAATAAATATT  1380

GTTTTCGCATAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1421
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Figure 3.7 Nucleotide and deduced amino acid sequences of AcMRJP1. Initiation and termination of translational codons and putative polyadenylation signal are boldfaced. The signal peptide sequence is underlined. *N*-linked glycosylation sites are boxed.

```
AmMRJP1         ATGACAAGAT---TGTTTATGCTGGTATGCCTTGGCATAGTTTGTCAAGGTACGACAGGC 57
AcMRJP1         ATGACAAGGTGGTTGTTCATGGTGGTATGCCTTGGCATAGTTTGTCAAGGTACGACAAGC 60
                ******** *   **** *** ************************************ **

AmMRJP1         AACATTCTTCGAGGAGAGTCTTTAAACAAATCATTACCCATCCTTCACGAATGGAAATTC 117
AcMRJP1         AGCATTCTTCGAGGAGAATCTTTAAACAAATCATTAAGCGTCCTTCACGAATGGAAATTC 120
                * *************** ****************  * ********************

AmMRJP1         TTTGATTATGATTTCGGTAGCGATGAAAGAAGACAAGATGCAATTCTATCTGGCGAATAC 177
AcMRJP1         TTTGATTATGATTTCGATAGCGATGAAAGAAGACAAGATGCAATTCTATCTGGCGAATAC 180
                *************** ********************************************

AmMRJP1         GACTACAAGAATAATTATCCATCCGACATTGACCAATGGCATGATAAGATTTTTGTCACC 237
AcMRJP1         GACTACAGGAAAAATTATCCATCCGACGTTGATCAATGGCATGGTAAGATTTTTGTCACC 240
                ******* *** *************** **** ********** ***************

AmMRJP1         ATGCTGAGATACAATGGCGTACCTTCCTCTTTGAACGTGATATCTAAAAAGGTCGGTGAT 297
AcMRJP1         ATGCTAAGATACAATGGCGTACCTTCCTCTTTGAACGTGATATCTAAAAAGATCGGTGAT 300
                ***** ********************************************** ********

AmMRJP1         GGTGGTCCTCTTCTACAACCTTATCCCGATTGGTCGTTTGCTAAATATGACGATTGCTCT 357
AcMRJP1         GGTGGACCTCTTCTTCAACCTTATCCCGATTGGTCGTTTGCTAAATATGACGATTGCTCT 360
                ***** ******** **********************************************

AmMRJP1         GGAATCGTGAGCGCCTCAAAACTTGCGATCGACAAATGCGACAGATTGTGGGTTCTGGAC 417
AcMRJP1         GGAATCGTGAGCGCCACAAAACTTGCGATCGACAAATGCGACAGATTGTGGGTTCTGGAC 420
                *************** **********************************************

AmMRJP1         TCAGGTCTTGTCAATAATACTCAACCCATGTGTTCTCCAAAACTGCTCACCTTTGATCTG 477
AcMRJP1         TCAGGTCTTGTCAATAATACTCAACCCATGTGTTCTCCAAAACTGCTCACCTTTGATCTG 480
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Figure 3.8

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AmMRJP1         ACTACCTCGCAATTGCTCAAGCAAGTTGAAATACCACATGATGTTGCCGTAAATGCCACT 537
AcMRJP1         ACTACCTCGCAATTGCTCAAGCAAGTCGAAATACCGCATGATGTTGCCGTAAATGCCACC 540
                ************************** ******** ************************

AmMRJP1         ACAGGAAAGGGAAGATTATCATCTCTAGCTGTTCAATCTTTAGATTGCAATACAAATAGC 597
AcMRJP1         ACAGGAAAGGGAAGACTATCATCTCTAGCTGTTCAACCTTTAGATTGCAATATAAATGGT 600
                *************** ******************** *************** **** *

AmMRJP1         GATACTATGGTGTACATAGCAGACGAGAAAGGTGAAGGTTTAATCGTGTATCATAATTCT 657
AcMRJP1         GATACTATGGTATACATAGCAGACGAGAAAGGTGAAGGTTTAATCGTGTATCATGATTCT 660
                *********** ********************************************* *****

AmMRJP1         GATGATTCCTTCCATCGATTGACTTCCAACACTTTCGATTACGATCCTAAATTTACCAAA 717
AcMRJP1         GATAATTCTTTCCATCGATTGACTTCCAAAACTTTCGATTACGATCCTAAATTTACCAAA 720
                *** **** ******************** ******************************

AmMRJP1         ATGACGATCGATGGAGAAAGTTACACAGCCCAAGATGGAATTTCTGGAATGGCTCTTAGT 777
AcMRJP1         ATGACGATCAATGGAGAAAGTTTCACAACGCAAAGTGGAATTTCTGGAATGGCTCTTAGT 780
                ********* ************ **** * ***  *************************

AmMRJP1         CCCATGACTAACAATCTCTATTACAGTCCTGTAGCTTCCACCAGTTTGTATTATGTTAAC 837
AcMRJP1         CCCATGACTAACAATCTCTATTACAGTCCTGTAGCTTCTACCAGTTTGTATTATGTTAAC 840
                ************************************ ********************

AmMRJP1         ACGGAACAATTCAGAACATCCGATTATCAACAGAATGACATACATTACGAAGGAGTCCAA 897
AcMRJP1         ACGGAACAATTCAGAACATCCAATTATGAACAAAATGCCGTACATTATGAAGGAGTTCAA 900
                ********************* ***** **** **** * ******* ******** ***

AmMRJP1         AATATTTTGGATACCCAATCGTCCGCTAAAGTAGTATCAAAGAGTGGCGTTCTCTTCTTC 957
AcMRJP1         AATATTTTGGATACCCAATCGTCTGCTAAAGTAGTATCGAAAAGTGGCGTCCTCTTCTTC 960
                ********************** ************** ** ******** *********

AmMRJP1         GGATTGGTGGGCGATTCAGCTCTTGGCTGCTGGAACGAACATCGAACACTTGAAAGACAC 1017
AcMRJP1         GGACTGGTGGGCGATTCAGCTCTTGGCTGCTGGAACGAACATCGATCACTTGAAAGACAC 1020
                *** ****************************************** **************

AmMRJP1         AATATCCGTACCGTCGCTCAAAGTGATGAGACTCTTCAAATGATCGCTAGCATGAAGATT 1077
AcMRJP1         AATATCCGTACCGTCGCTCAAAGTGATGAGACACTTCAAATGATCGTTGGCATGAAGATT 1080
                ********************************** ************* * ***********

AmMRJP1         AAGGAAGCTCTNCCACACGTGCCTATATTCGATAGGTATATAAACCGTGAATACATATTG 1137
AcMRJP1         AAGGAAGCCCTTCCACACGTGCCCATATTCGATAGATATATAAACCGTGAATACATATTG 1140
                ******** ** *********** *********** ************************

AmMRJP1         GTTTTAAGTAACAAAATGCAAAAAAATGGTGAATAATGACTTCAACTTCGACGATGTTAAC 1197
AcMRJP1         GTTTTAAGTAACAGAATGCAAAAAAATGGCGAATAATGACTATAACTTCAACGATGTAAAC 1200
                ************** ************** ***********  ****** ******* ***

AmMRJP1         TTCAGAATTATGAACGCGAATGTAAACGAATTGATATTGAACACTCGTTGCGAAAATCCC 1257
AcMRJP1         TTCAGAATTATGGACGCTAATGTAAATGACTTGATATTGAACACTCGTTGCGAAAATCCT 1260
                ************ **** ******** ** ******************************

AmMRJP1         GATAATGATCGAACACCTTTCAAAATTTCAATCCATTTGTAA 1299
AcMRJP1         AATAATGATAACACCCCTTTCAAAATTTCAATACATCTGTAA 1302
                 ********   ** ***************** *** *****
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Figure 3.8 An alignment of nuclotide sequence of AcMRJP1 (GenBank accession number AF525776) and AmMRJP1 (AF00062).

```
AcMRJP1         MTRWLFMVVCLGIVCQGTTSSILRGESLNKSLSVLHEWKFFDYDFDSDERRQDAILSGEY 60
AmMRJP1         MTR-LFMLVCLGIVCQGTTGNILRGESLNKSLPILHEWKFFDYDFGSDERRQDAILSGEY 59
                *** ***:***********..***********.:***********.**************

AcMRJP1         DYRKNYPSDVDQWHGKIFVTMLRYNGVPSSLNVISKKIGDGGPLLQPYPDWSFAKYDDCS 120
AmMRJP1         DYKNNYPSDIDQWHDKIFVTMLRYNGVPSSLNVISKKVGDGGPLLQPYPDWSFAKYDDCS 119
                **::*****:****.**********************:**********************

AcMRJP1         GIVSATKLAIDKCDRLWVLDSGLVNNTQPMCSPKLLTFDLTTSQLLKQVEIPHDVAVNAT 180
AmMRJP1         GIVSASKLAIDKCDRLWVLDSGLVNNTQPMCSPKLLTFDLTTSQLLKQVEIPHDVAVNAT 179
                *****:************************************************************

AcMRJP1         TGKGRLSSLAVQPLDCNINGDTMVYIADEKGEGLIVYHDSDNSFHRLTSKTFDYDPKFTK 240
AmMRJP1         TGKGRLSSLAVQSLDCNTNSDTMVYIADEKGEGLIVYHNSDDSFHRLTSNTFDYDPKFTK 239
                ************.**** *.******************:**:********:***********

AcMRJP1         MTINGESFTTQSGISGMALSPMTNNLYYSPVASTSLYYVNTEQFRTSNYEQNAVHYEGVQ 300
AmMRJP1         MTIDGESYTAQDGISGMALSPMTNNLYYSPVASTSLYYVNTEQFRTSDYQQNDIHYEGVQ 299
                ***:***:*:*.*************************************:*:** :*******

AcMRJP1         NILDTQSSAKVVSKSGVLFFGLVGDSALGCWNEHRSLERHNIRTVAQSDETLQMIVGMKI 360
AmMRJP1         NILDTQSSAKVVSKSGVLFFGLVGDSALGCWNEHRTLERHNIRTVAQSDETLQMIASMKI 359
                ************************************:*******************..***

AcMRJP1         KEALPHVPIFDRYINREYILVLSNRMQKMANNDYNFNDVNFRIMDANVNDLILNTRCENP 420
AmMRJP1         KEALPHVPIFDRYINREYILVLSNKMQKMVNNDFNFDDVNFRIMNANVNELILNTRCENP 419
                *************************:****.***:**:*******:****:**********

AcMRJP1         NNDNTPFKISIHL 433
AmMRJP1         DNDRTPFKISIHL 432
                :**.*********
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Figure 3.9 An alignment of deduced amino sequences of AcMRJP1 (GenBank accession number AF525776) amd AmMRJP1 (AF00062).

3.1.6 Characterization of cDNA sequences of AcMRJP2

The 1,600 bp cDNA fragment inserted in recombinant plasmid, pRT-AcMRJP2-Taq and pRT-AcMRJP2-Pfu, was expected to be AcMRJP2 cDNA as analyzed from *Ssp* I and *Bam*H I digestion. The recombinant plasmid was initially sequenced using M13 forward and M13 reverse primers. Internal sequencing primers (2F850 and 2R850) were then designed and used for sequencing along the entire length. The nucleotide sequences derived from both strands were assembled and compared with the DNA sequence deposited in GenBank database using Blast*N* and Blast*X* (http://www.ncbi.nlm.nih.gov).

The nucleotide sequence of pRT-AcMRJP1-Taq and pRT-AcMRJP1-Pfu showed an identical insert sequence of 1,565 bp insert fragment representing the complete open reading frame of 1,392 nucleotides encoding a polypeptide of 463 amino acid residues (accession number AF525777). The putative multiple (AATAAATAAAATAAA) polyadenylation signals was found at 14 nucleotides upstream from the poly (A) tail. The AcMRJP2 also contained a consensus AATAAA at 73 bp upstream from the multiple polyadenylation signal sequence. (Figure 3.10). The nucleotide and deduced amino acid sequence comparison showed significant similarity of this sequence to the homologues *A. mellifera* MRJP2 (designated as AmMRJP2). Sequence analysis revealed 92% nucleotide identity and 86% deduced amino acid similarity to AmMRJP2. The alignment of nucleotide sequences and deduced amino acid residues of MRJP2 was shown in Figure 3.11 and 3.12. This sequence was most likely to be AcMRJP2 cDNA. The sequence analysis of deduced AcMRJP2 revealed putative cleavage site of signal peptidase located between G_{17} - A_{18}. The deduced amino acid (without putative signal peptide) composition of AcMRJP2 comprised of 41.3% hydrophobic, 31.6% neutral and 27.1% hydrophilic amino acid residues. The essential amino acid content was 45%. The estimated molecular weight was 50.6 kDa. The pI-value of AcMRJP2 was estimated to be 7.7. Two putative *N*-link glycosylation sites were found at 145^{th} and 178^{th} amino acid residues (Figure 3.10). The interesting character of the AcMRJP2 protein compared to AmMRJP2 is the direct repeat region located at C-terminal (amino acid residue 423 to 457). The repeat unit consists of tandemly arranged NQKNN pentapeptide unit encoded by AATCAGAA(A/G)AATAAC, pentadecanucleotide. This motif appears

in 6 copies making this part of the sequence were nitrogen-rich domain. The nucleotide and deduced amino acid sequence of the repeat region was searched in the GenBank and the SwissProt databases. No region of significant identity was detected suggested this decapeptide motif is unique to AcMRJP2. The feature of the repeat regions is the appearance of polar uncharged, asparagine (N), glutamine (Q) and positively charge lysine (K) residue.

```
ATGACAAGGTGGTTGTTCATGGTGGCATGCCTTGGCATAGCTTGTCAAGGCGCCATTATT    60
M  T  R  W  L  F  M  V  A  C  L  G  I  A  C  Q  G  A  I  I

CGACAAAATTCTGCAAAAAACTTGGAAAATTCGTTGAACGTAATTCACGAATGGAAATAT   120
R  Q  N  S  A  K  N  L  E  N  S  L  N  V  I  H  E  W  K  Y

ATCGATTATGATTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCTGGCGAATAC   180
I  D  Y  D  F  G  S  E  E  R  R  Q  A  A  I  Q  S  G  E  Y

GATCATACGAAAAATTATCCCTTCGATGTCGATCAATGGCATGATAAGACTTTTGTCACC   240
D  H  T  K  N  Y  P  F  D  V  D  Q  W  H  D  K  T  F  V  T

ATACTAAAGTACGATGGTGTGCCTTCTACTTTGAACATGATATCTAACAAAATCGGTAAG   300
I  L  K  Y  D  G  V  P  S  T  L  N  M  I  S  N  K  I  G  K

GGTGGACGCCTTCTACAACCATATCCTGATTGGTCGTGGGCAGAGAATAAAGATTGCTCT   360
G  G  R  L  L  Q  P  Y  P  D  W  S  W  A  E  N  K  D  C  S

GGAATCGTGAGCGCTTTCAAAATTGCGATTGACAAATTCGACAGATTGTGGGTTTTGGAT   420
G  I  V  S  A  F  K  I  A  I  D  K  F  D  R  L  W  V  L  D

TCAGGTCTTATCAATAGAACTGAACCTATATGTGCTCCAAAGTTGCATGTCTTTGATCTG   480
S  G  L  I  N  R  T  E  P  I  C  A  P  K  L  H  V  F  D  L

AAAAACACAAAGCACCTTAAGCAAATCGAAATACCGCATGATATTGCCGTAAATGCCACC   540
K  N  T  K  H  L  K  Q  I  E  I  P  H  D  I  A  V  N  A  T

ACAGGAAAGGGAGGGCTAGTCTCTCTAGTTGTTCAAGCCATGGATCCTATGAATACTTTA   600
T  G  K  G  G  L  V  S  L  V  V  Q  A  M  D  P  M  N  T  L

GTATACATAGCAGACCATAAGGGTGATGCTTTGATCGTCTATCAAAATTCCGATGATTCC   660
V  Y  I  A  D  H  K  G  D  A  L  I  V  Y  Q  N  S  D  D  S

TTCCATCGAATGACTTCCAACACTTTCGATTACGATCCCAGATATGCCAAAATGACGATC   720
F  H  R  M  T  S  N  T  F  D  Y  D  P  R  Y  A  K  M  T  I

AATGGAGAAAGTTTCACATTGAAAAATGGAATTTGTGGAATGGCTCTTAGTCCCGTGACG   780
N  G  E  S  F  T  L  K  N  G  I  C  G  M  A  L  S  P  V  T

AACAATCTTTATTACAGTCCTCTCGCTTCTCACGGTTTGTATTATGTCAACACGGAACCA   840
N  N  L  Y  Y  S  P  L  A  S  H  G  L  Y  Y  V  N  T  E  P
```

Figure 3.10

```
TTTATGAAATCACAATTTGGAGACAATAATAACGTGCAATATGAAGGATCCCAAGATACT   900
F  M  K  S  Q  F  G  D  N  N  N  V  Q  Y  E  G  S  Q  D  T

TTGAACACGCAATCATTGGCTAAAGCAGTATCGAAAGATGGCGTCCTCTTCGTCGGACTT   960
L  N  T  Q  S  L  A  K  A  V  S  K  D  G  V  L  F  V  G  L

GTGGGTAATTCAGCTCTTGGATGCTTGAACGAGCATCAACCACTTCAGAGAGAAAATTTA  1020
V  G  N  S  A  L  G  C  L  N  E  H  Q  P  L  Q  R  E  N  L

GAACTGGTCGCCCAAAATGAAAAAACACTTCAAATGATCGCAGGTATGAAAATTAAGGAA  1080
E  L  V  A  Q  N  E  K  T  L  Q  M  I  A  G  M  K  I  K  E

GAGCTTCCACATTTCGTAGGAAGTAACAAACCTGTAAAGGACGAATATATGTTAGTTTTA  1140
E  L  P  H  F  V  G  S  N  K  P  V  K  D  E  Y  M  L  V  L

AGTAACAAAATGCAGAAAATAGTAAATAATGATTTTAATTTCAACGACGTAAACTTCCGA  1200
S  N  K  M  Q  K  I  V  N  N  D  F  N  F  N  D  V  N  F  R
ATTTTGGGTGCGAATGTAAAGGAATTAATGAGAAATACTCATTGCGCAAATTTTAACAAT  1260
I  L  G  A  N  V  K  E  L  M  R  N  T  H  C  A  N  F  N  N

AAAAATAATCAGAAGAATAACAATCAGAAGAATAACAATCAGAACAATAACAATCAGAAG  1320
K  N  N  Q  K  N  N  N  Q  K  N  N  N  Q  N  N  N  N  Q  K

AATAACAATCAGAAAAATAACAATCAGAAGAATAACAATCAGAAGAATAACAATCAGAAT  1380
N  N  N  Q  K  N  N  N  Q  K  N  N  N  Q  K  N  N  N  Q  N

ACTAACAATTAGAATGATAATCAAGTTCGTCGTTCTTCAAAATCGCATTAAAATCAATAA  1440
T  N  N  *

ACCAAATTATTTTTTAAAATATTTTTTCGATGTAAACAAAATTTTTTAAAATATTTCATT  1500

ATATTATAAATAAATAAAATAAATATCGTTTTCGCATAAAAAAAAAAAAAAAAAAAAAAA  1560

AAAAA 1565
```

Figure 3.10 Nucleotide and deduced amino acid sequences of AcMRJP2. Initiation and termination of translational codons and putative polyadenylation signal are boldfaced. The signal peptide was underlined. *N*-linked glycosylation sites are boxed.

```
AmMRJP2         ATGACAAGGTGGTTGTTCATGGTGGCATGCCTCGGCATAGCTTGTCAAGGCGCCATTGTT 60
AcMRJP2         ATGACAAGGTGGTTGTTCATGGTGGCATGCCTTGGCATAGCTTGTCAAGGCGCCATTATT 60
                ********************************** ************************ **

AmMRJP2         CGAGAAAATTCTCCAAGAAACTTGGAAAAATCATTGAACGTAATTCACGAATGGAAGTAT 120
AcMRJP2         CGACAAAATTCTGCAAAAAACTTGGAAAATTCGTTGAACGTAATTCACGAATGGAAATAT 120
                *** ******** *** ************ ** ************************ ***

AmMRJP2         TTTGATTATGACTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCTGGCGAATAT 180
AcMRJP2         ATCGATTATGATTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCTGGCGAATAC 180
                 * ******** ************************************************

AmMRJP2         GACCATACGAAAAATTATCCCTTCGACGTCGATCAATGGCGTGATAAGACTTTTGTCACC 240
AcMRJP2         GATCATACGAAAAATTATCCCTTCGATGTCGATCAATGGCATGATAAGACTTTTGTCACC 240
                ** ************************ ************* ********************

AmMRJP2         ATACTAAGATACGATGGTGTTCCTTCTACTTTGAACGTGATATCTGGTAAAACTGGTAAG 300
AcMRJP2         ATACTAAAGTACGATGGTGTGCCTTCTACTTTGAACATGATATCTAACAAAATCGGTAAG 300
                ******* *********** *************** ********   ****  ******

AmMRJP2         GGTGGACGACTTTTAAAACCATATCCTGATTGGTCGTTTGCAGAGTTTAAAGATTGCTCT 360
AcMRJP2         GGTGGACGCCTTCTACAACCATATCCTGATTGGTCGTGGGCAGAGAATAAAGATTGCTCT 360
                ******** *** ** ********************  ******  **************

AmMRJP2         AAAATTGTGAGCGCTTTCAAAATTGCGATTGACAAATTCGACAGATTGTGGGTTTTGGAT 420
AcMRJP2         GGAATCGTGAGCGCTTTCAAAATTGCGATTGACAAATTCGACAGATTGTGGGTTTTGGAT 420
                  *** ************************************************

AmMRJP2         TCAGGTCTTGTCAATAGAACTGTACCTGTATGTGCTCCAAAGTTGCACGTCTTTGATCTG 480
AcMRJP2         TCAGGTCTTATCAATAGAACTGAACCTATATGTGCTCCAAAGTTGCATGTCTTTGATCTG 480
                ********* ************ **** ******************* ************

AmMRJP2         AAAACCTCAAATCACCTTAAGCAAATCGAGATACCGCATGATATTGCCGTGAATGCCACC 540
AcMRJP2         AAAAACACAAAGCACCTTAAGCAAATCGAAATACCGCATGATATTGCCGTAAATGCCACC 540
                **** * **** ***************** ******************** *********

AmMRJP2         ACAGGAAAGGGAGGGCTAGTGTCTTTGGCTGTTCAAGCTATAGATCTTGCAAATACTTTA 600
AcMRJP2         ACAGGAAAGGGAGGGCTAGTCTCTCTAGTTGTTCAAGCCATGGATCCTATGAATACTTTA 600
                ******************** *** * * ********* ** **** *   *********

AmMRJP2         GTGTACATGGCAGACCATAAAGGTGATGCTTTAATCGTCTACCAAAATGCCGATGATTCC 660
AcMRJP2         GTATACATAGCAGACCATAAGGGTGATGCTTTGATCGTCTATCAAAATTCCGATGATTCC 660
                ** ***** *********** *********** ******** ****** ************

AmMRJP2         TTCCATCGATTGACTTCCAACACTTTCGACTACGATCCCAGATATGCCAAAATGACGATC 720
AcMRJP2         TTCCATCGAATGACTTCCAACACTTTCGATTACGATCCCAGATATGCCAAAATGACGATC 720
                ********* ******************* ********************************

AmMRJP2         GATGGAGAAAGTTTCACACTGAAAAATGGAATTTGTGGAATGGCTCTTAGTCCCGTGACG 780
AcMRJP2         AATGGAGAAAGTTTCACATTGAAAAATGGAATTTGTGGAATGGCTCTTAGTCCCGTGACG 780
                 ***************** ******************************************

AmMRJP2         AACAATCTTTATTACAGTCCTCTCGCTTCTCACGGTTTGTATTATGTTAACACGGCACCA 840
AcMRJP2         AACAATCTTTATTACAGTCCTCTCGCTTCTCACGGTTTGTATTATGTCAACACGGAACCA 840
                ************************************************ ******* ****

AmMRJP2         TTTATGAAATCACAATTTGGAGAAAATAA---CGTCCAATACCAAGGATCCGAAGATATT 897
AcMRJP2         TTTATGAAATCACAATTTGGAGACAATAATAACGTGCAATATGAAGGATCCCAAGATACT 900
                ************************ *****   *** *****  ******** ****** *

AmMRJP2         TTGAACACGCAATCATTGGCTAAAGCAGTATCGAAAAATGGCGTCCTCTTCGTCGGACTT 957
AcMRJP2         TTGAACACGCAATCATTGGCTAAAGCAGTATCGAAAGATGGCGTCCTCTTCGTCGGACTT 960
                ************************************ ************************

AmMRJP2         GTAGGTAATTCAGCTGTTGGCTGCTGGAACGAGCATCAATCACTTCAGAGACAAAATTTA 1017
AcMRJP2         GTGGGTAATTCAGCTCTTGGATGCTTGAACGAGCATCAACCACTTCAGAGAGAAAATTTA 1020
                ** ************ **** **** ************ *********** ********

AmMRJP2         GAAATGGTCGCTCAAAATGACAGAACACTTCAAATGATCGCAGGTATGAAAATTAAGGAA 1077
AcMRJP2         GAACTGGTCGCCCAAAATGAAAAAACACTTCAAATGATCGCAGGTATGAAAATTAAGGAA 1080
                *** ******** ******** * ************************************
```

Figure 3.11

```
AmMRJP2         GAGCTTCCACATTTCGTAGGAAGCAACAAACCTGTAAAGGATGAATATATGTTAGTTTTA 1137
AcMRJP2         GAGCTTCCACATTTCGTAGGAAGTAACAAACCTGTAAAGGACGAATATATGTTAGTTTTA 1140
                ************************ ***************** ******************

AmMRJP2         AGTAACAGAATGCAGAAAATAGTAAATGATGATTTTAATTTCGACGATGTAAACTTCCGA 1197
AcMRJP2         AGTAACAAAATGCAGAAAATAGTAAATAATGATTTTAATTTCAACGACGTAAACTTCCGA 1200
                ******* ******************* ************** **** ************

AmMRJP2         ATTTTGGGTGCAAATGTAAAGGAATTAATAAGAAATACTCATTGCGTAAAT--------- 1248
AcMRJP2         ATTTTGGGTGCGAATGTAAAGGAATTAATGAGAAATACTCATTGCGCAAATTTTAACAAT 1260
                *********** ***************** ***************** ****

AmMRJP2         ---AACAATCAGAATGATAACATTCAAAATACTAACAATCAGAATGATAACAATCAGAAG 1305
AcMRJP2         AAAAATAATCAGAAGAATAACAATCAGAAGAATAACAATCAGAACAATAACAATCAGAAG 1320
                   ** ********  ****** *** ** * *************  **************

AmMRJP2         AATAACAAGAAAAATGCTAACAATCAAAAGAATAACAATCAGAATGATAATTAA------ 1359
AcMRJP2         AATAACAATCAGAAAAATAACAATCAGAAGAATAACAATCAGAAGAATAACAATCAGAAT 1380
                ********  * **   ********* ******************  ****  *

AmMRJP2         ------------
AcMRJP2         ACTAACAATTAG 1392
```

Figure 3.11 An alignment of nucleotide sequence of AcMRJP2 (AF525777) and AmMRJP2 (AF00063).

```
AmMRJP2         MTRWLFMVACLGIACQGAIVRENSPRNLEKSLNVIHEWKYFDYDFGSEERRQAAIQSGEY 60
AcMRJP2         MTRWLFMVACLGIACQGAIIRQNSAKNLENSLNVIHEWKYIDYDFGSEERRQAAIQSGEY 60
                ********************:*:**.:***:**********:******************

AmMRJP2         DHTKNYPFDVDQWRDKTFVTILRYDGVPSTLNVISGKTGKGGRLLKPYPDWSFAEFKDCS 120
AcMRJP2         DHTKNYPFDVDQWHDKTFVTILKYDGVPSTLNMISNKIGKGGRLLQPYPDWSWAENKDCS 120
                *************:********:*********:**.* *******:******:** ****

AmMRJP2         KIVSAFKIAIDKFDRLWVLDSGLVNRTVPVCAPKLHVFDLKTSNHLKQIEIPHDIAVNAT 180
AcMRJP2         GIVSAFKIAIDKFDRLWVLDSGLINRTEPICAPKLHVFDLKNTKHLKQIEIPHDIAVNAT 180
                 ***********************:*** *:***********.::****************

AmMRJP2         TGKGGLVSLAVQAIDLANTLVYMADHKGDALIVYQNADDSFHRLTSNTFDYDPRYAKMTI 240
AcMRJP2         TGKGGLVSLVVQAMDPMNTLVYIADHKGDALIVYQNSDDSFHRMTSNTFDYDPRYAKMTI 240
                *********.***:*  *****:*************:******:****************

AmMRJP2         DGESFTLKNGICGMALSPVTNNLYYSPLASHGLYYVNTAPFMKSQFGENN-VQYQGSEDI 299
AcMRJP2         NGESFTLKNGICGMALSPVTNNLYYSPLASHGLYYVNTEPFMKSQFGDNNNVQYEGSQDT 300
                :************************************ ********:** ***:**:*

AmMRJP2         LNTQSLAKAVSKNGVLFVGLVGNSAVGCWNEHQSLQRQNLEMVAQNDRTLQMIAGMKIKE 359
AcMRJP2         LNTQSLAKAVSKDGVLFVGLVGNSALGCLNEHQPLQRENLELVAQNEKTLQMIAGMKIKE 360
                ************:************:** ****.***:***:****:;************

AmMRJP2         ELPHFVGSNKPVKDEYMLVLSNRMQKIVNDDFNFDDVNFRILGANVKELIRNTHCV---- 415
AcMRJP2         ELPHFVGSNKPVKDEYMLVLSNKMQKIVNNDFNFNDVNFRILGANVKELMRNTHCANFNN 420
                ***********************:******:****:**************:******.

AmMRJP2         -----NNNQNDNIQNTNNQNDNNQKNNKKNANNQKNNNQNDN- 452
AcMRJP2         KNNQKNNNQKNNNQNNNNQKNNNQKNNNQKNNNQKNNNQNTNN 463
                     ****:;* **.***:;*******::: ********* *
```

Figure 3.12 An alignment of deduced amino acid sequences of AcMRJP2 (AF525777) and AmMRJP2 (AF00063).

3.1.7 Genetic distance and phylogenetic relationships of AcMRJPs

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

A bootstrapped NJ tree constructed from sequence divergence of nucleotides and deduced amino acids (Figure 3.13) revealed close relationships between AcMRJP1 - AmMRJP1 and AcMRJP2 - AmMRJP2 from different bee species typically found in genes born from gene duplication process (Mitsuo *et al.*, 2001). Albert *et al.* (1999a) determined 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.

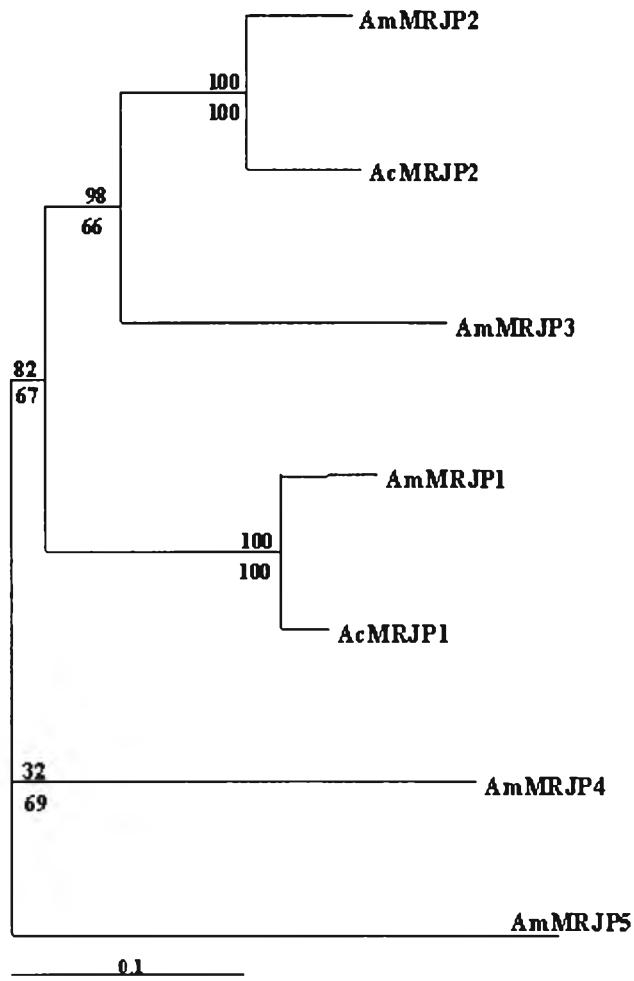

Figure 3.13 A bootstrapped neighbor-joining tree illustrating relationships between different families of AmMRJPs (1 - 5) and AcMRJP1 and AcMRJP2. Values at the node (nucleotides, above and deduced amino acid, below) indicated the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original nucleotide or deduced protein sequences.

3.2 Cloning and characterization of AcMRJP1 and AcMRJP2 genes

3.2.1 Genomic DNA extraction

Genomic DNA was extracted from thorax of each *A. cerana* individual using phenol-chloroform-SDS method. The genomic DNA was further analyzed by 0.7% agarose gel electrophoresis. A single band of a high-molecular weight genomic DNA at the size above 23.1 kb was observed (Figure 3.14). The total DNA concentration was determined by measuring the optical density at 260 nm. An OD_{260}/OD_{280} ratio in the range of 1.8 to 2.0 indicated pure prepared DNA. An amount of extracted DNA was approximately 1.5-2.0 μg per honeybee individual.

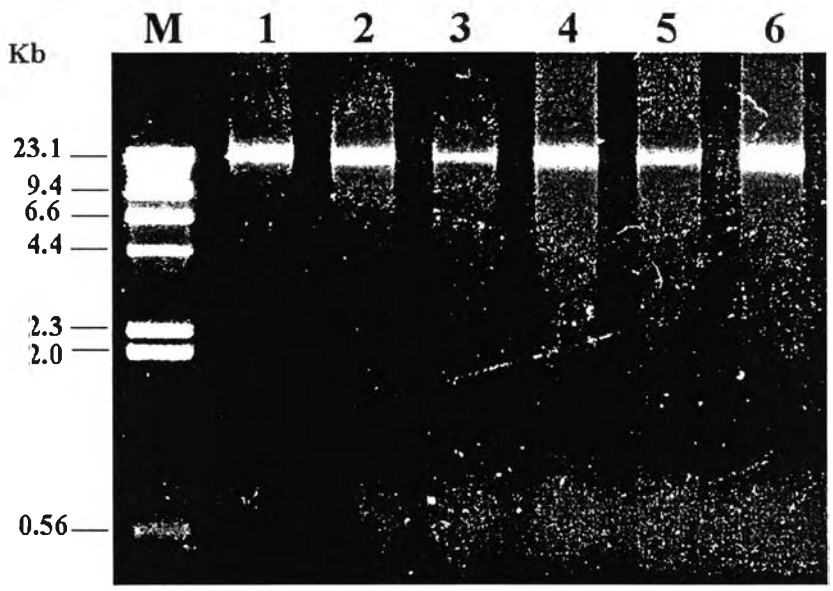

Figure 3.14 A 0.7 % ethidium bromide stained - agarose gel showing the quality of total DNA extracted from thorax of *A. cerana*

lane M = λ / *Hin*dIII

lanes 1-6 = Total DNA extracted from six individuals of *A. cerana*

3.2.2 Amplification of AcMRJP1 and AcMRJP2 genes

The genomic organization of AcMRJP1 and AcMRJP2 genes were determined by Polymerase Chain Reaction (PCR). The several of specific and degenerated primers were designed from cDNA sequences of AcMRJP1 and AcMRJP2 obtained from RT-PCR experiment. Including the cDNA of AmMRJP1 and AmMRJP2 deposited in GenBank. Gene sequences were deduced from nucleotide sequences of three overlapping regions. The genomic structures were determinined by comparison between the genomic sequences and the cDNA sequences.

3.2.2.1 Isolation of AcMRJP1 gene

Genomic DNA of *A. cerna* was initially amplified using degenerated FMRJP and RMRJP primers (Table 2.2) designed from conserved cDNA sequence at location 28^{th}-48^{th} nucleotides, 767^{th}-787^{th} nt in AcMRJP1 cDNA and 28^{th}-48^{th} nt, 758^{th}-778^{th} nt in AcMRJP2 cDNA. The PCR product was checked on 1.2% agarose gel electrophoresis. A band of PCR product with the size around 1,500 bp was purified from agarose gel. To improve specificity, the nested gene specific primer; *n*M1F (Table 2.2), was designed and subsequently employed in a second round of amplification. Semi-nested PCR (*n*M1F and RMRJP) was carried out using the gel-eluted PCR product as template. The PCR products were analyzed on 1.2% agarose gel electrophoresis. After amplification, approximately 1,500 bp of semi-nested PCR product were obtained (Figure 3.15, A). The PCR product was purified from agarose gel and phosphorelated by T4 polynucleotide kinase. The DNA fragment was ligated to dephosphorylated / *Sma* I - digested pGEM®-3Zf(+) and subsequently electrotransformed to *E. coli* XL1-Blue. Three recombinant clones were selected for sequencing in both directions by M13 forward and reverse primers using and automated DNA sequencing. Analysis of the DNA sequence of all clones revealed that they contained 1,501 nucleotide sequences corresponding to part of exon 1 to exon 4 of the AcMRJP1 gene encoding 242 amino acid residues.

In the second overlapping region, the degenerated primer 3M1/2F designed from AcMRJP1 cDNA at location 660th - 687th nt and at 654th - 678th nt of AcMRJP2 cDNA sequence together with AcMRJP1 gene-specific primer (3M1R, located at 1,364th - 1,387th nt) were used to amplified the genomic DNA. The PCR product of about 1,600 bp was obtained followed by eluting from agarose gel. Nested primer (3*n*M1F, located at 685th -710th nt of AcMRJP1 cDNA) was designed and used to amplified the gel-eluted first PCR product with the same reverse primer; 3M1R. The result showed approximately 1,600 bp of nested PCR products were obtained (Figure 3.15, B). The amplification product was eluted and subjected to further analysis by DNA cloning and sequencing. Three recombinant plasmids were subjected to sequencing. DNA sequence analysis revealed that the nucleotides sequence of the recombinant clones contained the 1,596 bp product correspoded to part of the exon 4 to exon 6 of AcMRJP1 gene encoding 205 amino acid residues.

In the third region; the upstream 5' region of AcMRJP1 gene was performed using primer designed from the 5' upstream of AmMRJP1 which deposited in GenBank (AF388203). The 5M1F primer designed from 604 bp upstream region of AmMRJP1 and the gene-specific primer; 5M1R designe was based on the known nucleotide sequence of the AcMRJP1 gene previously obtained from the first amplification. These primers were used to amplify the genomic DNA. After amplification, a band of approximately 1,200 bp of PCR product (Figure 3.15, C) was purified from agarose gel and subjected to clone. The colony was analyzed by colony PCR amplification. Three clones were identified to have the expected insert fragment. The recombinant plasmid was chosen for further analysis by DNA sequencing. Nucleotide sequence analysis revealed the inserted fragment of 1,196 bp corresponded to 5' upstream region, exon 1 and exon 2 encoding for 129 amino acid residues.

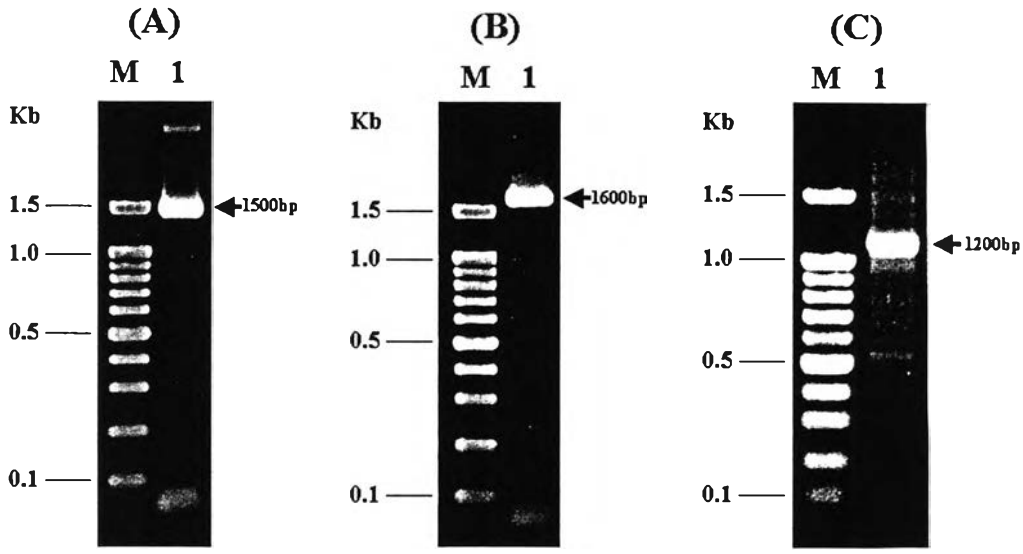

Figure 3.15 The amplification products of the 1st and 2nd regions (A and B) and 3rd region (C) of AcMRJP1 gene.

Lane M = 100 bp DNA ladder

(A): Lane 1 = Semi-nested PCR product of AcMRJP1 amplified with *n*M1F and RMRJP primers

(B): Lane 1 = Semi-nested PCR product of AcMRJP1 amplified with 3*n*M1F and 3M1R primers

(C): Lane 1= PCR products of AcMRJP1 amplified with 5M1F and 5M1R primers

3.2.2.2 Isolation of AcMRJP2 gene

The 1,500 bp major band of PCR product amplified from degenerated FMRJP and RMRJP primers was extracted from agarose gel. Nested primer, *n*M2F (Table 2.2), was designed and subsequently used in PCR reaction together with RMRJP primer. Semi-nested PCR (*n*M2F and RMRJP) was carried out using the gel-eluted PCR product as template. The PCR product was analyzed on 1.2% agarose gel. After amplification, approximately 1,500 bp of semi-nested PCR product were obtained (Figure 3.16, A). The PCR product was purified form agarose gel. The DNA fragment was further analyzed by cloning and sequencing. Ten recombinant clones were subjected to colony PCR. Three clones revealed the expected insert DNA fragment. Plasmid DNA was extracted from each recombinant clone and double-strand sequenced. Analysis of the DNA sequence of all clones revealed that they contain 1,480 nucleotide sequences corresponding to part of exon 1 to exon 4 of the AcMRJP2 gene encoding 232 amino acid residues.

In the second overlapping region, the degenerated primer 3M1/2F designed from AcMRJP1 cDNA at location 660^{th} - 687^{th} nt and at 654^{th} - 678^{th} nt of AcMRJP2 cDNA sequence together with AcMRJP2 gene-specific primer (3M2R, located at $1,425^{th}$ - $1,449^{th}$ nt) were used to amplified the genomic DNA. The PCR product of 1,800 kb was obtained, followed by eluting from agarose gel. The gel-eluted PCR product was used as template in the second semi-nested PCR. Nested primer (3M2F, located at 682^{th} -706^{th} of AcMRJP2 cDNA) was designed and used to amplified the gel-eluted first PCR product with the same reverse primer; 3M2R. The result showed approximately 1,800 bp of nested PCR products were observed (Figure 3.16, B). The amplification product was eluted and subjected to further analysis by DNA cloning and sequencing. DNA sequence analysis revealed that the nucleotides sequence of the recombinant clones contained the 1,811 bp product corresponded to part of the exon 4 to exon 6 of AcMRJP2 gene encoding 236 amino acid residues.

In the third region; the upstream 5' region of AcMRJP2 gene was performed using primer designed from the 5' upstream of AmMRJP2 which deposited in GenBank (AY078399). The 5M2F primer designed from 859 bp upstream region of AmMRJP2 and the gene-specific primer; 5M2R designe was based on the known nucleotide sequence of the AcMRJP2 gene previously obtained from the first

amplification were used to amplified the genomic DNA. After amplification, a band of approximately 1,200 bp of PCR product was purified from agarose gel and subjected to second round of PCR. Semi-nested PCR was carried out using the original forward 5M2F primer and 5*n*M2R-1 (133 rd -160 th nt). The PCR product was analyzed on 1.2% agarose gel electrophoresis. The result showed the amplified PCR product of approximately 800 bp (Figure 3.16, C). The PCR product was then eluted and subjected to clone and sequence. The recombinant plasmid were selected for further analysis by DNA sequencing. Nucleotide sequence analysis revealed the inserted fragment of 790 bp correspoded to 5' upstream region and part of the exon 1 encoding for 53 amino acid residues.

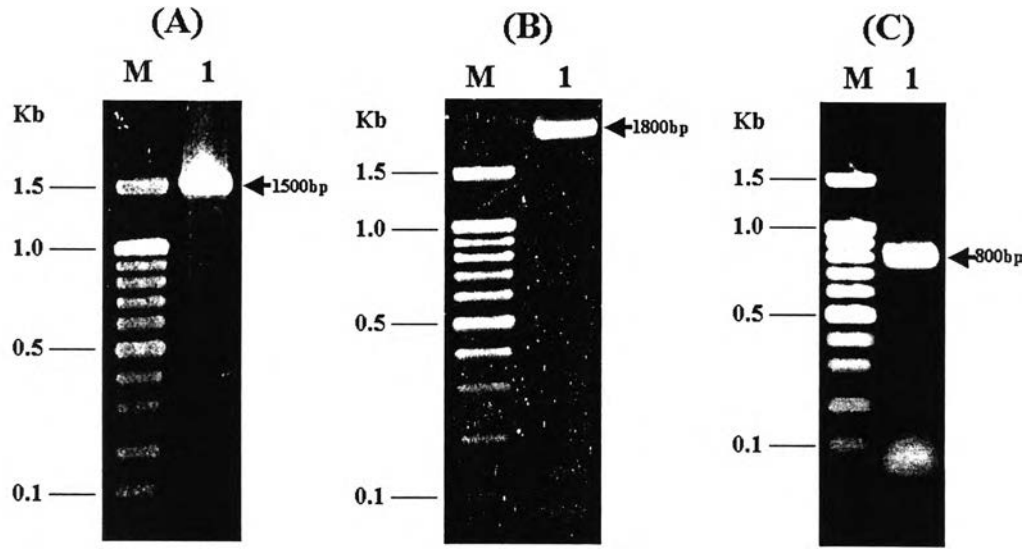

Figure 3.16 The amplification products of the 1st and 2nd regions (A and B) and 3rd region (C) of AcMRJP2 gene.

Lane M = 100 bp DNA ladder

(A): Lane 1 = Semi-nested PCR product of AcMRJP2 amplified with *n*M2F and RMRJP primers

(B): Lane 1 = Semi-nested PCR product of AcMRJP2 amplified with 3M1/2F and 3M2R primers

(C): Lane 1= Semi-nested PCR product of AcMRJP2 amplified with 5M2F and 5*n*M2R-1 primers

3.2.3 Characterization of AcMRJP1 gene

To analyze the AcMRJP1 gene, a Polymerase Chain Reaction (PCR) approach was used. After sequencing of three overlapping recombinant clones, the sequence datas were assembled into a single contig of 3,663 bp in length (Figure 3.17; GenBank accession number AY515688). The intron-exon organization of AcMRJP1 gene was performed by comparison of the 3,663 bp sequence with the cDNA coding for AcMRJP1. The coding region of the AcMRJP1 gene spans over 2,968 bp. The sequences around the exon/intron boundaries were determined and are shown in Table 3.2. Six exons were found in the coding region and all intron-exon boundaries followed the GT-AG rule (Figure 3.18, Table3.2). The continuous ORF encoded the AcMRJP1 protein, which is 433 amino acid residues in length.

The length of each exon varied from 133 bp (exon 5) - 284 bp (exon 4) whereas introns varied between 84 bp (intron 2) and 563 bp (intron 5) in length (Table 3.3). The ATG translation initiation codon is located in exon 1 and the TAG termination codon is in exon 6. The GC content reflects a slightly greater thermal stability in exons (34% - 42%) than in introns (15% - 29%) (Table 3.3). Introns 2, 3, and 5 interrupt ORFs between two codons (type 0 intron) whereas the remaining introns interrupt ORFs after the 1^{st} or 2^{nd} of the codons (type 1 and type 2 intron, respectively). The sequence of AMRJP1 gene was in good agreement with the cDNA sequence.

```
ACATCACTATTCTCATTGCATCAGACCTGCAAAAGAAATTGAATTTTTTGGATTTTTGTA      60
TCTACATCTTTTTTTTTAATTGCAATAATTTCCAAATAAATTAAATAAACATAATATTTT     120
TCAATATAATTATTCTAATTTTTAAAAAATTACACTACATATTTTTTTTTTTTGAATTAA     180
TCATTATCTCAATTAACATTTCTCCAGAACTATAGATGAAGCGAGACACAAAAAAAATAG     240
TGTGACATAATAGATAAACAAAATTTTGTAAAATTTCTACTCAAACAATATCTTTTTAGG     300
GAATATAAATTATAGACCTGTCACATTCTAATTCTTTACAACATATCTACAAGATAAATA     360
AGATAATTTCAAGAATTTCAAAGGAATTTTAAAATACAATTTACTTTATCTCTATAAAGT     420
ATACGTCATTACCGCCTATATCATTGACCGATCGTTGGTGAAAATTCAACAGCTCCTGCA     480
GTTCACGTACAATATCCATTGCTTCGTTACTCGCAGCCTAGGTAAGTGTTCCAAATATCT     540
CAATTGTAATACTCCTATACGAAACACCTTATTATTTTCTGACAAGACGAAATATTTTGT     600
AGAAAAATGACAAGGTGGTTGTTTATGGTGGTATGCCTTGGCATAGTTTGTCAAGGTACG     660
      M  T  R  W  L  F  M  V  V  C  L  G  I  V  C  Q  G  T

ACAAGCAGCATTCTTCGAGGAGAATCTTTAAACAAATCATTAAGCGTCCTTCACGAATGG     720
T  S  S  I  L  R  G  E  S  L  N  K  S  L  S  V  L  H  E  W

AAATTCTTTGATTATGATTTCGATAGCGATGAAAGAAGACAAGATGCAATTCTATCTGGC     780
K  F  F  D  Y  D  F  D  S  D  E  R  R  Q  D  A  I  L  S  G

GAATACGACTACAGGAAAAATTATCCATCCGACGTTGATCAATGGCATGgtaaattagat     840
E  Y  D  Y  R  K  N  Y  P  S  D  V  D  Q  W  H  G
cataaaatattttaatattgcattttacttgtccaaaattcttaatatccaatgattaca     900
atttaaaaaatattaaacatttttcatttcttattcaagGTAAGATTTTTGTCACCATGC     960
                                        K  I  F  V  T  M  L

TAAGATACAATGGCGTACCTTCCTCTTTGAACGTGATATCTAAAAAGATCGGTGATGGTG    1020
  R  Y  N  G  V  P  S  S  L  N  V  I  S  K  K  I  G  D  G  G

GACCTCTTCTTCAACCTTATCCCGATTGGTCGTTTGCTAAATATGACGATTGCTCTGGAA    1080
  P  L  L  Q  P  Y  P  D  W  S  F  A  K  Y  D  D  C  S  G  I
TCGTGAGCGCCACAAAACTTGCGgtaattgaacattgtctttatgattatatcttcacaa    1140
  V  S  A  T  K  L  A
ttaattttccaaagaaaaagaagattcatttgttatgtgatatttagATCGACAAATGCG    1200
                                              I  D  K  C  D

ACAGATTGTGGGTTCTGGACTCAGGTCTTGTCAATAATACTCAACCCATGTGTTCTCCAA    1260
  R  L  W  V  L  D  S  G  L  V  N  N  T  Q  P  M  C  S  P  K

AACTGCTCACCTTTGATCTGACTACCTCGCAATTGCTCAAGCAAGTCGAAATACCGCATG    1320
  L  L  T  F  D  L  T  T  S  Q  L  L  K  Q  V  E  I  P  H  D

ATGTTGCCGTAAATGCCACCACAGGAAAGGGAAGACTATCATCTCTAGCTGTTCAACCTT    1380
  V  A  V  N  A  T  T  G  K  G  R  L  S  S  L  A  V  Q  P  L

TAGATTGCAATATAAATGGTGATACTATGgtgagtttataattataaagtaggcaactta    1440
  D  C  N  I  N  G  D  T  M
cttttcttggaatttcattcactttgtgtatttctagcgatgctaagcgatgaataatt     1500
catatggaaatataacttaaattagaaaagtaatatcgcagaatgataaaatatccaaaa    1560
taataccctcttaaataattctacctgaaatcttaagacaaagaattagaatgtctctta    1620
cgtattacttcgctcttatataaaaaaaaatgcatcttgtaatttttttttgatgttttc    1680
tttggcaaaaagatataaaatataaagttctctctctatatatatatgtgatattagact    1740
ttctcaataaatcgttaatctttgaaactaaaattgaaaaatattagcaaaaatgaatgt    1800
attttcaatatttttttatttaaatatatttataattatatattgagagaaataaaattc    1860
ttctcaacgcattaggatttataaaaaaaaaaacatctatgaaagttacaaaatcaaaaa    1920
aaaaagttgaaatttatcggacaatattattaattacaatataatcataccatgataatg    1980
atataacagGTATACATAGCAGACGAGAAAGGTGAAGGTTTAATCGTGTATCATGATTCT    2040
         V  Y  I  A  D  E  K  G  E  G  L  I  V  Y  H  D  S
```

Figure 3.17

```
GATAATTCTTTCCATCGATTGACTTCCAAAACTTTCGATTACGATCCTAAATTTACCAAA   2100
D  N  S  F  H  R  L  T  S  K  T  F  D  Y  D  P  K  F  T  K

ATGACGATCAATGGAGAAAGTTTCACAACGCAAAGTGGAATTTCTGGAATGGCTCTTAGT   2160
M  T  I  N  G  E  S  F  T  T  Q  S  G  I  S  G  M  A  L  S

CCCATGACTAACAATCTCTATTACAGTCCTGTAGCTTCTACCAGTTTGTATTATGTTAAC   2220
P  M  T  N  N  L  Y  Y  S  P  V  A  S  T  S  L  Y  Y  V  N

ACGGAACAATTCAGAACATCCAATTATGAACAAAATGCCGTACATTATGAAGGgtaaata   2280
T  E  Q  F  R  T  S  N  Y  E  Q  N  A  V  H  Y  E  G
taaaattaagtttacttttaaataatgttactatattcagtggagaattgattctaaaat   2340
ataacgtttttcaacttgtctgaatcgtgattaagataattttaagtatttctaattaaa   2400
aatattcaaatattgaaacattgttaaatgaaataaggctgaaaatatagaattgtatct   2460
ctgctaacgcaaatttaaattacttacaatttaataaataaaacttgaaattattataca   2520
acaatatttcctatagcttttagaatcgatatcacagatttgttgcatttttttagttaaa   2580
tccacatttttttactgattccagAGTTCAAAATATTTTGGATACCCAATCGTCTGCTAAA   2640
                        V  Q  N  I  L  D  T  Q  S  S  A  K

GTAGTATCGAAAAGTGGCGTCCTCTTCTTCGGACTGGTGGGCGATTCAGCTCTTGGCTGC   2700
V  V  S  K  S  G  V  L  F  F  G  L  V  G  D  S  A  L  G  C

TGGAACGAACATCGATCACTTGAAAGACACAATATCgttagcaactgcgaatgatttttg   2760
W  N  E  H  R  S  L  E  R  H  N  I
atttttgttatattttcgtgtcacatttcttcctaccatatgttatgactactaagcatg   2820
aacttctcgcagggacaaaacaatccactactagattgacagggaacaagtcacgtgatg   2880
cgaagctgcttgggccagtgacgtaccctgtgtatgtagtgggataatgtggatatcatt   2940
tcaggttcaggcaggaattgaatataatgttagtaataaaggaagaaatgtctctacgat   3000
ttatattagtacatcttttctctatatatatattaataatattttcgttttcatcttaat   3060
ttttaaaattatgtttatcttcaattatctcttaatggtccaatcgaaatgtcacaatcg   3120
ctatcaattgattagcatttgcacttttcgttaattaataagaaatatttcgatttgatc   3180
gaaattcgataatcgaactaaaaattatttaatatgaatgcggtttctgtaaataagttg   3240
atcatatatatttcttaaaaaaggatataaataaaaagaaaattattttgaaattacagC   3300
                                                              R
GTACCGTCGCTCAAAGTGATGAGACACTTCAAATGATCGTTGGCATGAAGATTAAGGAAG   3360
  T  V  A  Q  S  D  E  T  L  Q  M  I  V  G  M  K  I  K  E  A

CCCTTCCACACGTGCCCATATTCGATAGATATATAAACCGTGAATACATATTGGTTTTAA   3420
  L  P  H  V  P  I  F  D  R  Y  I  N  R  E  Y  I  L  V  L  S

GTAACAGAATGCAAAAAATGGCGAATAATGACTATAACTTCAACGATGTAAACTTCAGAA   3480
  N  R  M  Q  K  M  A  N  N  D  Y  N  F  N  D  V  N  F  R  I

TTATGGACGCTAATGTAAATGACTTGATATTGAACACTCGTTGCGAAAATCCTAATAATG   3540
  M  D  A  N  V  N  D  L  I  L  N  T  R  C  E  N  P  N  N  D

ATAACACCCCTTTCAAAATTTCAATACATCTGTAATAATCTGTTTTTTTTCGATATATATTA   3600
  N  T  P  F  K  I  S  I  H  L

AATATTGTTCGAAATTTCTTATGAATGTATTATGAATGTATAAAATAAATATTGTTTTCG   3660
CAT                                                            3663
```

Figure 3.17 Organization of 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. Signal peptide sequence and the poly A additional signal site are underlined.

A

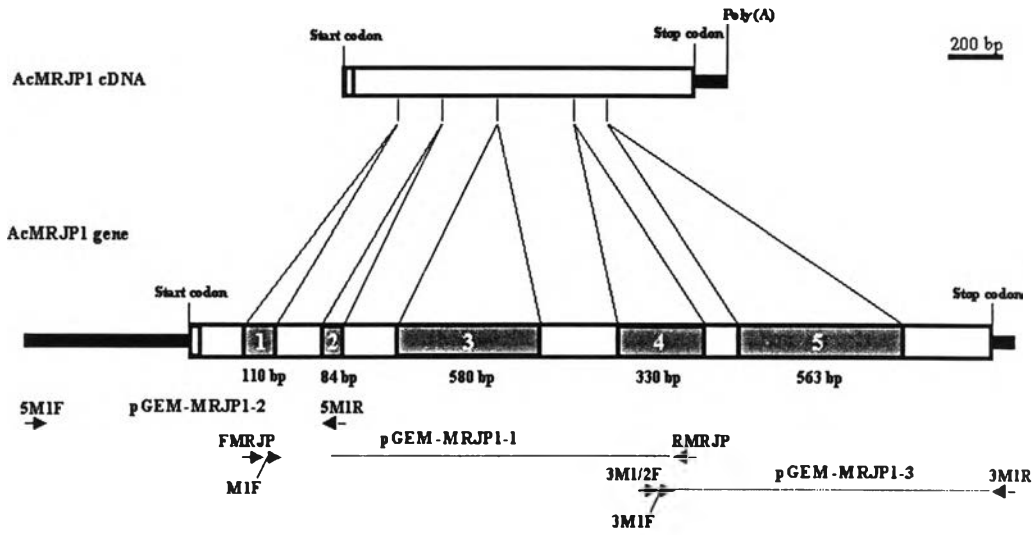

Figure 3.18 Schematic diagrams of *A. cerana* MRJP1 cDNA and gene. Complete cDNA was obtained by RT–PCR whereas genomic DNA fragment of AcMRJP1 was obtained from overlapping PCR amplification products. Non–coding regions are represented by solid bars. Introns (with numbers) are gray–shaded. Primers used for amplification of genomic AcMRJP1 and corresponding clones are illustrated.

Table 3.2 Exon-intron splice junctions of the AcMRJP1 gene

Exon no.	Exon size (bp)	Sequence at exon-intron junction			Intron no.	Intron size (bp)	Amino interrupted
		5'splice donor		3'splice acceptor			
1	223	CAA TGG CAT G Q W H G	gtaaattagat cata.....cattt cttattcaag	GT AAG ATT TTT GTC K I F V	1	110	Gly-75
2	164	ACA AAA CTT GCG T K L A	gtaattgaaca ttgt.....ttatg tgatatttag	ATC GAC AAA TGC I D K C	2	84	Ala-129
3	222	GGT GAT ACT ATG G D T M	gtgagtttata atta.....ataat gatataacag	GTA TAC ATA GCA V Y I A	3	580	Met-203
4	284	CAT TAT GAA GG H Y E G	gtaaatataaa atta.....tttta ctgattccag	A GTT CAA AAT ATT V Q N I	4	330	Gly-298
5	133	AGA CAC AAT ATC R H N I	gttagcaactg cgaa.....atttt gaaattacag	CGT ACC GTC GCT R T V A	5	563	Ile-342
6	276						

The exon sequences are shown in upper-case letters and the intron sequences are shown in lower-case letters.

Table 3.3 GC content and length of exons and introns in AcMRJP1 gene

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			

3.2.4 Characterization of AcMRJP2 gene

The PCR approach was used to analyze AcMRJP2 gene. After sequencing of three overlapping recombinant clones, the sequence datas were assembled into a single contig of 3,963 bp in length (Figure 3.19; GenBank accession number AY515689). The intron-exon organization of AcMRJP2 gene was performed by comparison of the 3,663 bp sequence with the cDNA coding for AcMRJP2. The coding region of the AcMRJP2 gene spans over 3,187 bp. The sequences around the exon/intron boundaries were determined and are shown in Table 3.4. Six exons were found in the coding region and all intron-exon boundaries followed the GT-AG rule (Figure 3.20, Table 3.4). The continuous ORF encoded the AcMRJP2 protein, which is 463 amino acid residues in length.

The length of each exon varied from 133 bp (exon 5) - 372 bp (exon 6) whereas introns varied between 88 bp (intron 2) and 868 bp (intron 5) in length. The ATG translation initiation codon is located in exon 1 and the TAG termination codon is in exon 6. The GC content reflects a slightly greater thermal stability in exons (28% - 42%) than in introns (16% - 24%) (Table 3.5). Introns 2, 3, and 5 interrupt ORFs between two codons (type 0 intron) whereas the remaining introns interrupt ORFs after the 1^{st} or 2^{nd} of the codons (type 1 and type 2 intron, respectively). The sequence of AMRJP2 gene was in good agreement with the cDNA sequence, except for A_8 in cDNA sequence that was corrected to G from this result.

```
TGAGAATGAATTGCAGAATATGGTCGCTAATAATAAGACTTTTCAAATTATTAGTTCTAC      60
AATTAAGATTTTCCAATTTTAATTCCGTATTTAACTCATCATATGTGAATATATATATAT     120
TGATTTTGAATAGAAAATATTACAAAATGTTAAATGGGAATTTAAATCTCGATAACATGA     180
ATCCCTAAATTTTGAATGAGAATATAAAAACATTAACATATAATTATTCTATTTTTAAAA     240
AATTGCTCTTCATATTTTTTTTGGAACTAAATATTATCTCTCTTAATAGTTTTTTAGAAC     300
TCCGTATGAAGCTAAACACACACGCAAAAATAGAGGTGTGGCAAAGCATTTTTAGGGAAT     360
ATAAATTATAACCCGTCACATTCTAATCCTTTAAGAAATATCTACAAGATAAACGAAAAT     420
TTTGAAAATTCAAAAAAACAATTTACTTTATCTCTGTAAAGTACGTACCATTACCACCTA     480
TTTTTTGACCAACCGTAGTCAAAATTCAACAGTTTGTACAGTTCACTTACATTCTGCAGT     540
ATCCTAAGTAAGTTTCTTGATTATCTTGATTATAATATTTATTTGCAATCTTTCATTTAT     600
CTGGAAAATGAAATATTTTATTTTAGAAAAATGACAAAGTGGTTGTTTATGGTGGCATGC     660
                              M  T  K  W  L  F  M  V  A  C

CTTGGCATAGCTTGTCAAGGCGCCATTATTCGACAAAATTCTGCAAAAAACTTGGAAAAT     720
L  G  I  A  C  Q  G  A  I  I  R  Q  N  S  A  K  N  L  E  N

TCGTTGAACGTAATTCACGAATGGAAATATATCGATTATGATTTCGGTAGCGAAGAAAGA     780
S  L  N  V  I  H  E  W  K  Y  I  D  Y  D  F  G  S  E  E  R

AGACAAGCTGCGATTCAATCTGGCGAATACGATCATACGAAAAATTATCCCTTCGATGTC     840
R  Q  A  A  I  Q  S  G  E  Y  D  H  T  K  N  Y  P  F  D  V

GATCAATGGCATGgtaaaattttcttattttaaactattaatagcattttaatcgtcgaa     900
D  Q  W  H  D
acacttaatattcaataattttcatcgctcgtatttcttcatttttgaataattaaaaag     960
atattccacgttttgtatttcttgtttaagATAAGACTTTTGTCACCATACTAAAGTACG    1020
                                 K  T  F  V  T  I  L  K  Y  D

ATGGTGTGCCTTCTACTTTGAACATGATATCTAACAAAATCGGTAAGGGTGGACGCCTTC    1080
  G  V  P  S  T  L  N  M  I  S  N  K  I  G  K  G  G  R  L  L

TACAACCATATCCTGATTGGTCGTGGGCAGAGAATAAAGATTGCTCTGGAATCGTGAGCG    1140
  Q  P  Y  P  D  W  S  W  A  E  N  K  D  C  S  G  I  V  S  A

CTTTCAAAATTGCGgtaattgaacatttttttctatatttatctttaaaattaattttcc    1200
  F  K  I  A
tcttcatagaaaaagaagattcatttgtcgtatgatatttagATTGACAAATTCGACAGA    1260
                                          I  D  K  F  D  R

TTGTGGGTTTTGGATTCAGGTCTTATCAATAGAACTGAACCTATATGTGCTCCAAAGTTG    1320
L  W  V  L  D  S  G  L  I  N  R  T  E  P  I  C  A  P  K  L

CATGTCTTTGATCTGAAAAACACAAAGCACCTTAAGCAAATCGAAATACCGCATGATATT    1380
H  V  F  D  L  K  N  T  K  H  L  K  Q  I  E  I  P  H  D  I

GCCGTAAATGCCACCACAGGAAAGGGAGGGCTAGTCTCTCTAGTTGTTCAAGCCATGGAT    1440
A  V  N  A  T  T  G  K  G  G  L  V  S  L  V  V  Q  A  M  D

CCTATGAATACTTTAgtgagtttaaattacattaaaatttaaattagagattaaattaga    1500
P  M  N  T  L
cattgcatatgataaaaataaaattcataactttcgaaatagtaaaatagttaaatttag    1560
attaaaaaattgaaatttacaatatatactaattgtactatttttttgtataaaaaaaat    1620
gattttataactttctctgatgttttactttagaaaaattgatcatattaaattagattt    1680
```

Figure 3.19

```
ttttatcattaatttttataaaatttttaaaataactatatttttgatgttttttttattt   1740
gaattattacttaagcatatttaaatatttataatcaattttattatgatcgagtgagtt   1800
aattttaaagaacaactgaatgactattatttcaaaagctatgtattctagaaaaaaaag   1860
atctccagaatctgggcaaaatttataaagcatacatttaatgaaagttataaaatcaag   1920
aaaaatttaaaatttatcgaacaatattattatgatatgatataatgattaatcatataa   1980
tagGTATACATAGCAGACCATAAGGGTGATGCTTTGATCGTCTATCAAAATTCCGATGAT   2040
   V  Y  I  A  D  H  K  G  D  A  L  I  V  Y  Q  N  S  D  D

TCCTTCCATCGAATGACTTCCAACACTTTCGATTACGATCCCAGATATGCCAAAATGACG   2100
S  F  H  R  M  T  S  N  T  F  D  Y  D  P  R  Y  A  K  M  T

ATCAATGGAGAAAGTTTCACATTGAAAAATGGAATTTGTGGAATGGCTCTTAGTCCCGTG   2160
I  N  G  E  S  F  T  L  K  N  G  I  C  G  M  A  L  S  P  V

ACGAACAATCTTTATTACAGTCCTCTCGCTTCTCACGGTTTGTATTATGTCAACACGGAA   2220
T  N  N  L  Y  Y  S  P  L  A  S  H  G  L  Y  Y  V  N  T  E

CCATTTATGAAATCACAATTTGGAGACAATAATAACGTGCAATATGAAGGgtaaatataa   2280
P  F  M  K  S  Q  F  G  D  N  N  N  V  Q  Y  E  G
aaataatttcttaaattttatgaaatagaataatgttaaataatatgttaatatgttcgt   2340
gaaatttttttgtaaagttaattcaagaatagcgtttcaatttatttattcttgcacagc   2400
atcttgcacattcttagttagttgcctattccttattaatttcagATCCCAAGATACTTT   2460
                                                S  Q  D  T  L

GAACACGCAATCATTGGCTAAAGCAGTATCGAAAGATGGCGTCCTCTTCGTCGGACTTGT   2520
 N  T  Q  S  L  A  K  A  V  S  K  D  G  V  L  F  V  G  L  V

GGGTAATTCAGCTCTTGGATGCTTGAACGAGCATCAACCACTTCAGAGAGAAAATTTAgt   2580
 G  N  S  A  L  G  C  L  N  E  H  Q  P  L  Q  R  E  N  L
tagtagtttttattgtgtctcttttcttcacatttttgttatatttttcttggtacat   2640
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cctgttcgtgaattgcgagtgtggtattagtatagatgacgtagttaagtcccttctaga   2880
tttatgagaaatttttcttaaatatatataattataattcgtttatttcattatttttc   2940
ctttaaaaatcttaaattttaaattaataaaatttttaacggcaacgaaaaacctgaaaag   3000
tagatatataattaaatttataatagtgccgtagttaaatttttctgcttttgcgttttt   3060
tctctttaaaaataatcataatattaatcatttcgtttcatttctatcgttttttttctt   3120
ttataaaatctgttctttttttttaaaagtttatatttaagatcttttatcgacaatcgaa   3180
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gaatttatgtcaatttatgttctcaaagcaacaaatgttttatatgatgaaatataaata   3420
aaaatgaaactatttcgaaattacagGAACTGGTCGCCCAAAATGAAAAAACACTTCAAA   3480
                          E  L  V  A  Q  N  E  K  T  L  Q  M
TGATCGCAGGTATGAAAATTAAGGAAGAGCTTCCACATTTCGTAGGAAGTAACAAACCTG   3540
  I  A  G  M  K  I  K  E  E  L  P  H  F  V  G  S  N  K  P  V

TAAAGGACGAATATATGTTAGTTTTAAGTAACAAAATGCAGAAAATAGTAAATAATGATT   3600
  K  D  E  Y  M  L  V  L  S  N  K  M  Q  K  I  V  N  N  D  F
```

Figure 3.19

```
TTAATTTCAACGACGTAAACTTCCGAATTTTGGGTGCGAATGTAAAGGAATTAATGAGAA   3660
  N  F  N  D  V  N  F  R  I  L  G  A  N  V  K  E  L  M  R  N

ATACTCATTGCGCAAATTTTAACAATAAAAATAATCAGAAGAATAACAATCAGAAGAATA   3720
  T  H  C  A  N  F  N  N  K  N  N  Q  K  N  N  N  Q  K  N  N

ACAATCAGAACAATAACAATCAGAAGAATAACAATCAGAAAAATAACAATCAGAAGAATA   3780
  N  Q  N  N  N  N  Q  K  N  N  N  Q  K  N  N  N  Q  K  N  N

ACAATCAGAAGAATAACAATCAGAATACTAACAATTAGAATGATAATCAAGTTCGTCGTT   3840
  N  Q  K  N  N  N  Q  N  T  N  N
CTTCAAAATCGCATTAAAATCAATAAACCAAATTATTTTTTAAAATATTTTTTCGATGTA   3900
AACAAAATTTTTTAAAATATTTCATTATATTATAAATAAATAAAATAAATATCGTTTTCG   3960
CAT                                                            3963
```

Figure 3.19 Organization of 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. 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

B

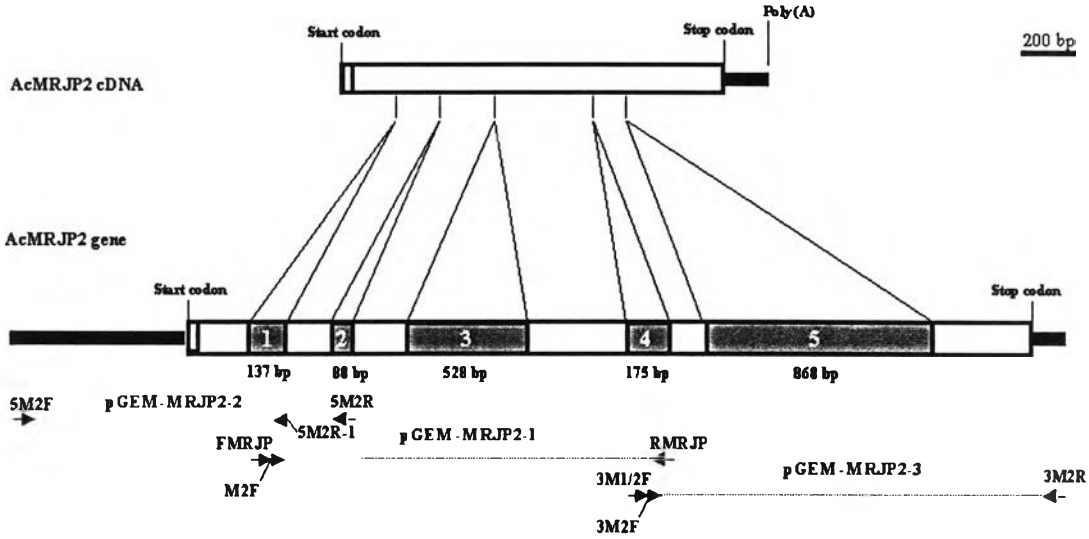

Figure 3.20 Schematic diagrams of *A. cerana* MRJP2 cDNA and gene. Complete cDNA was obtained by RT–PCR whereas genomic DNA fragment of AcMRJP2 was obtained from overlapping PCR amplification products. Non–coding regions are represented by solid bars. Introns (with numbers) are gray–shaded. Primers used for amplification of genomic AcMRJP2 and corresponding clones are illustrated.

Table 3.4 Exon-intron splice junctions of the AcMRJP2 gene

Exon no.	Exon size (bp)	Sequence at exon-intron junction										Intron no.	Intron size (bp)	Amino interrupted
		5'splice donor acceptor									3'splice			
1	223	CAA	TGG	CAT	G	gtaaaattttc ttat.....tattt cttgtttaag	AT	AAG	ACT	TTT	GTC	1	137	Asp-75
		Q	W	H	D			K	T	F	V			
2	164	TTC	AAA	ATT	GCG	gtaattgaaca tttt.....tcgta tgatatttag		ATT	GAC	AAA	TTC	2	88	Ala-129
		F	K	I	A			I	D	K	F			
3	213	ATG	AAT	ACT	TTA	gtgagtttaaa ttac.....ttaat catataatag		GTA	TAC	ATA	GCA	3	528	Leu-200
		M	N	T	L			V	Y	I	A			
4	287	CAA	TAT	GAA	GG	gtaaatataaa aata.....ccta ttaatttcag	A	TCC	CAA	GAT	ACT	4	175	Gly-296
		Q	Y	E	G			S	Q	D	T			
5	133	AGA	GAA	AAT	TTA	gttagtagttt ttta.....atttc gaaattacag		GAA	CTG	GTC	GCC	5	868	Leu-340
		R	E	N	L			E	L	V	A			
6	372													

The exon sequences are shown in upper-case letters and the intron sequences are shown in lower-case letters.

Table 3.5 GC content and length of exons and introns in AcMRJP2 gene

Exon	Genomic DNA (No. of nucleotides)	GC content (%)	Intron	Genomic DNA (No. of nucleotides)	GC content (%)
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			

3.2.5 Identification of the 5' upstream (the putative promoter sequence, transcription initiation start site and upstream regulatory elements) of AcMRJP1 and AcMRJP2 genes

The Neural Network Promoter Prediction (NNPP2.1) software of the Berkeley drosophila Project on www. Server (http://www.fruitfly.org/seq-tools/promoter.html) was used in order to find putative promoter regions in the putative regulatory sequences of AcMRJP1 and AcMRJP2 genes.

In AcMRJP1 gene, a nucleotide at position 468 (A) was predicted to be a potential transcription initiation start site with a score cut off 0.94. In AcMRJP2 gene, a nucleotide at position 511 (A) was predicted to be a potential transcription initiation start site with a score cut off 0.62 (Figure 3.21 and 3.22). The putative promoter sequence of both genes were detected with the sequence TATATATT that was highly homologous to the TATA box like element (TATAAA consensus sequence). The putative TATA box of AcMRJP1 and AcMRJP2 were found at -31 and -32 nucleotides upstrem from the transcription initiation sites, respectively. The putative CAAT box (CCAAT) was found in AcMRJP1 (CAAAT) at an identical position to the CCAAT in AmMRJP1, but the consensus sequence was not found in AcMRJP2 gene.

Potential transcription factor binding site within the 5' upstream region of the AcMRJP1 and AcMRJP2 genes were identifed by Genomatixsuit on www. Server (http://genometix.de). The TF (transcription factors) binding sites for dead ringer (Dri) (binding site core sequence: GATTA or AATTA) and ultraspiracle (USP) (binding site core GGTCA) were detected in both AcMRJP1 and AcMRJP2 putative regulatory sequences (Figure 3.21 and 3.22). The distribution of the predicted regulatory elements found in the putative regulatory sequences of AcMRJP1 and AcMRJP2 genes was shown in Figure 3.21 and 3.22. The ultraspiracle transcriptional factor (USP-TF) is a member of the ligand-modulated transcription factors that regulate cell homeostasis, reproduction, differentiation and development (Sergaves, 1991). In AcMRJP1 and AcMRJP2 contained a single USP-TF binding site at the 5' UTR immediately following the TATA box.

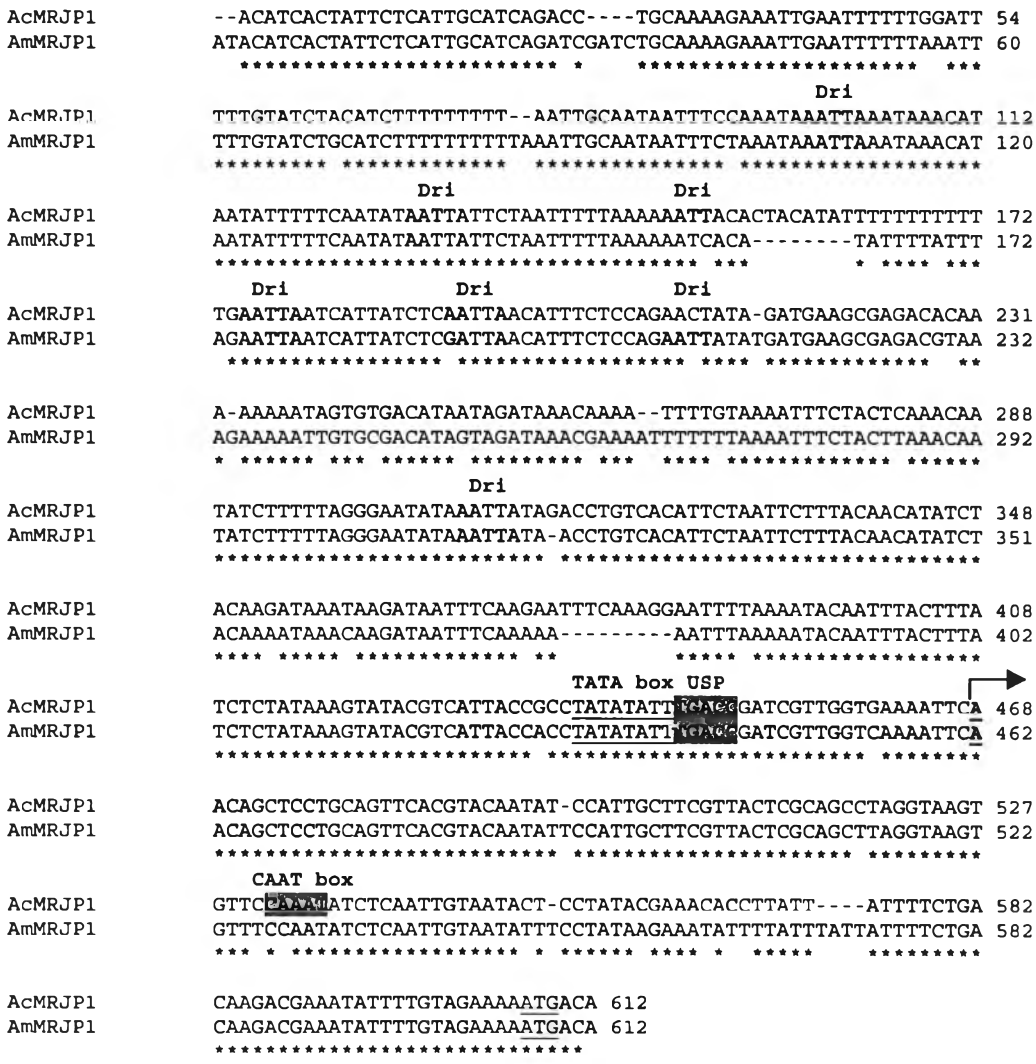

Figure 3.21 Putative regulatory elements in the 5' upstream region of the AcMRJP1 gene. The position are relative to the predicted transcription start site. The putative TATA box was underlined and highlighted. The binding sites for transcription factors were highlighted. The predicted transcription initation start site was indicated by arrow.

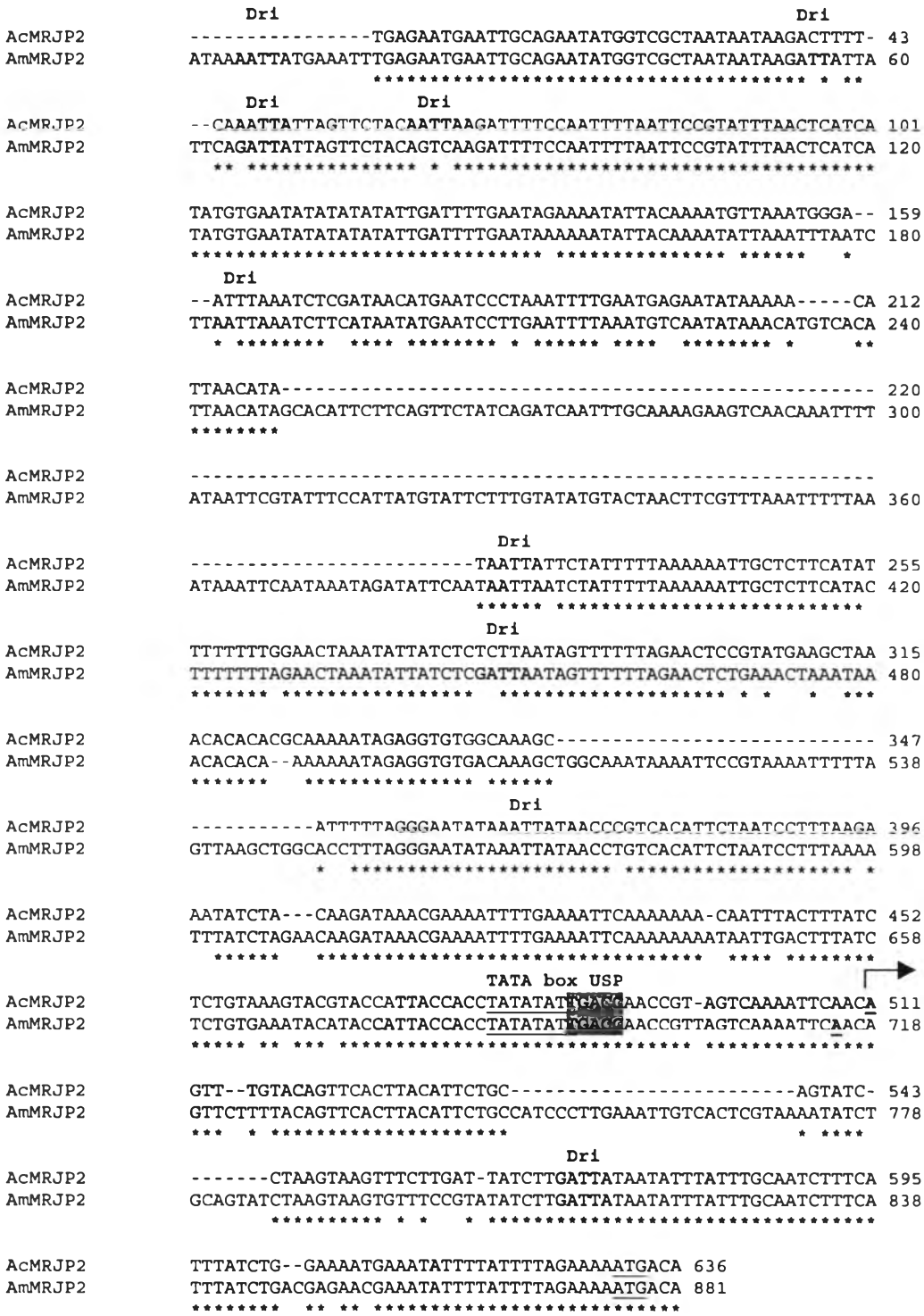

Figure 3.22 Putative regulatory elements in the 5' upstream region of the AcMRJP2 gene. The position are relative to the predicted transcription start site. The putative TATA box was underlined and highlighted. The binding sites for transcription factors were highlighted. The predicted transcription initation start site was indicated by arrow.

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

3.3.1 Expression of AcMRJP1 and AcMRJP2 under control of Lac promoter of pGEM®-3Zf(+) vector

3.3.1.1 Construction of AcMRJP1 and AcMRJP2 in pGEM®-3Zf(+) expression vector

To verify that the obtained AcMRJP1 and AcMRJP2 cDNAs actually encoded for AcMRJP1 and AcMRJP2, the preliminary *E. coli* expression under the lac promoter of pGEM®-3Zf(+) was constructed. The fragment of mature AcMRJP1 and AcMRJP2 cDNA of *A.cerana* were amplified from the plasmid pRT-AcMRJP1-Taq and pRT-AcMRJP2-Taq as a template using Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA). The AcMRJP1 and AcMRJP2 were amplified as an intense band of 1,253 bp and 1,352 bp, respectively. The amplified fragments were purified, digested with *Pst* I and *Kpn* I and cloned into the corresponding site of pTrcHis2c. After transformation to *E. coli* XL1-Blue, the recombinant clones were identified by colony PCR and the recombinant plasmids were extracted. The recombinant plasmids were digested with *Sal* I and *Kpn* I and cloned into the *Xho* I and *Kpn* I of pGEM®-3Zf(+) to generate the translational reading frame of AcMRJP1 and AcMRJP2 under the lac promoter. The recombinant plasmids were subsequently transformed to *E. coli* DH5α. Three white colonies were random picked for plasmid extraction and double digested with *Hin*d III and *Eco*R I. The result of electrophoretically analyzed show that all three recombinant plasmids contained the expected size of either AcMRJP1 or AcMRJP2 fragments (Figure 3.23). These recombinant plasmids were designated as pGEM®-3Zf(+)-AcMRJP1-Taq or pGEM®-3Zf(+)-AcMRJP2-Taq, respectively.

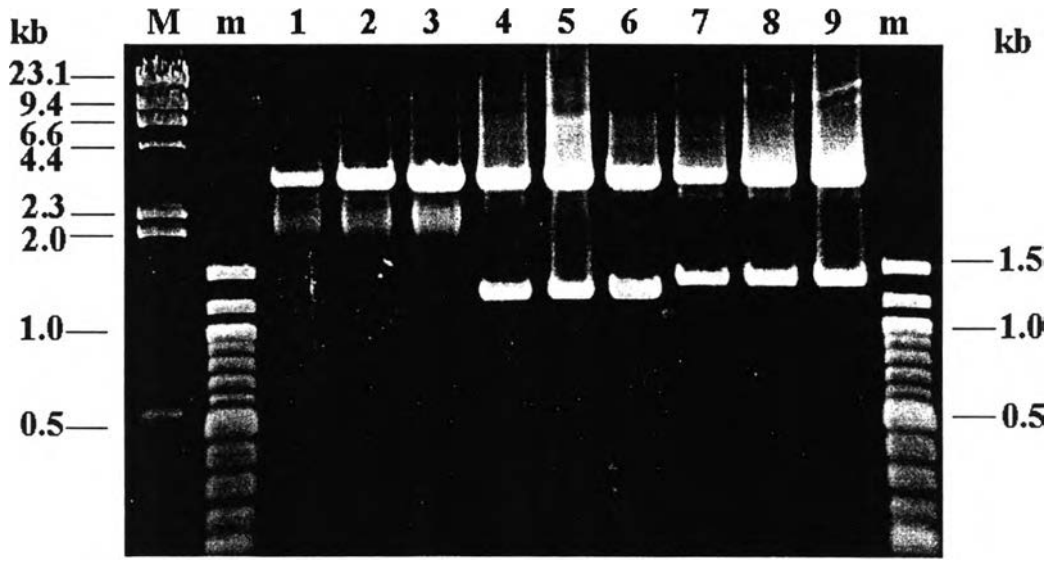

Figure 3.23 Restriction endonuclease analysis of recombinant plasmids pGEM®-3Zf (+)-AcMRJP1-Taq and pGEM®-3Zf(+)-AcMRJP2-Taq analyzed on 1.2% agarose gel

Lane M = λ / *Hin*d III standard marker

Lane m = 100 bp DNA ladder

Lane 1-3 = pGEM®-3Zf(+) vector digested with *Hin*d III and *Eco*R I

Lane 4-6 = pGEM®-3Zf(+)-AcMRJP1-Taq vector digested with *Hin*d III and *Eco*R I

Lane 7-9 = pGEM®-3Zf(+)-AcMRJP2-Taq vector digested with *Hin*d III and *Eco*R I

3.3.1.2 Expression of AcMRJP1 and AcMRJP2 under control of Lac promoter of pGEM®-3Zf(+) vector

The expression of AcMRJP1 and AcMRJP2 was preliminarily performed under the control of lac promoter in pGEM®-3Zf(+) vector. The lac promoter in general purpose vector, pGEM®-3Zf(+) vector designed for blue/white screening of clones containing foreign DNA insert. It can be used to express a foreign protein, usually as a fusion protein with the amino terminus of the lacZ gene and/or the polylinker sequence. Although the lac promoter is not as strong as the tac or trc promoters, the high copy number of most general-purpose vectors allows expression of foreign proteins at acceptable level.

The constructed recombinant plasmids pGEM®-3Zf(+)-AcMRJP1-Taq and pGEM®-3Zf(+)-AcMRJP2-Taq were transformed to *E. coli* DH5α cell and expression of the AcMRJP1 and AcMRJP2 were initiated by addition of 1 mM IPTG at 37 °C for 3 hours. The time course of expression of AcMRJP1 and AcMRJP2 after induction with IPTG for 15 minute, 1 hour and 3 hours was monitored by SDS-PAGE. For comparison, the amount of lysate loaded on SDS-PAGE was normalized basing on optical density at each sample collection. The whole cell lysate of IPTG induced pGEM®-3Zf(+) was parallely analyzed as control.

A dense protein band with molecular mass approximately 43 or 55 kDa were observed at 0 hour of induction (Figure 3.24 and 3.25). This band was absent in negative control with induced pGEM®-3Zf(+) (Figure 3.24 and 3.25). The calculated molecular weight of recombinant AcMRJP1 and AcMRJP2 protein deduced from DNA sequences were 40.2 and 54.4 kDa. The amount of induced protein appeared to be maximum at 15 minutes of induction. The expressed protein band was not stable as it was decreased after 1 hour of induction.

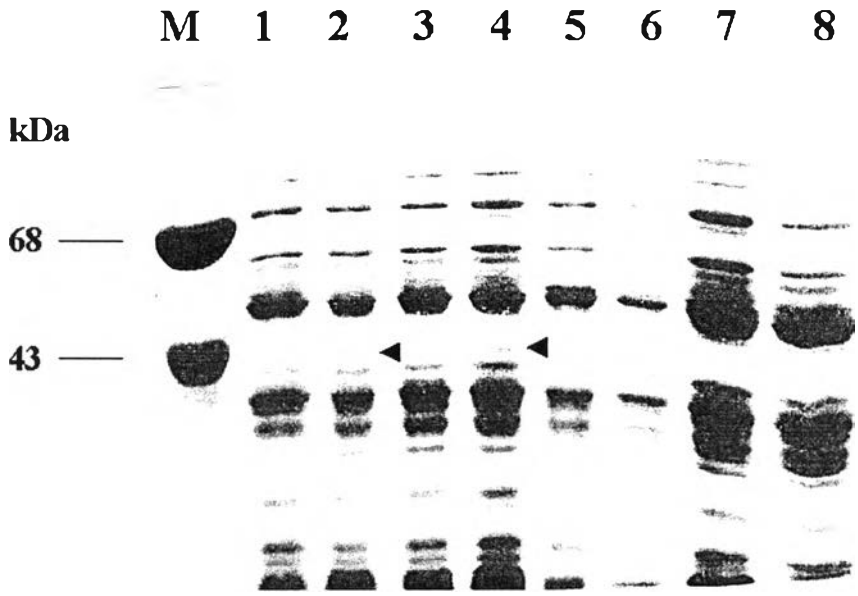

Figure 3.24 SDS-PAGE of AcMRJP1 expression under the control of Lac promoter of pGEM®-3Zf(+) vector. The *E. coli* DH5α harboring pGEM®-3Zf(+)-AcMRJP1-Taq was grown at 37 °C for 3 hours after induction with 1 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = Protein molecular weight markers (bovine serum albumin; 68 kDa, ovalbumin; 43 kDa)

Lane 1, 3, 5, 7 = Crude extract of the induced *E. coli* containing pGEM®-3Zf(+) vector at 0 hr, 15 min, 1 hr and 3 hr, respectively

Lane 2, 4, 6, 8 = Crude extract from induced *E. coli* containing pGEM®-3Zf(+)-AcMRJP1 at 0 hr, 15 min, 1 hr and 3 hr, respecitivey

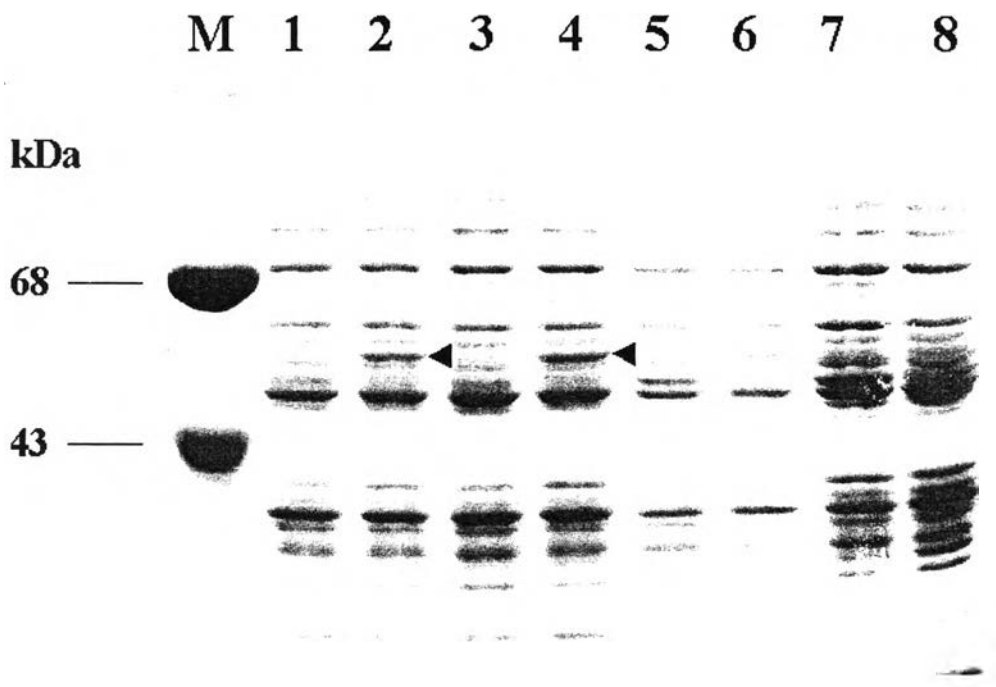

Figure 3.25 SDS-PAGE of AcMRJP2 expression under the control of Lac promoter of pGEM®-3Zf(+) vector. The *E. coli* DH5α harboring pGEM®-3Zf(+)-AcMRJP2-Taq was grown at at 37 °C for 3 hours after induction with 1 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = Protein molecular weight markers (bovine serum albumin; 68 kDa, ovalbumin; 43 kDa)

Lane 1, 3, 5, 7 = crude extract of the induced *E. coli* containing pGEM®-3Zf(+) vector at 0 hr, 15 min, 1 hr and 3 hr, respectively

Lane 2, 4, 6, 8 = crude extract from induced *E. coli* containing pGEM®-3Zf(+)-AcMRJP2 at 0 hr, 15 min, 1 hr and 3 hr, respecitivey

3.3.2 Expression of AcMRJP1 and AcMRJP2 under control of Trc promoter of pTrcHis2c vector

3.3.2.1 Construction of AcMRJP1 and AcMRJP2 under control of Trc promoter of pTrcHis2c vector

Attempts were made to express the AcMRJP1 and AcMRJP2 as fusion protein with C-terminal His-Tag of pTrcHis2c vector. The primers were designed to amplified the AcMRJP1 and AcMRJP2 cDNAs to translate in frame with the initiation ATG and a C-terminal six histidine fusion peptide in pTrcHis2c vector. The recognition sites for *Pst* I and *Kpn* I were introduced to facilitate further cloning of PCR products. The AcMRJP1 and AcMRJP2 fragments were amplified from pRT-AcMRJP1-Pfu and pRT-AcMRJP2-Pfu using *Pfu* DNA polymerase (Promega Corporation Medison, Wisconsin, USA). The AcMRJP1 and AcMRJP2 cDNAs were amplified as an intense band of 1,253 bp and 1,352 bp, respectively. The amplified fragments were purified, digested with *Pst* I and *Kpn* I and cloned into the corresponding site of pTrcHis2c. The ligated product was transformed to *E. coli* DH5α. White colonies were randomly picked for plasmid extraction. The recombinant plasmids were analyzed by restriction digestion with *Pst* I and *Kpn* I. The results showed that all recombinant plasmids contained the expected size of DNA insert of either AcMRJP1 or AcMRJP2 (Figure 3.26). These recombinant plasmids were designated as pTrcHis2c-AcMRJP1-Pfu or pTrcHis2c-AcMRJP2-Pfu, respectively.

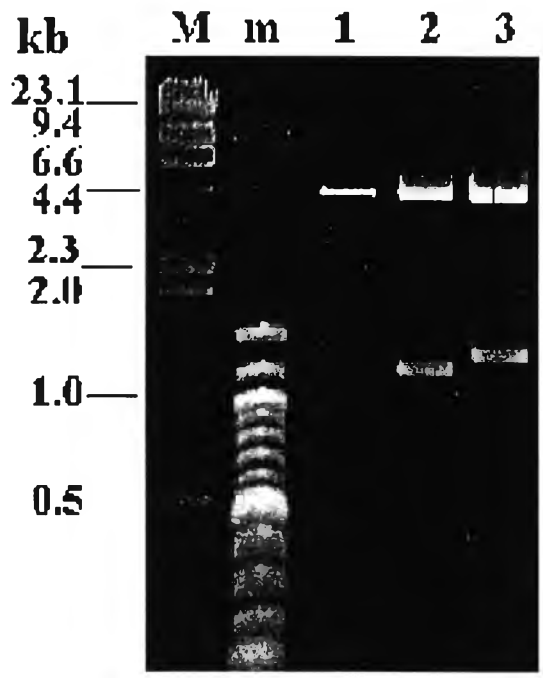

Figure 3.26 Restriction endonuclease analysis of recombinant plasmids pTrcHis2c-AcMRJP1-Pfu and pTrcHis2c-AcMRJP2-Pfu analyzed on 1.2% agarose gel

Lane M = λ / *Hin*d III standard marker

Lane m = 100 bp DNA ladder

Lane 1 = pTrcHis2c vector digested with *Pst* I and *Kpn* I

Lane 2 = pTrcHis2c-AcMRJP1-Pfu digested with *Pst* I and *Kpn* I

Lane 3 = pTrcHis2c-AcMRJP2-Pfu digested with *Pst* I and *Kpn* I

3.3.2.2 Expression of AcMRJP1 and AcMRJP2 under control of Trc promoter of pTrcHis2c vector

The AcMRJP1 and AcMRJP2 were expressed as fusion protein with C-terminal His-Tag of pTrcHis2c vector. The pTrcHis2c plasmid is pUC-derived expression vector designed for efficient recombinant protein expression and purification in *E. coli*. High level of expression is possible using trc (trp-lac) promoter; -35 region of the trp promoter together with the –10 region of the lac promoter. The DNA inserts are positioned downstream and in frame with the initiation ATG and a C-terminal six histidine fusion peptide that function as a metal binding site in the expressed protein.

The recombinant plasmids pTrcHis2c-AcMRJP1 and pTrcHis2c-AcMRJP2 were transformed into *E. coli* DH5α cells. Expression was initiated using 1 mM IPTG and the cells were incubated at 37 °C for 6 hours. Expression of AcMRJP1 and AcMRJP2 were performed according to the recommended procedure from the manufacturer. The time course of expression of AcMRJP1 and AcMRJP2 after induction with IPTG for 0-6 hours was determined by SDS-PAGE. The whole cell lysate of induced pTrcHis2c was parallely analyzed as control.

All cell lysates obtained from various time intervals contained many protein bands. However, the presence of protein bands of expected apparent molecular mass of 50 or 55 kDa were always observed (Figure 3.27 and 3.28). The molecular weight of induced protein band was corresponded well to the calculated molecular weight of recombinant AcMRJP1 (51.3 kDa) or AcMRJP2 (55.2 kDa) protein deduced from DNA sequence. The expressed proteins were observed within the first hour after induction and increased according to the incubation time. The amount of induced protein appeared to be maximum at 6 hours of induction.

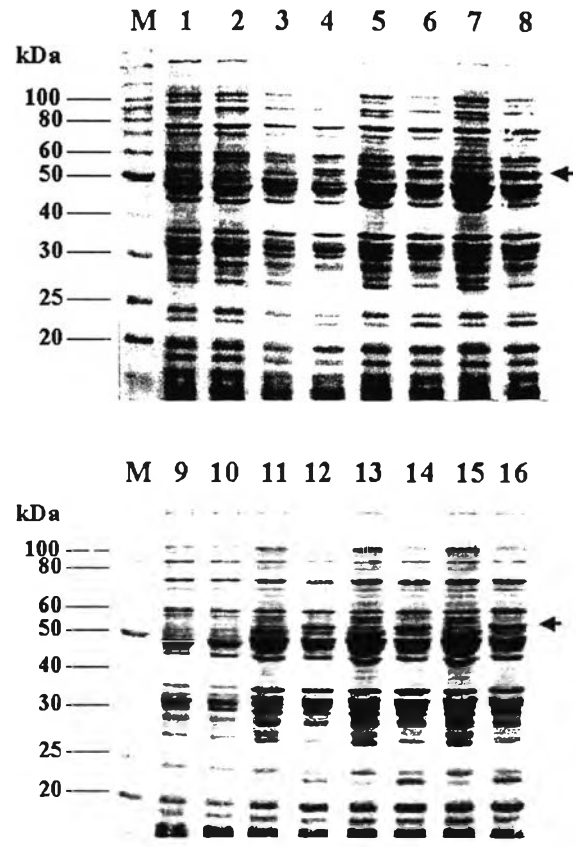

Figure 3.27 SDS-PAGE of AcMRJP1 expression under the control of Trc promoter of pTrcHis2C vector. The *E. coli* DH5α harboring pTrcHis2c-AcMRJP1 was grown at at 37 °C for 6 hours after induction with 1 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = BenchMark™ Protein Ladder

Lane 1, 9 = crude extract of the induced *E. coli* containing pTrcHis2C vector at 0 hr

Lane 2,10= crude extract from induced *E. coli* containing TrcHis2C-AcMRJP1 at 0 hr

Lane 3, 5, 7, 11, 13, 15 = crude extract of the induced *E. coli* containing pTrcHis2C vector at 1-6 hr, respectively

Lane 4, 6, 8, 12, 14, 16 = crude extract from induced *E. coli* containing TrcHis2C-AcMRJP1 at 1-6 hr, respecitivey

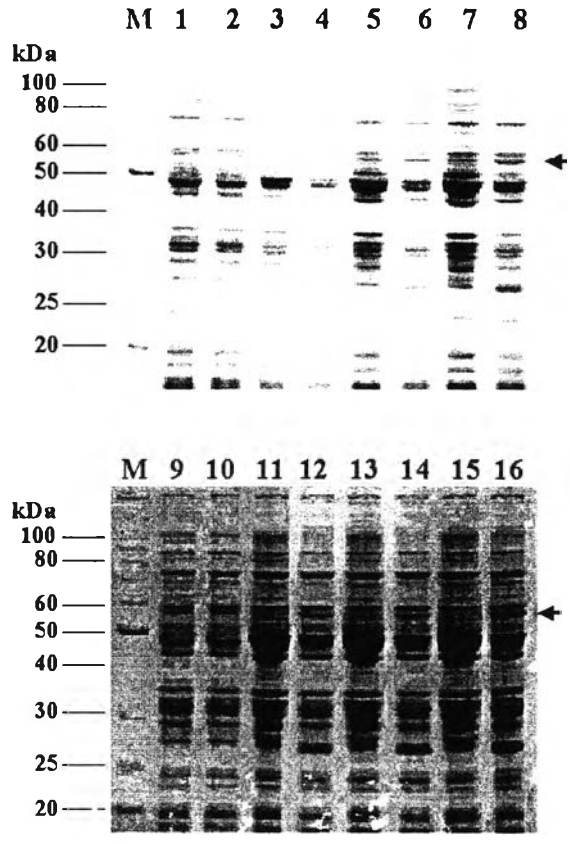

Figure 3.28 SDS-PAGE of AcMRJP2 expression under the control of Trc promoter of pTrcHis2C vector. The *E. coli* DH5α harboring pTrcHis2c-AcMRJP2 was grown at 37 °C for 6 hours after induction with 1 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = BenchMark™ Protein Ladder

Lane 1, 9 = crude extract of the induced *E. coli* containing pTrcHis2C vector at 0 hr

Lane 2,10= crude extract from induced *E. coli* containing TrcHis2C-AcMRJP1 at 0 hr

Lane 3, 5, 7, 11, 13, 15 = crude extract of the induced *E. coli* containing pTrcHis2C vector at 1-6 hr, respectively

Lane 4, 6, 8, 12, 14, 16 = crude extract from induced *E. coli* containing pTrcHis2c-AcMRJP2 at 1-6 hr, respectively

3.3.2.3 Purification of AcMRJP1 or AcMRJP2 from *E. coli* containing pTrcHis2c-AcMRJP1 or pTrcHis2c-AcMRJP2

The supernatant and pellet fractions of *E. coli* lysate harboured recombinant plsmid were analyzed for the presence of AcMRJP1 or AcMRJP2 by SDS-PAGE. As shown in Figure 3.29, most of the expressed AcMRJP1 and AcMRJP2 were express in inclusion body in the pellet fraction (Figure 3.29A and B). Therefore, the pellet fraction of induced samples (1 mM IPTG for 6 hours) were used for further purification. To remove the other contaminant proteins, which co-precipitate with AcMRJP1 and AcMRJP2, the pellet fraction was washed in washing buffer. The pellet fraction of AcMRJP1 and AcMRJP2 inclusion bodies was then solubilized in buffer containing 8 M urea and further purified by Ni^{+2}-affinity chromatography. After washing the column with washing buffer, proteins bound to Ni-NTA resin were eluted using washing buffer containing various concentration of imidazole (0, 20, 60, 100,150, 200, 250, 300 and 500 mM). The SDS-PAGE patterns of all samples (lysate, pellet, supernatant and eluate) are shown in Figure 3.30. The recombinant AcMRJP1 was eluted by washing buffer containg 60 mM imidazole from affinity column chromatography purification. There was minor protein band of approximately 38 kDa eluted with AcMRJP1. The recombinant AcMRJP2 was eluted by washing buffer containing 60 mM imidazole from affinity column chromatography.

3.3.2.4 Immunoblot analysis of AcMRJP1 and AcMRJP2 proteins

The purified rAcMRJP1 (recombinant *A. cerana* major royal protein 1) and rAcMRJP2 were analyzed by SDS-PAGE and Western blot analysis. For rAcMRJP1, two positive protein bands were detected. A major one around 50 kDa that corresponding with the predicted molecular weight of rAcMRJP1(51.3 kDa) and a minor one at about 38 kDa. For rAcMRJP2, one major protein band was observed with a molecular weight around 55 kDa, which is very close to the predicted molecular weight of rAcMRJP2 (Figure 3.30).

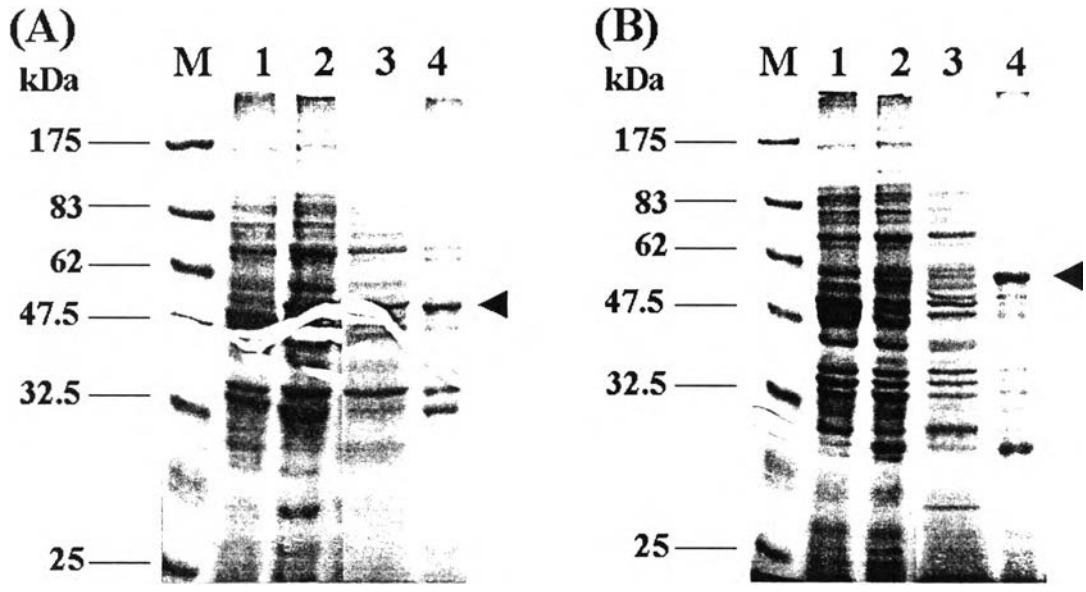

Figure 3.29 SDS-PAGE of *E. coli* DH5α harboring pTrcHis2c-AcMRJP1 (A) or pTrcHis2c-AcMRJP2 (B) grown at 37 °C for 6 hours after induction with 1 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = BenchMark™ Protein Ladder

Lane 1 = Crude extract of the non-induced *E. coli*

Lane 2 = Crude extract of the induced *E. coli*

Lane 3= Soluble fraction of the induced *E. coli*

Lane 4 =Insoluble fraction of the induced *E. coli*

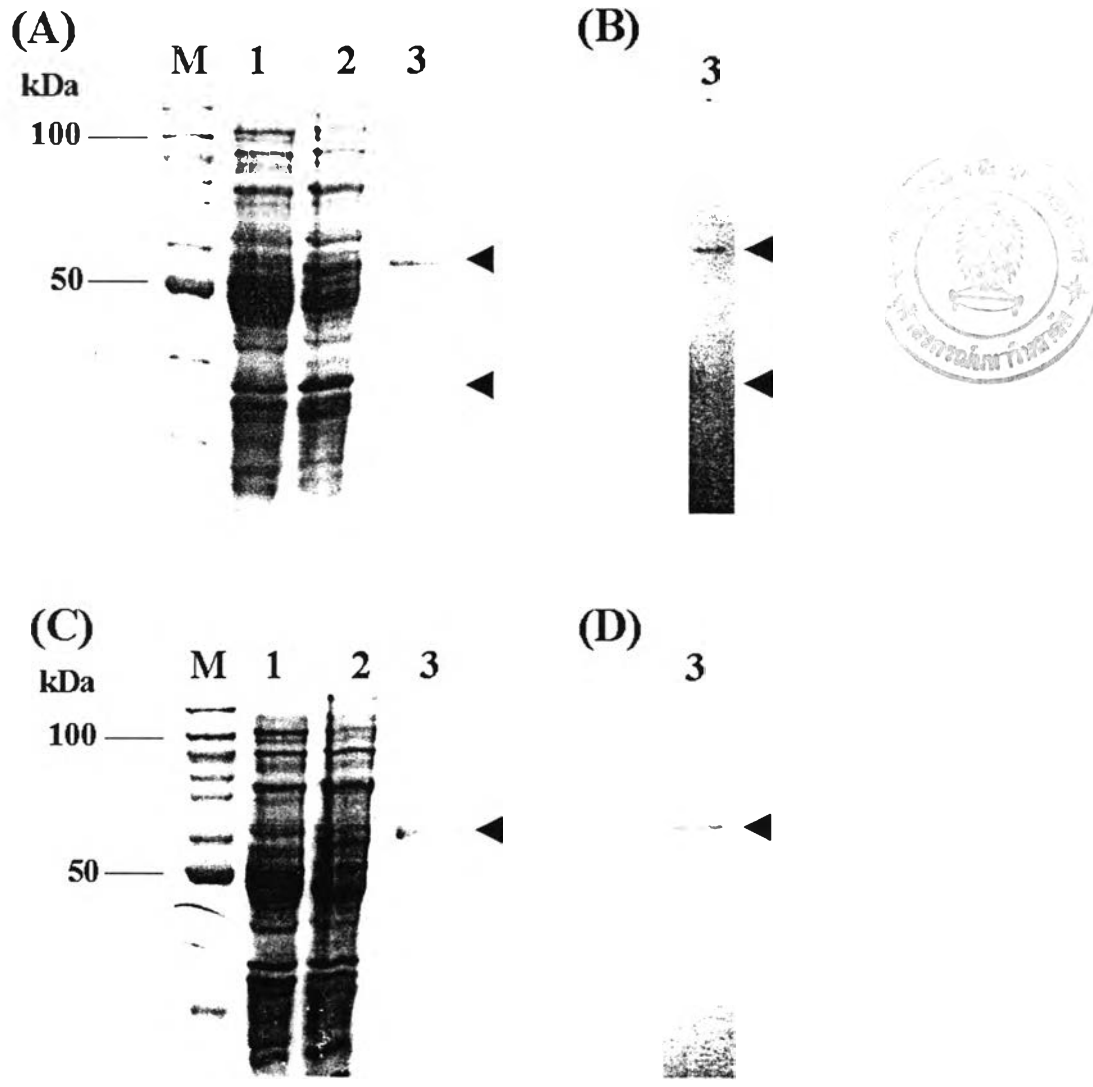

Figure 3.30 SDS-PAGE and Western blot analysis to examine the expression of AcMRJP1 (A and B) and AcMRJP2 (C and D). *E. coli* DH5α harboring pTrcHis2c-AcMRJP1 (A) or pTrcHis2c-AcMRJP2 (B) grown at 37 °C for 6 hours after induction with 1 mM IPTG.

Lane M = BenchMark™ Protein Ladder

Lane 1 = Crude extract of the non-induced *E. coli*

Lane 2 = Crude extract of the induced *E. coli*

Lane 3= Purified protein eluted with 60 mM imidazole

3.3.3 Expression of AcMRJP1 and AcMRJP2 under control of T7 promoter of pET17b vector

3.3.3.1 Construction of AcMRJP1 and AcMRJP2 cDNA under control of T7 promoter of pET17b vector

The AcMRJP1 and AcMRJP2 were expressed as fusion protein with N-terminal His-Tag of the pET17b. The forward primers contained nucleotide sequences encoding for six histidine were designed. The recognition site for *Nhe* I and *Kpn* I were introduced to facilitate further cloning of the PCR products. The AcMRJP1 and AcMRJP2 cDNA fragments were amplified from pRT-AcMRJP1-Pfu and pRT-AcMRJP2-Pfu using *Pfu* DNA polymerase (Promega Corporation Medison, Wisconsin, USA). The amplified products were purified, digested with *Nhe* I and *Kpn* I and cloned into the compatible sites of the pET17b. The ligated products were transformed to *E. coli* DH5α. Seven colonies of each transformation experiment were first screened by colony PCR using T7 and Exp1R or Ex2R primers. Six and five each transformants showed the expected insert fragment of 1,349 bp and 1,448 bp, respectively (Figure 3.31). These recombinant colonies were picked for plasmid extraction and then double digested with *Nhe* I and *Kpn* I. Restriction digestion analysis revealed the presence of the expected of ether AcMRJP1 or AcMRJP2 fragment in the recombinant plasmid. The nucleotide sequence of the insertion fragment was confirmed by sequencing using T7 primer. The obtained nucleotide and deduced amino acid showed that gene fragment had correct reading frame. The recombinant plasmids designated as pET17b-AcMRJP1 and pET17b-AcMRJP2 were electrotransformed to several *E. coli* host strain, *E. coli* BL21(DE3)pLysS, BL21 Star (DE3)pLysS and Rosetta (DE3)pLysS. The transformant were selected on plate containing ampicillin and chloramphenicol. The transformants of various *E. coli* host strain were random picked for screening by colony PCR. The result from electrophoretic analysis showed that all of these clones contained the correct insert fragment.

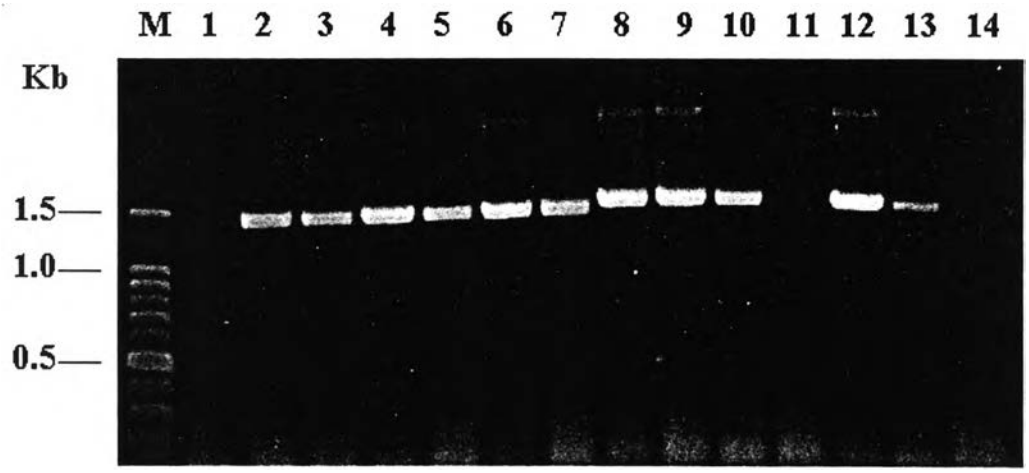

Figure 3.31 Colony PCR analysis of recombinant plasmids pET17b-AcMRJP1 and pET17b-AcMRJP2 analyzed on 1.2% agarose gel

Lane M = 100 bp DNA ladder

Lane 1-7 = Putative recombinant clone containing pET17b-AcMRJP1 (clone 1-7)

Lane 8-14 = Putative recombinant clone containing pET17b-AcMRJP2 (clone 1-8)

3.3.3.2 Expression of AcMRJP1 and AcMRJP2 under control of T7 promoter of pET17b vector

The pET17b plasmids are original and basic pET vectors developed for the cloning and expression recombinant protein in *E. coli*. The target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription promoter, expression is induced by providing a source of T7 RNA polymerase in the *E. coli* host cell. To express the AcMRJP1 and AcMRJP2 as fusion protein with *N*-terminal His-Tag of the pET17b, the forward primers containing nucleotide sequences encoding for six histidine were designed and used to amplify the AcMRJP1 and AcMRJP2 gene from pRT-AcMRJP1-Pfu and pRT-AcMRJP2-Pfu using *Pfu* DNA polymerase.

The recombinant plasmid of pET17b-AcMRJP1 or pET17b-AcMRJP2 were transformed into several *E. coli* host strains; BL21(DE3)pLysS, BL21(DE3)pLysS star, Rosetta(DE3)pLysS, to evaluate *in vivo* expression levels. The expression was performed according to the recommend procedure from the manufacturer. BL21(DE3) pLysS is a host strain used for high-stringency expression of genes constructed in expression vectors containing the bacteriophage T7 promoter. This host is a lysogen of bacteriophage DE3, a lambda derivative that carries the *lac I* gene and the gene for T7 RNA polymerase under *lacUV5* control. Addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase expression, which in turn transcribes the target genes under contol of T7 promoter of pET vectors (Novagen, Madison, WI, USA). BL21 Star (DE3)pLysS host strain is BL21 derivatives containing a mutation in the *rne* gene which encodes the endonuclease RNaseE, a key enzyme responsible for mRNA degradation, enhanced transcript stability and improved protein yield (Invitrogen, USA). Rosetta (DE3)pLysS host strain is BL21 derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA on a compatible chloramphenicol resistant plasmid (Novagen, Madison, WI, USA).

The *E. coli* containing pET17b-AcMRJP1 or pET-17b-AcMRJP2 was cultured, and the expression of AcMRJP1 or AcMRJP2 was achieved by induction with 0.4 mM IPTG at 37 °C. Cell lysate was analyzed for the presence of AcMRJP1 or AcMRJP2 by SDS-PAGE. Under the control of T7 promoter, AcMRJP1 and

AcMRJP2 were expressed as fusion protein of molecular mass approximately 50 and 52 kDa, respectively which corresponded to 47.8 and 51.7 kDa, the calculated molecular weight of recombinant AcMRJP1 and AcMRJP2 protein deduced from DNA sequences. The major protein of either AcMRJP1 or AcMRJP2 was found only in IPTG induced cell lysate but could not be detected in noninduced cell culture (Figure 3.32-3.34). All host strains expressed the AcMRJP1 and AcMRJP2 gene. The results in Figure 3.32 show that BL21(DE3)pLysS host cell expressed the AcMRJP1 and AcMRJP2 as observed expected size band at 1-7 hours after induction. The level of expression increased according to the incubation time and the highest expression level appeared to be at 4 hour after induction with IPTG.

In the BL21 Star (DE3)pLysS host strains, the expected protein band was observed after IPTG induction (Figure 3.33). The level of expression of AcMRJP1 was highest at 4 hours after induction with IPTG and 2 hours after induction in AcMRJP2. The expression level in this host strain were higher than that of BL21 (DE3)pLysS.

The Rosetta (DE3)pLysS host strains expressed the AcMRJP1 and AcMRJP2 as observed expected size band at 1-5 hours after induction (Figure 3.34). The level of expression increased according to the incubation time and the highest expression level appeared to be at 4 hours after induction with IPTG. The expression level in this host strain was higher than that of BL21(DE3)pLysS and BL21 Star (DE3)pLysS host strains.

The results in Figure 3.32-3.34 showed that all host strains could express the AcMRJP1 and AcMRJP2 gene but Rosetta host cell showed the highest expression yield. Therefore, the Rosetta host strain was chosen to carry on further experiments of purification. Based on the above observation, scale-up of expresssion experiment was performed using Rosetta cells induced with 0.4 mM IPTG for 4 hours. One hundred millilitres culture expressing AcMRJP1 or AcMRJP2 was grown and induced with 0.4 mM IPTG for 4 hours and these proteins were purified using a Ni/NTA column.

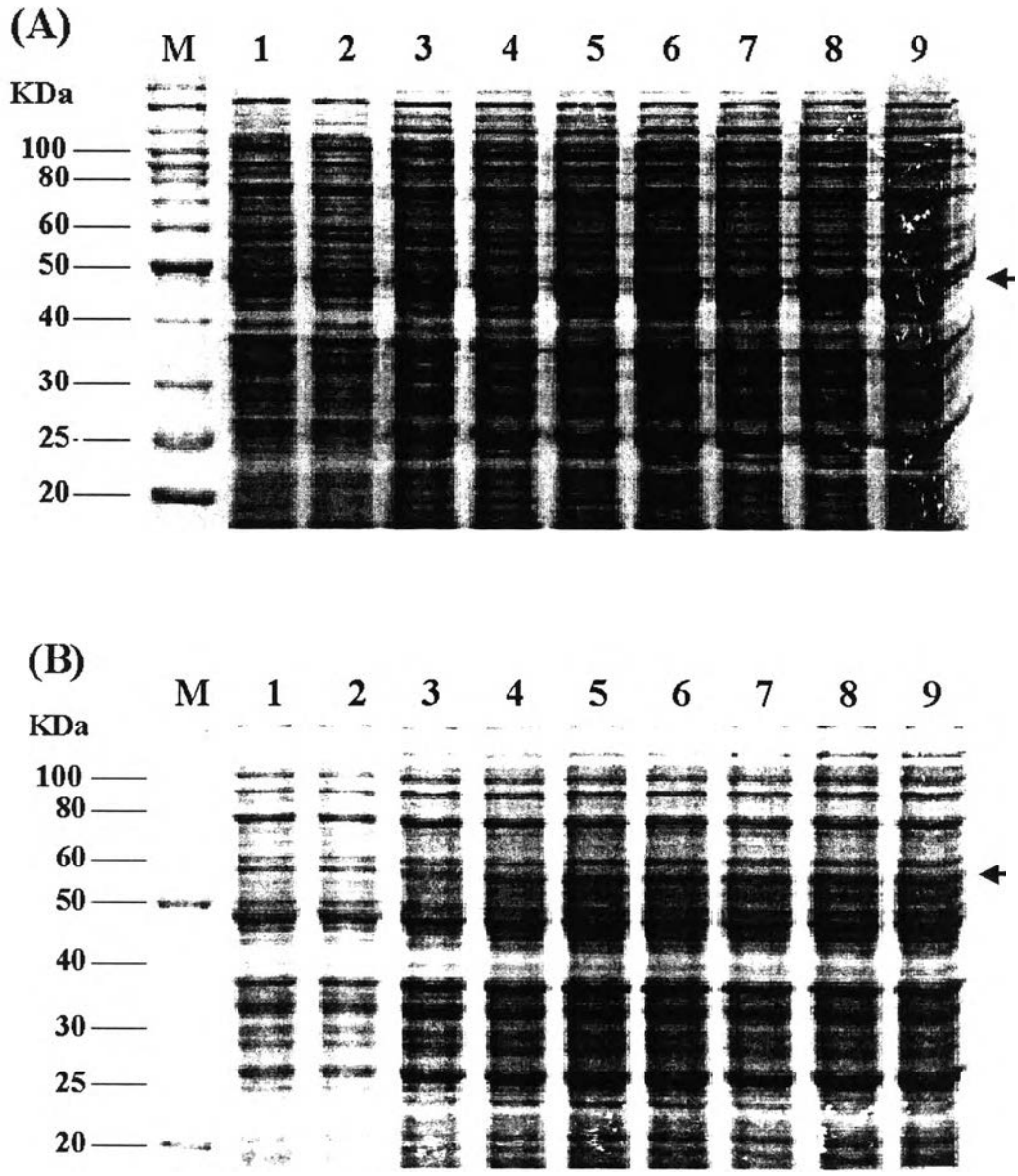

Figure 3.32 SDS-PAGE of AcMRJP1 (A) and AcMRJP2 (B) expression in *E. coli* BL21(DE3)pLysS under the control of T7 promoter of pET17b vector. The BL21 (DE3)pLysS harboring pET17b-AcMRJP1 (A) and pET17b-AcMRJP2 (B) was grown at at 37 °C for 7 hours after induction with 0.4 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = BenchMark™ Protein Ladder

Lane 1 = Crude extract of the non-induced *E. coli* containing pET17b vector

Lane 2-9 = Crude extract from induced *E. coli* at 0, 1, 2, 3, 4, 5, 6 and 7 hour, respectively

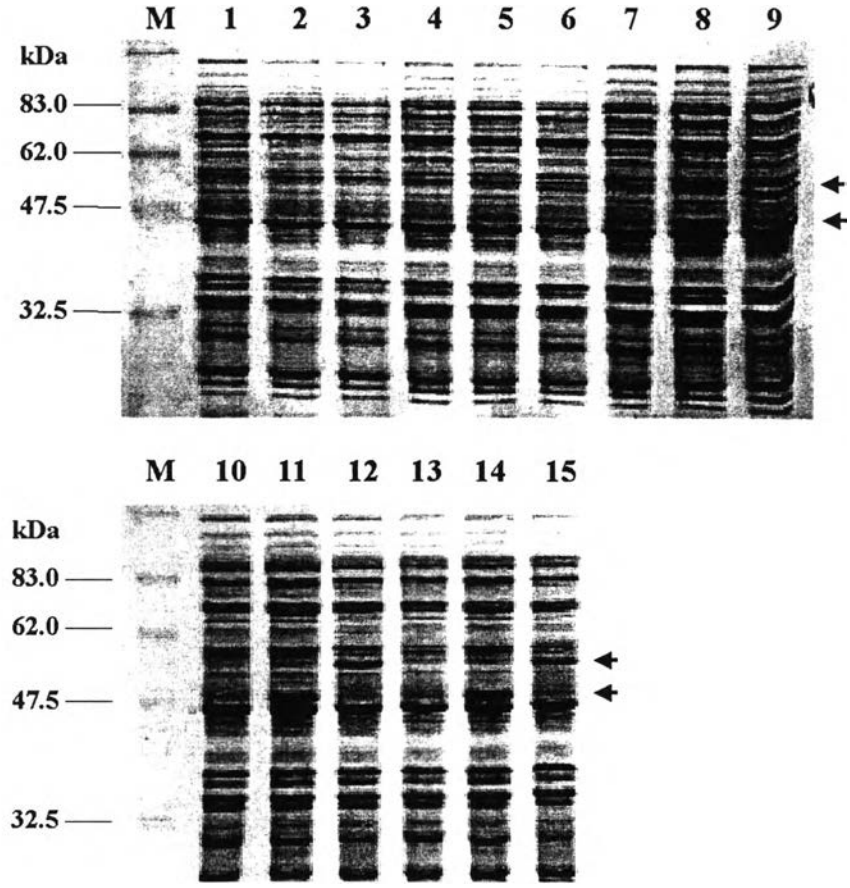

Figure 3.33 SDS-PAGE of AcMRJP1 and AcMRJP2 expression in *E. coli* BL21 Star (DE3)pLysS under the control of T7 promoter of pET17b vector. The BL21 Star (DE3)pLysS harboring pET17b-AcMRJp1 and pET17b-AcMRJP2 was grown at 37 C for 8 hours after induction with 0.4 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = Protein marker

Lane 1, 4, 7, 10, 13 = Crude extract of the induced *E. coli* containing pET17b vector at 0, 1, 2, 4 and 8 hour, respectively

Lane 2, 5, 8, 11, 14 =Crude extract of the induced *E. coli* containing pET17b-AcMRJP1 at 0, 1, 2, 4 and 8 hour, respectively

Lane 3, 6, 9, 12, 15 =Crude extract of the induced *E. coli* containing pET17b-AcMRJP2 at 0, 1, 2, 4 and 8 hour, respectively

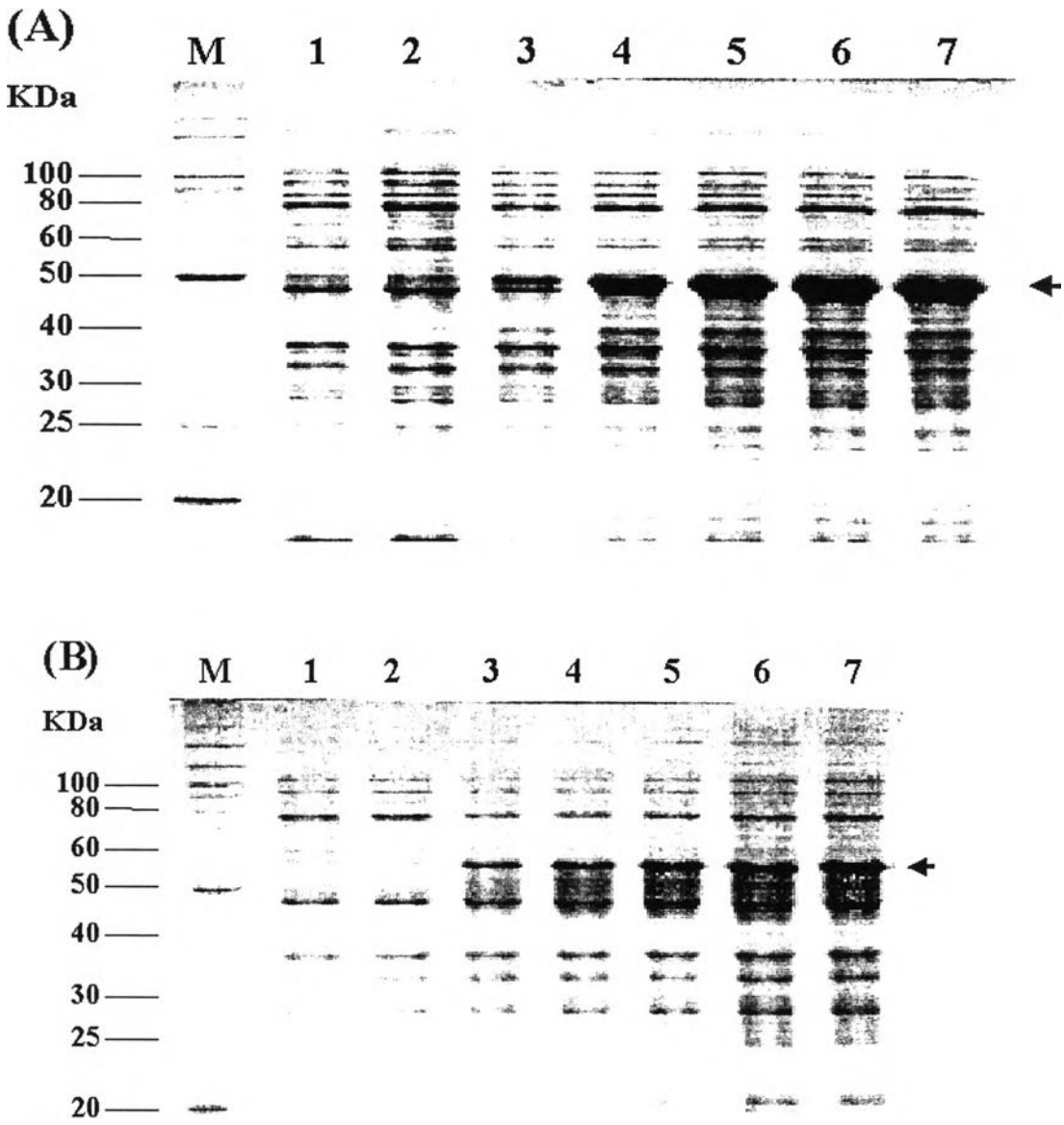

Figure 3.34 SDS-PAGE of expressed AcMRJP1 (A) and AcMRJP2 (B) in *E. coli* Rosetta (DE3)pLysS under the control of T7 promoter of pET17b vector. The Rosetta (DE3)pLysS harboring pET17b-AcMRJP1 (A) and pET17b-AcMRJP2 (B) was grown at at 37 °C for 7 hours after induction with 0.4 mM IPTG. The crude extract was prepared and analyzed by SDS-PAGE.

Lane M = BenchMark™ Protein Ladder

Lane 1 = Crude extract of the non-induced *E. coli* containing pET17b vector

Lane 2-7 =Crude extract of the induced *E. coli* at 0, 1, 2, 3, 4 and 5 hour, respectively

3.3.3.3 Purification of AcMRJP1 or AcMRJP2 from recombinant clone harboring pET17b-AcMRJP1 or pET17b-AcMRJP2

Aliquots of supernate and pellet fractions were analyzed for the presence of AcMRJP1 and AcMRJP2 by SDS-PAGE. The results showed that most of the expressed AcMRJP1 and AcMRJP2 were detected in the precipitate of induced sample but not present in supernatant fraction (Figure 3.35A and B). From the result, it was concluded that the AcMRJP1 and AcMRJP2 were expressed as inclusion bodies in pellet fraction. Therefore, the pellet fraction of induced samples were used for further purification. To remove the other contaminant proteins, which co-precipitate with AcMRJP1 and AcMRJP2, the pellet fraction was washed in washing buffer. The pellet fraction of AcMRJP1 and AcMRJP2 inclusion bodies were then solubilized in buffer containing 8 M urea and further applied to Ni^{+2}-affinity chromatography for purification. After washing the column with washing buffer, proteins bound to Ni-NTA resin were eluted using washing buffer containing various concentration of imidazole (0, 20, 60, 100,150, 200, 250, 300 and 500 mM), respectively. The SDS-PAGE patterns of all samples (lysate, pellet, supernatant and eluate) were shown in Figure 3.35. The recombinant AcMRJP1 and AcMRJP2 was eluted by washing buffer containing 250 mM imidazole. After affinity column chromatography purification, the protein fractions was dialyzed to remove imidazole. The yield of purified AcMRJP1 and AcMRJP2 from the 1 liter flask culture was approximately 20 mg and 8 mg, respectively.

3.3.3.4 Immunoblot analysis and *N*-terminal protein sequence of AcMRJP1 and AcMRJP2

molecular weight of purified rAcMRJP1 and rAcMRJP2 were 47.9 and 51.7 kDa determined by SDS–PAGE and positively identified by Western blot analysis (Figure 3.35). The purified AcMRJP1 and AcMRJP2 separated on the SDS-PAGE were transferred to PVDF membrane by electroblotting. The *N*-terminal sequence of AcMRJP1 and AcMRJP2 were determined by amino acid sequencing. Twenty residues were obtained from the *N*-terminus of both AcMRJP1 and AcMRJP2, which were ASHHHHHHSILRGESLNKSL and ASHHHHHHAIIRQN(S/N)(S/A)KNL, respectively. The sequences were identical with that predicted from the corresponding open reading frame of AcMRJP1 and AcMRJP2.

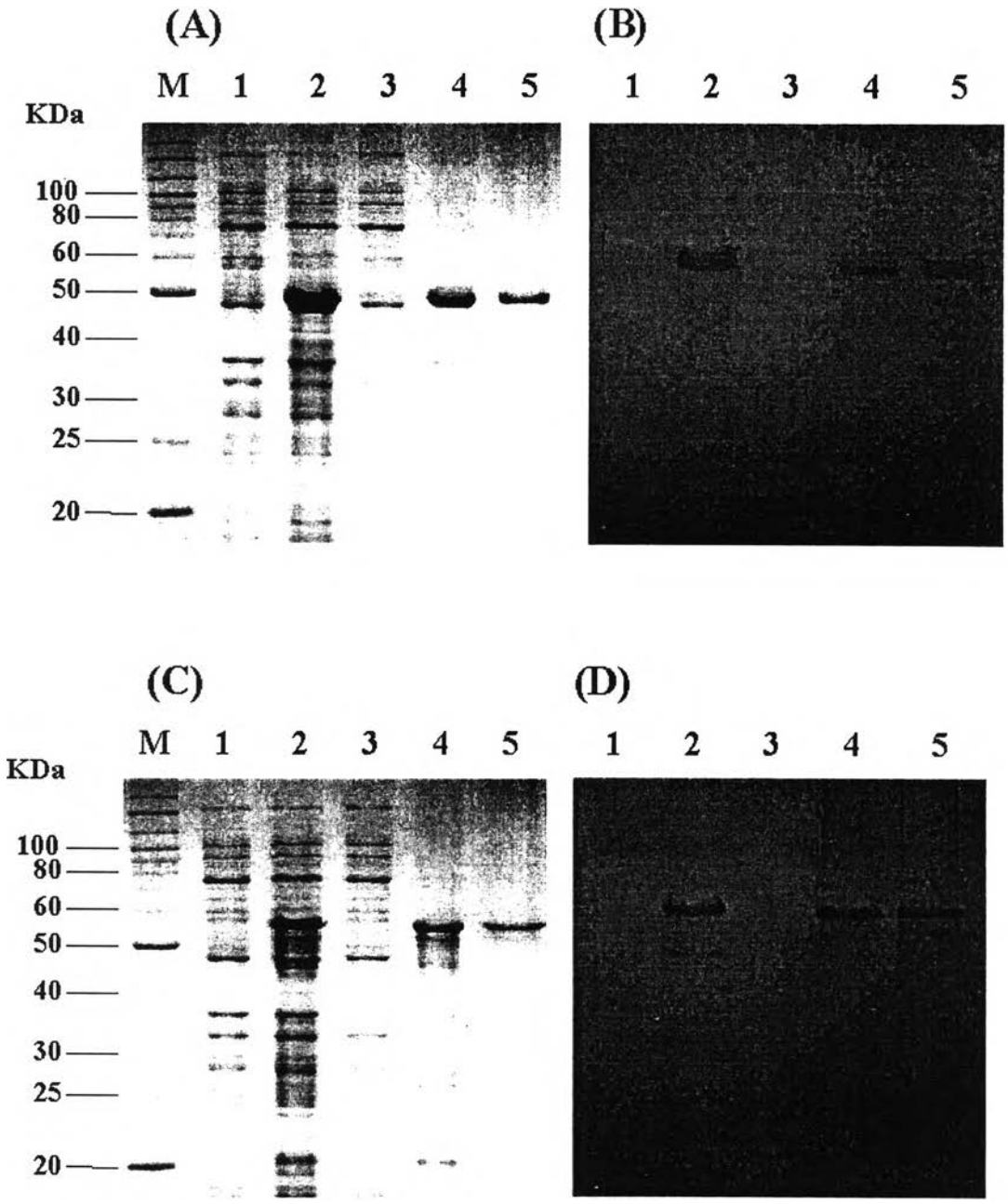

Figure 3.35 SDS-PAGE (A and C) and Western blot (B and D) analyses to examine the expression of AcMRJP1 (A and B) and AcMRJP2 (C and D)

Lane M = BenchMark™ Protein Ladder

Lane 1 = Crude extract of the non-induced *E. coli*

Lane 2 = Crude extract of the IPTG induced *E. coli*

Lane 3= Soluble fraction of the IPTG induced *E. coli*

Lane 4 =Insoluble fraction of the IPTG induced *E. coli*

Lane 5 = Purified AcMRJP

3.4 Expression of AcMRJP1 in plant expression system

3.4.1 Construction of expression vector

3.4.1.1 Amplification of AcMRJP1 gene for cloning into plant expression cassette

The AcMRJP1 gene was amplified by PCR from pET17b-AcMRJP1 using F1500SalKosak and R1500Exp primers. The oligonucleotide sequence surrounding the translation initation codon of the AcMRJP1 gene was changed to a preferred nucleotide context for translation in eukaryotic cells (Kozak, 1981). The oligoncleotide sequence encoding the ER retention signal (KDEL) and the hexa-histidine coding sequence were included at the 3' end of the coding sequence of the AcMRJP1 gene. The restriction site of *Sal* I and *Kpn* I was introduced to the 5'end of primers to facilitate cloning.

The strong DNA fragment of approximately 1.3 kb, which was the expected size of AcMRJP1 gene, was obtained (Figure 3.36). The PCR product was extracted and purified from agarose gel. The PCR-amplified fragment of AcMRJP1 was digested with *Sal* I and *Kpn* I and used for subsequence cloning.

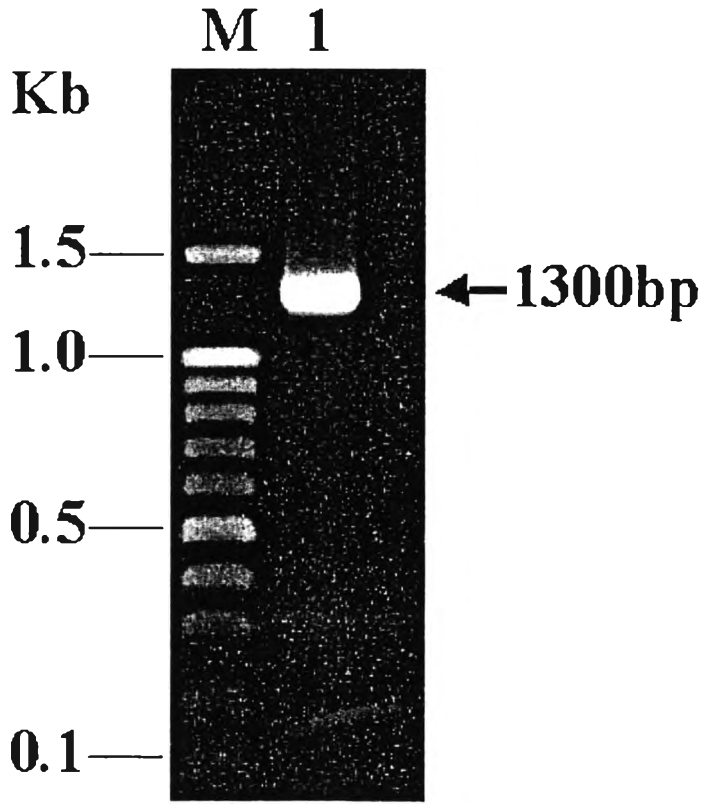

Figure 3.36 Amplification of the AcMRJP1 gene from pET17b-AcMRJP1 using specific primers (F1500SalKosak and R1500Exp primers). PCR product was analyzed by 1.2% agarose gel electrophoresis and stained with ehtidium bromide.

Lane M = 100 bp DNA ladder

Lane 1 = PCR amplification product

3.4.1.2 Amplification of Nopaline synthase (Nos) terminator fragment

The Nos terminator was amplified by PCR using FNosHis and RNosHis primers. 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 328 bp of PCR-amplified fragment of Nos was purified and digested with *Kpn* I and *Eco*R I and and used for subsequence cloning (Figure 3.37).

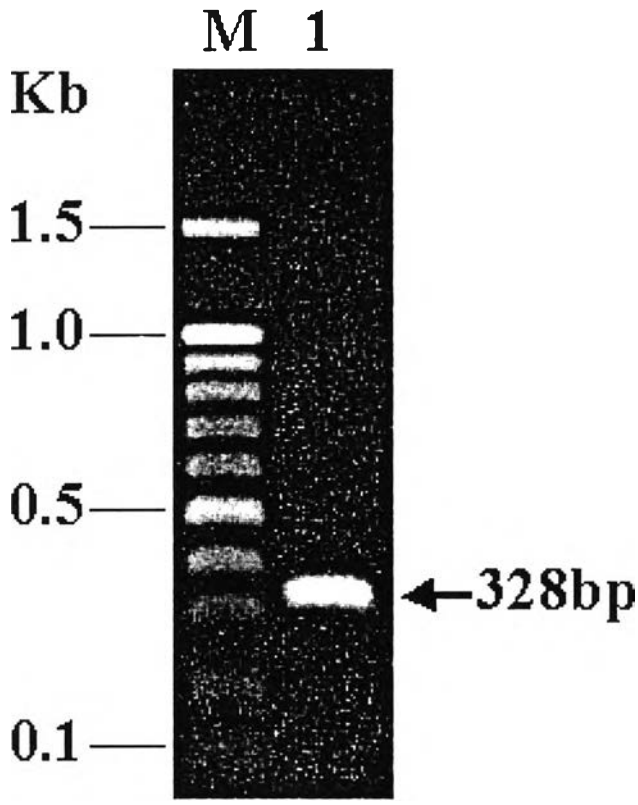

Figure 3.37 Amplification of the Nos terminator from pCMABIA2301 using specific primers (FNoshis and RNoshis primers). PCR product was analyzed by 1.2% agarose gel electrophoresis and stained with ehtidium bromide.

Lane M = 100 bp DNA ladder

Lane 1 = PCR amplification product

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

The expression cassette containing the GBSS gene promoter, AcMRJP1 and the nos terrminator was constructed in pGEM®-GBSS-AcMRJP1-Nos as described in 2.6.1.1. The cassette was isolated by *Hin*d III and *Eco*R I digestion. The 2.4 kb fragment was ligated into *Hin*d III and *Eco*R I site of plant transformation vector, pCAMBIA2301 (Appendix A) and transformed into *E. coli* DH5α strain. The pCAMBIA2301 contains the GUS (*uid*A) with an intron as a reporter gene, the kanamycin-resistant gene (*npt II*) as a plant and bacterial selectable marker within the T-DNA. Each gene was under the control of an 35S promotor. After transformation, six kanamycin resistant colonies were randomly picked and analyzed by colony PCR screening. The recombinant plasmids were extracted and then digested with *Hin*d III and *Eco*R I to determine the corrected insert fragment. The insert fragment size was determined as approximately 2.4 kb. Restriction endonuclease analysis of this recombinant plasmid with *Kpn* I release a 14 kb linear fragment corresponding to the size of this vector (11.6 kb) plus insert fragment of 2.4 kb (Figure 3.38). The sequence at the 5' end of the AcMRJP1 gene was verified by sequencing. The result showed that the AcMRJP1 gene in recombinant plasmids contained the ATG initiation codon in-frame (translatable) with ORF of AcMRJP1 gene. The resulting plasmid was designated as pCAMBIA2301-GBSS-AcMRJP1-Nos.

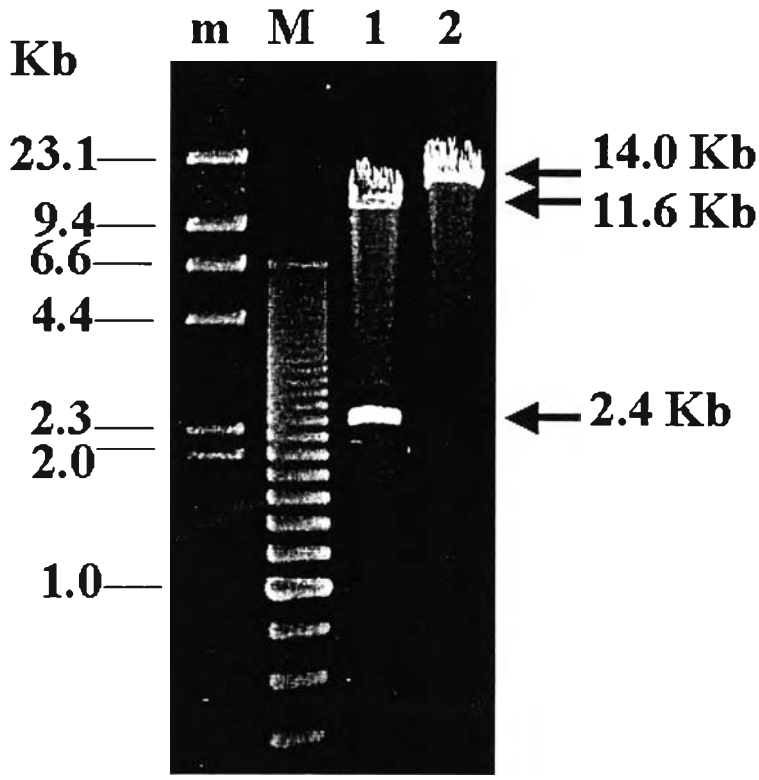

Figure 3.38 Restriction analysis of recombinant plasmid pCAMBIA2301-GBSS-AcMRJP1-Nos analyzed on 1.2% agarose gel.

Lane m = λ / *Hin*d III standard marker

Lane M = 200 bp DNA ladder

Lane 1 = pCAMBIA2301-GBSS-AcMRJP1-Nos digested with *Hin*d III and *Eco*RI

Lane 2 = pCAMBIA2301-GBSS-AcMRJP1-Nos digested with *Kpn* I

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

The expression cassette containing the B33 patatin gene promoter, AcMRJP1 and the nos terrminator was constructed in pGEM®-Nos-AcMRJP1-B33 as described in 2.6.1.2. The cassete was isolated by *Hin*d III and *Eco*R I digestion. The 3.1 kb fragment was ligated into *Hin*d III and *Eco*R I site of plant transformation vector, pCAMBIA2301 and transformed into *E. coli* DH5α strain. After transformation, six kanamycin resistant colonies were randomly picked and analyzed by colony PCR screening. The recombinant plasmids were extracted and then digested with *Hin*d III and *Eco*R I to determine the corrected insert fragment. The insert fragment size was determined as approximately 3.1 kb. Restriction endonuclease analysis of this recombinant plasmid with *Kpn* I release two fragments of 2.7 and 11.9 kb (Figure 3.39). The sequence at the 5' end of the AcMRJP1 gene was verified by sequencing. The result showed that the AcMRJP1 gene in recombinant plasmids contained the ATG initiation codon in-frame (translatable) with ORF of AcMRJP1 gene. The resulting plasmid was designated as pCAMBIA2301-B33-AcMRJP1-Nos.

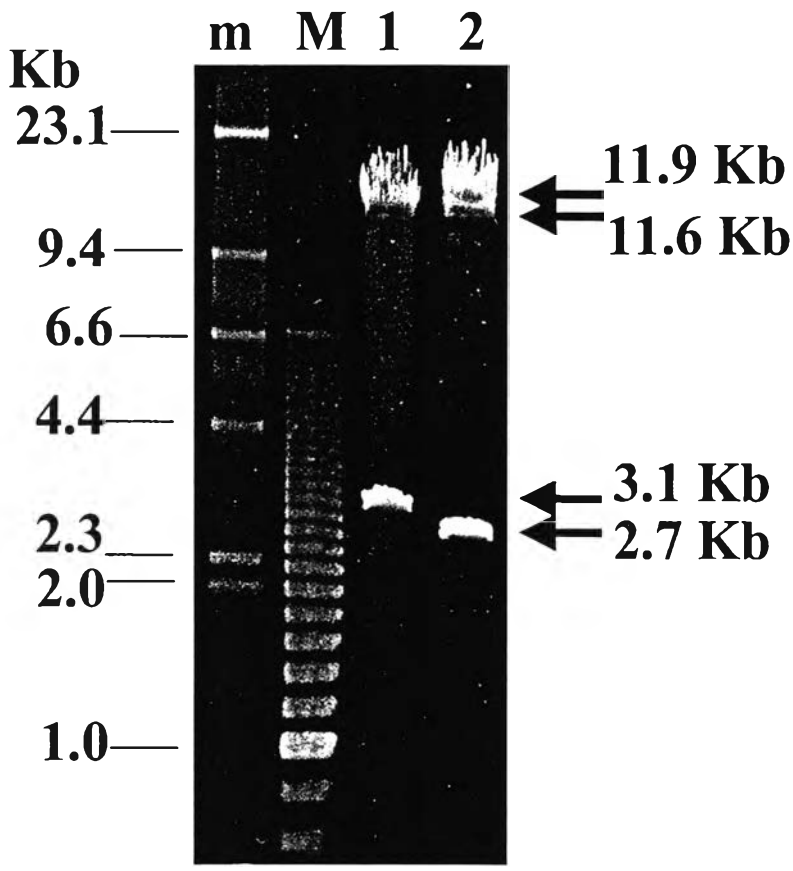

Figure 3.39 Restriction analysis of recombinant plasmid pCAMBIA2301-B33-AcMRJP1-Nos analyzed on 1.2% agarose gel.

Lane m = λ / *Hin*d III standard marker

Lane M = 200 bp DNA ladder

Lane 1 = pCAMBIA2301-B33-AcMRJP1-Nos digested with *Hin*d III and *Eco*RI

Lane 2 = pCAMBIA2301-B33-AcMRJP1-Nos digested with *Kpn* I

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

The CaMV 35S promoter was introduced into the binary transformation vector pCAMBIA2301 as a *Hin*d III / *Xho* I fragment and ligated into *Hin*d III / *Sal* I of plasmid pCAMBIA2301-AcMRJP1-Nos as described in 2.6.1.3. The AcMRJP1 in the construct was flanked by strong constitutive CaMV 35S promoter and a nos terminator. The ligated product was transformed into *E. coli* DH5α strain. After transformation, six kanamycin resistant colonies were randomly picked and analyzed by colony PCR screening. The recombinant plasmids were extracted and then digested with *Kpn* I. Restriciton endonuclease analysis of this recombinant plasmid released two fragments of 2.3 and 11.9 kb (Figure 3.40). The sequence at the 5' end of the AcMRJP1 gene was verified by sequencing. The result showed that the AcMRJP1 gene in recombinant plasmids contained the ATG initiation codon in-frame (translatable) with ORF of AcMRJP1 gene. The resulting plasmid was designated as pCAMBIA2301-35S-AcMRJP1-Nos.

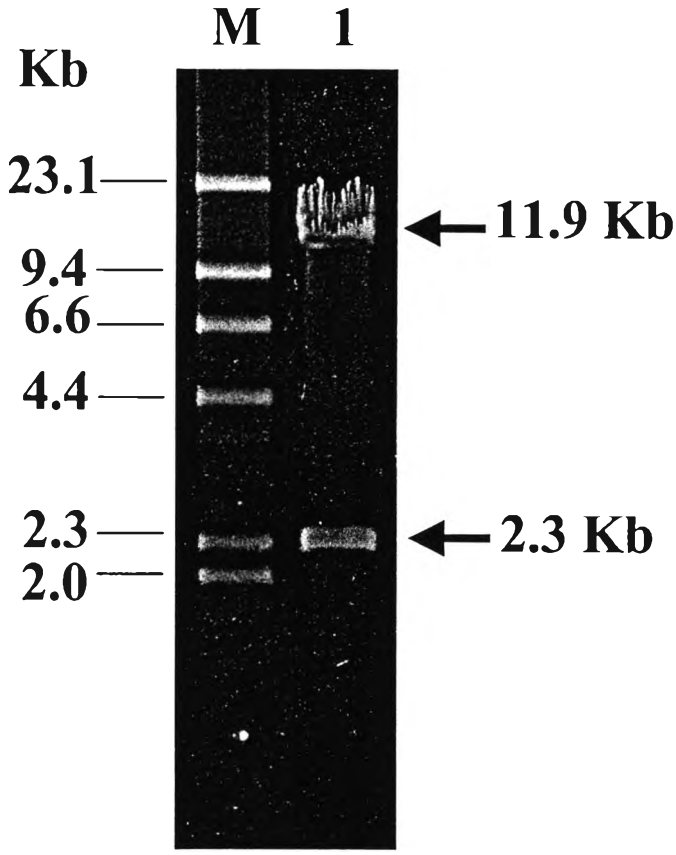

Figure 3.40 Restriction analysis of recombinant plasmid pCAMBIA2301-35S-AcMRJP1-Nos analyzed on 1.2% agarose gel.

Lane M = λ / *Hin*d III standard marker

Lane 1 = pCAMBIA2301-35S-AcMRJP1-Nos digested with *Kpn* I

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

For rice transformation, pCAMBIA1301 was employed. The CaMV 35S promoter was introduced into the binary transformation vector pCAMBIA1301 (Appendix A) as a *Hin*d III / *Xho* I fragment and ligated into *Hin*d III / *Sal* I of plasmid pCAMBIA1301-AcMRJP1-Nos as described in 2.6.1.4. The AcMRJP1 in the construct was flanked by strong constitutive CaMV 35S promoter and a nos terminator. The ligated product was transformed into *E. coli* DH5α strain. The pCAMBIA1301 contains the GUS (*uid*A) with an intron as a reporter gene, the kanamycin-resistant gene (*npt II*) as a bacterial selectable marker, the hygromycin-resistant gene (*hpt II*) as a plant selectable marker within the T-DNA. Each gene was under the control of an 35S promotor. After transformation, six kanamycin resistant colonies were randomly picked and analyzed by colony PCR screening. The recombinant plasmids were extracted and then digested with *Kpn* I to determine the corrected insert fragment. Restriciton endonuclease analysis of this recombinant plasmid release two fragments of approximately 2.3 and 12.2 kb (Figure 3.41). The sequence at the 5' end of the AcMRJP1 gene was verified by sequencing. The result showed that the AcMRJP1 gene in recombinant plasmids contained the ATG initiation codon in-frame (translatable) with ORF of AcMRJP1 gene. The resulting plasmid was designated as pCAMBIA1301-35S-AcMRJP1-Nos.

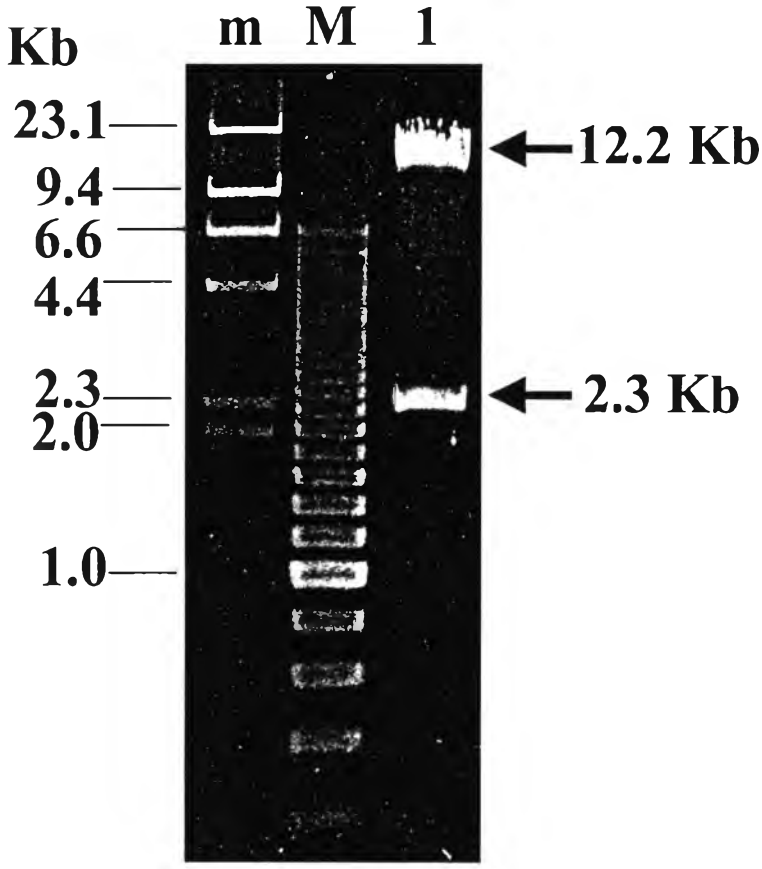

Figure. 3.41 Restriction analysis of recombinant plasmid pCAMBIA1301-35S-AcMRJP1-Nos analyzed on 1.2% agarose gel.

Lane m = λ / *Hin*d III standard marker

Lane M = 200 bp DNA ladder

Lane 1 = pCAMBIA1301-35S-AcMRJP1-Nos digested with *Kpn* I

3.4.2 Plant transformation

3.4.2.1 Transformation of potato

Agrobacterium-mediated T-DNA transformation was used to introduced the AcMRJP1 construct into potato *Solanum tuberosum* cultivar Atlantic. Three constructs were produced for transformation of AcMRJP1 in potato. The binary plasmid pCAMBIA2301- GBSS -AcMRJP1-Nos, pCAMBIA2301-B33-AcMRJP1-Nos and pCAMBIA2301-35S-AcMRJP1-Nos, carrying the translatable of AcMRJP1, β-glucuronidase (*gus*) reporter gene and a kanamycin-resistance selectable marker (*npt* II) genes, were constructed and transformed into *A. tumefaciens* EHA105 by electroporation.

The sprouting shoot of potato was sterilized and grown on shoot culture media for two to three weeks (Figure 3.42). The leaves of *in vitro* grown were used as starting material for leaf disks transformation by co-cultivation with *A. tumefaciens* EHA105 carrying the recombinant plasmid (Figure 3.43). After the co-cultivation, potato segments were transferred to the selective regeneration medium supplemented with 50 mg/l kanamycin and 250 mg/l cefotaxime to select the transformed shoots and eliminated Agrobacterial overgrow, respectively (Figure 3.44). The calli on the cut edges of the explants were observed after 2-3 weeks of cocultivation. These explant were transferred to fresh selection medium, shoots were regenerated form resistant calli within 8 weeks. There were 9 shoots regenerated from 110 explants (8.2%) transformed with pCAMBIA2301-GBSS-AcMRJP1-Nos, 10 shoots regenerated from 100 explants (10%) transformed with pCAMBIA2301-B33-AcMRJP1-Nos and 6 shoots regenerated from 100 explants (6%) transformed with pCAMBIA2301-35S-AcMRJP1-Nos (Figure 3.42). Shoot were elongated on regeneration media until they were 1-1.5 cm long, then excised from the leaf explant and subcultured on the RIM or selective root induction media for 2 weeks. The nodal segments of *in vitro* grown plantlet were subsequently transferred to microtuber induction medium (Figure 3.45). No differences were observed in the morphology between transformed and nontransformed plants.

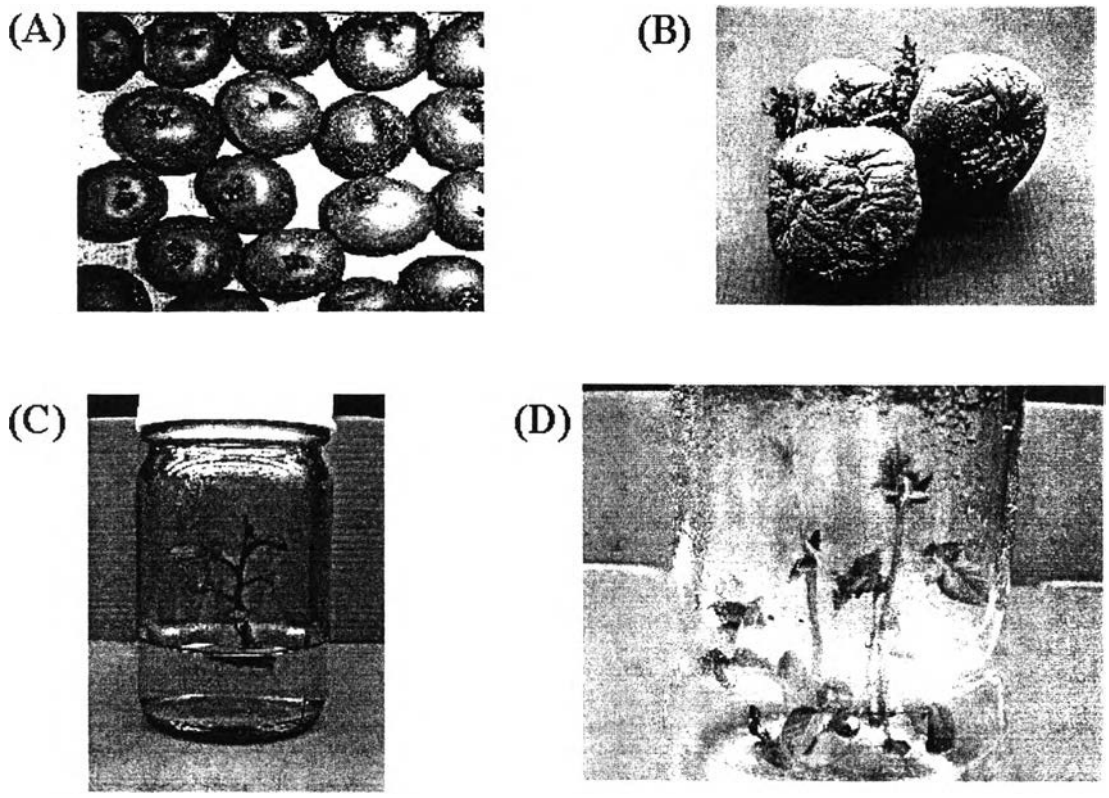

Figure 3.42 Tissue culture of potato (*S. tuberosum*) cultivar Atlantic

(A) Tuber of potato used in tissue culture to produce potato plantlet *in vitro*

(B) Sprouting shoot formation

(C and D) Potato plantlet *in vitro*

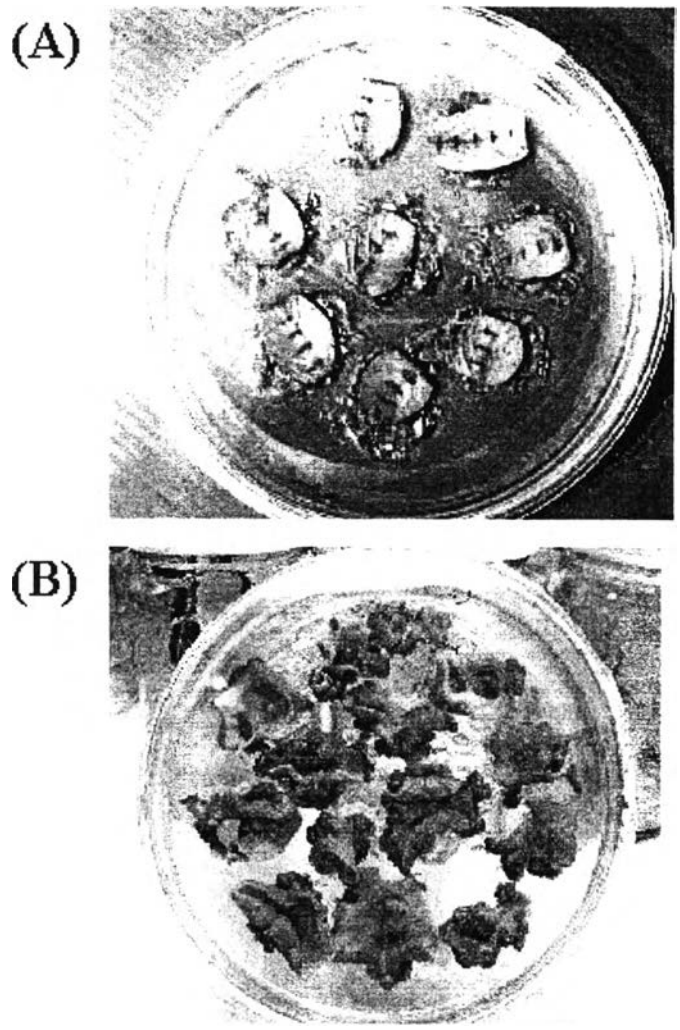

Figure 3.43 Callus formation of transgenic potato plant

(A) Culture of potato leaf discs after co-cultivation with *Agrobacterium*

(B) Callus formation on selective regeneration medium for 4 weeks

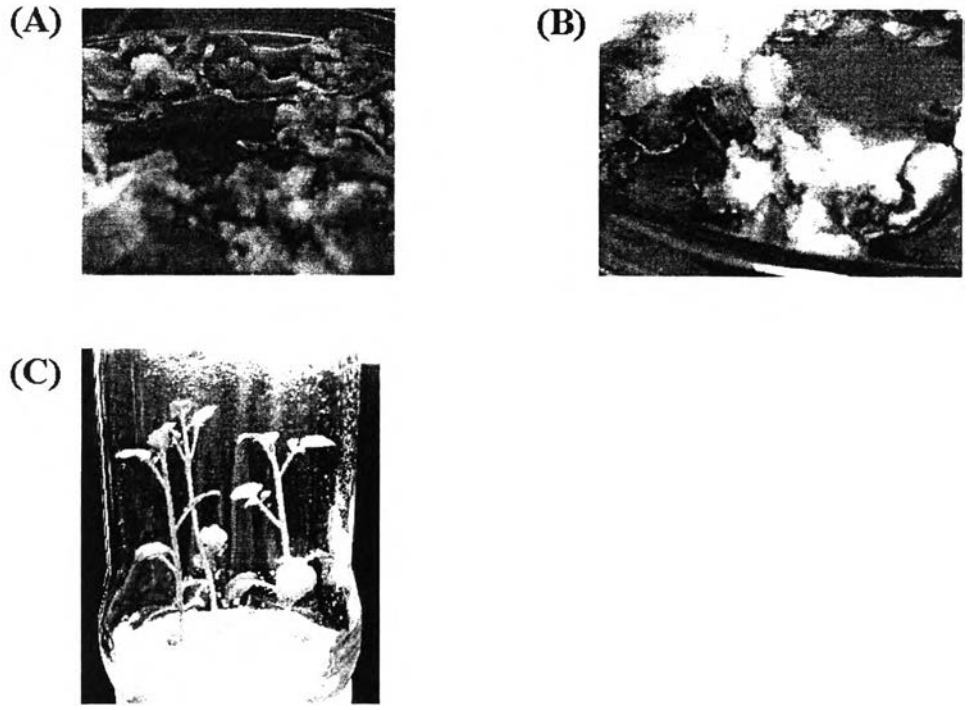

Figure 3.44 Regeneration of transformed potato.

(A) Callus formation of potato

(B) Shoot induction on selective regeneration medium for 8 weeks

(C) Transformed shoot on selective root induction medium

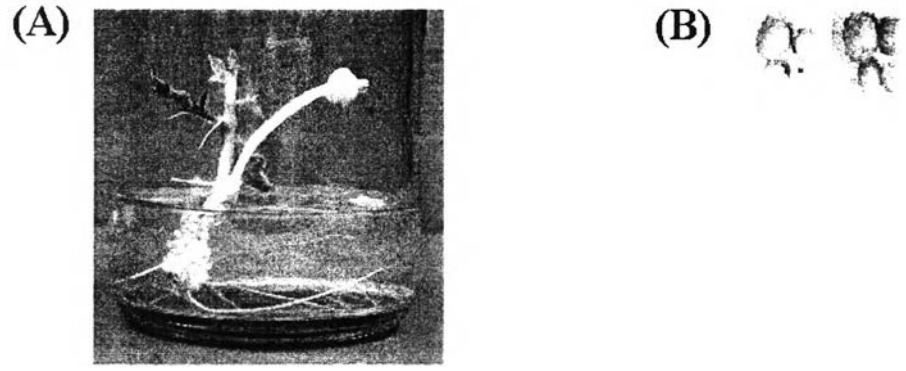

Figure 3.45 *In vitro* microtuberization of transformed potato

(A) Microtuber formation of transformed plant grown *in vitro* for 8 weeks in the dark on medium containing 8% sucrose

(B) Microtuber from transformed (left) and non-transformed control potato (right)

3.4.2.2 Transformation of rice

A. tumefaciens strain EHA105 habouring pCAMBIA1301-AcMRJP1-Nos, a strong constitutive 35S-AcMRJP1-Nos expression construct, was used for transformation of rice. The seeds of rice were sterilized and grown in callus induction NB medium. Callus is a mass of undifferentiated plant cells which, depending on the presence of different growth substances, can be induced to form shoots or roots. The six weeks old of rice calli were used as target tissues for *Agrobacterium*-mediated transformation (Figure 3.46).

After co-cultivation with *Agrobacterium* carrying the recombinant plasmid, the transformed calli were transferred to the selection medium containing 50 mg/l hygromycin to inhibit growth of non-transformed rice cells and supplemented with 500 mg/l cefotaxime to inhibit *A. tumefaciens* growth. Hygromycin resistant calli were obtained after 4 weeks selection. These growing calli were excised and transferred to fresh selection medium and incubated in the dark at 28 °C for 4 weeks. The uninoculated control calli did not show continuous growth, turned brown and died in selection medium (Figure 3.47). The resistant calli were transferred to selective regeneration medium. Shoots were regenerated form resistanat calli within four weeks on regeneration medium. When the shoot were 1.0 to 1.5 cm height, shoots were cut from the calli and subcultured on root induction media. A total of 6 hygromycin-resistant putative transformants were obtained from transformation of 80 explants for an apparent transformation efficient of 7.5%. Plantlets with extensive root systems were established on root induction medium after another 2-3 weeks (Figure 3.48). No differences were observed in the morphology between transformed and untransformed plants. These putative transformant were subsequently transferred to soil.

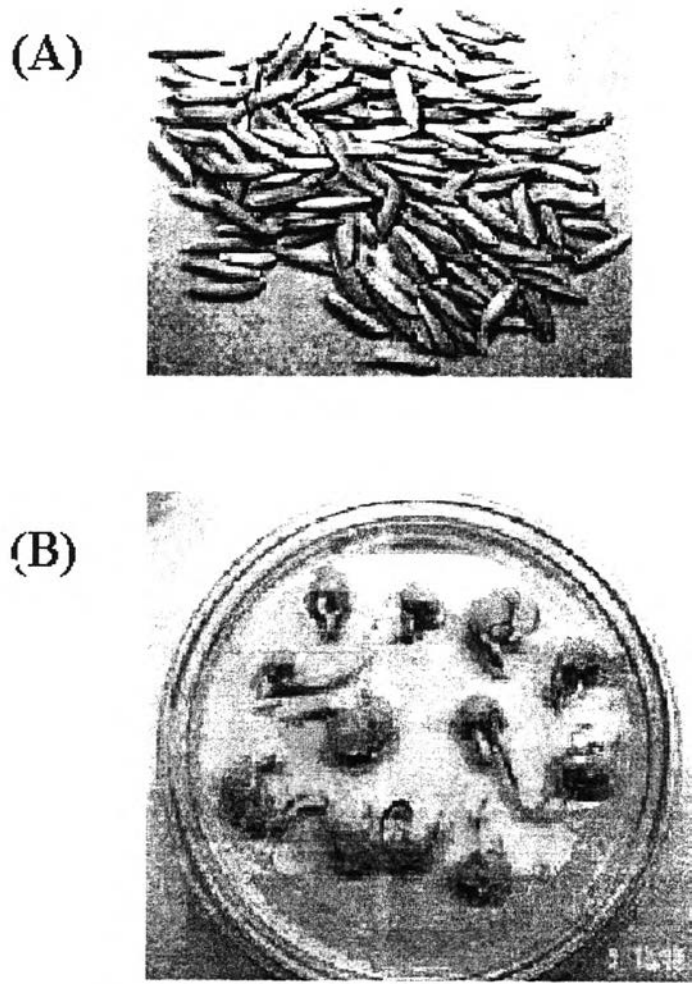

Figure 3.46 Tissue culture of rice *O. sativa* cutivar KDML105 used in tissure culture

(A) Mature seed of rice

(B) Callus formation of rice on callus induction medium

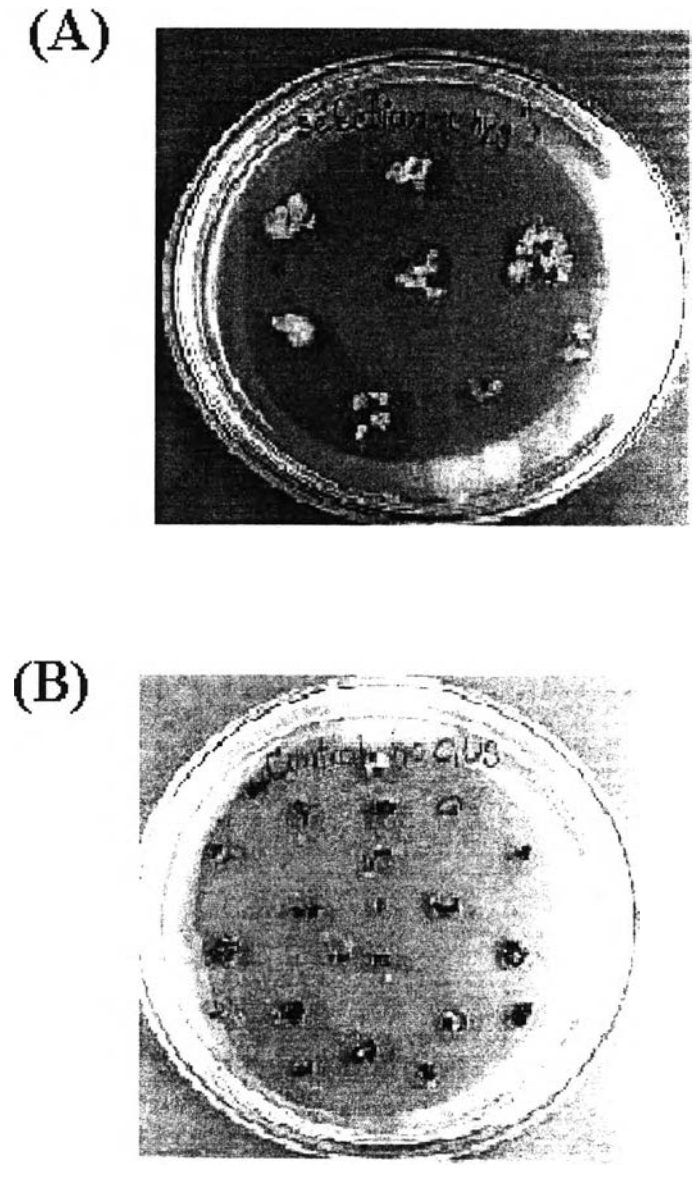

Figure 3.47 Selection of transformed rice callus on selection medium containing 50 mg/l hygromycin and 500 mg/l cefotaxime

(A) Resistant callus growing on selective hygromycin medium after 8 weeks

(B) Brown zone of non-transformed callus

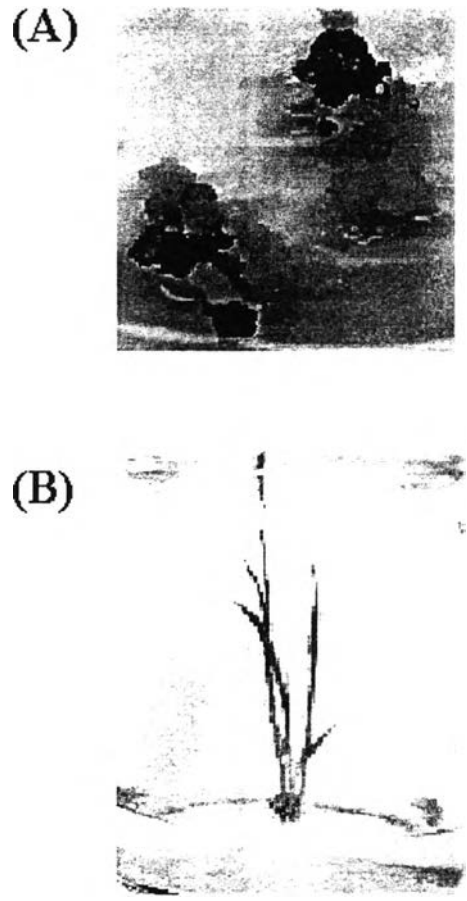

Figure 3.48 Regeneration of transformed rice plant

(A) Resistant callus formation

(B) Plantlet with root system

3.4.3 Molecular analysis of transgenic plants

3.4.3.1 Histochemical analysis of GUS expression in putative transformants

The binary plasmid pCAMBIA2301-GBSS-AcMRJP1-Nos, pCAMBIA2301-B33-AcMRJP1-Nos, pCAMBIA2301-35S-AcMRJP1-Nos and pCAMBIA1301-AcMRJP1-Nos carried β-glucuronidase (*gus*) reporter gene within the T-DNA. This reporter gene provided an indication that genetic transformation did take place. Both transformed potato and rice were subjected to analysis for GUS activity. The blue staining by the activity of β-glucuronidase enzyme was observed on the transformed tissue.

The histochemical assay for GUS activity was analyzed in callus, leaves, roots and tubers of putative transformed potato (Figure 3.49 and 3.50). The potato transformed with pCAMBIA2301-GBSS-AcMRJP1-Nos (7/9), pCAMBIA2301-B33-AcMRJP1-Nos (7/10) and pCAMBIA2301-35S-AcMRJP1-Nos (5/6) showed GUS activity. The intensity of blue color production was different from plant to plant. No GUS enzyme activity was observed in tissues from non-transformed control plants.

The GUS activity was analyzed by histochemical analysis in rice transformed with pCAMBIA1301-35S-AcMRJP1-Nos. Difference organs of rice tissues were submerged in staining solution. Of the six rice plants regenerated, all tested positive for GUS (Figure 3.51-3.52). The histochemical assay for GUS activity in different organs revealed intense blue staining in callus, roots (Figure 3.52), embryo and at the cut surfaces of the leaf pieces. The intensity of blue color production was different from plant to plant. Tissues from non-transformed control plants did not show GUS expression.

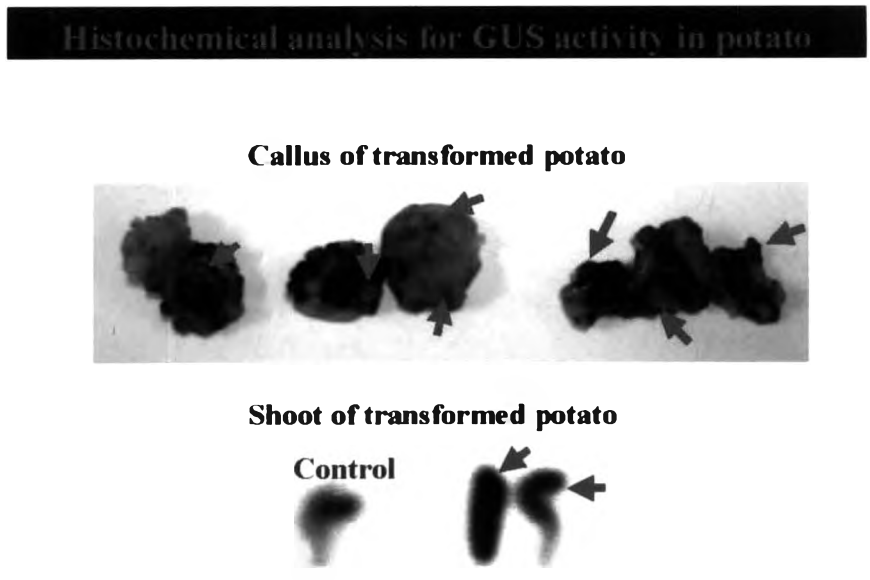

Figure 3.49 Histochemical analysis of primary transformants of potato

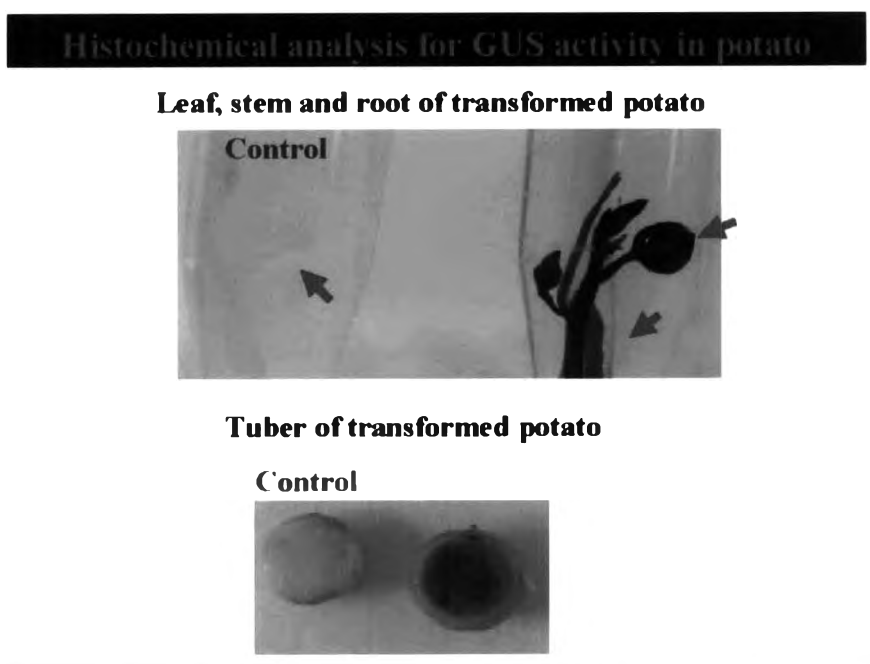

Figure 3.50 Histochemical analysis of GUS expression in leaf, stem, root and tuber of primary transformants of potato. Non-transformed potato was used as control.

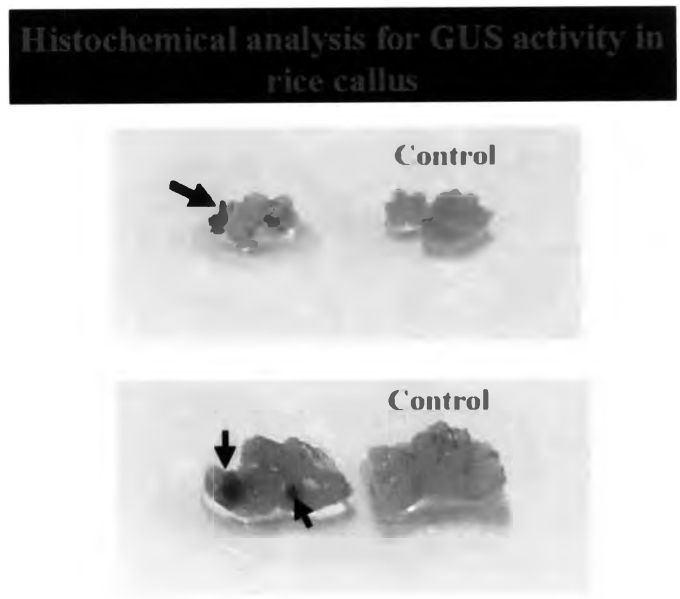

Figure 3.51 GUS histochemical staining of hygromycin resistant callus of transformed rice after 8 weeks on selection medium

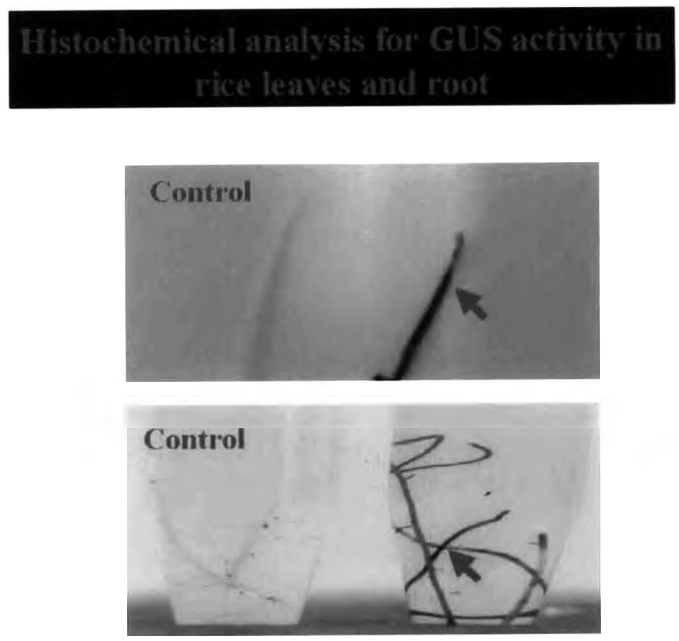

Figure 3.52 GUS histochemical staining of leaf and root of transformed rice plants. Non-transformed potato was used as control.

3.4.3.2 PCR analyses of putative transformants

To confirm the integration of AcMRJP1 gene into plant genome, the transformants were screened by PCR amplification for the AcMRJP1 gene. The genomic DNA was isolated from *in vitro* grown leaves from either non-transformed control plants or kanamycin resistant putative potato transformants or hygromycin resistant putative rice transformants using a modified CTAB extraction method. The DNA concentrations were spectrophotometrically determined by measuring the optical density at 260 nm. An OD_{260} of 1.0 corresponds to a concentration of 50 μg DNA/ml double stranded DNA. Approximately 1-5 μg of nucleic acids were obtained from 100 mg starting plant tissue. The ratio of OD_{260} / OD_{280} was 1.8 - 2.0 indicates pure prepared DNA.

The existence of AcMRJP1 gene in the chromosomal DNA of 25 transformed potato plants and 6 transformed rice plants was verified by PCR. The DNA of a non-transformed plant was used as a negative control and the AcMRJP1-binary vector, pCAMBIA2301-35S-AcMRJP1-Nos, was used as a positivie control. The presence of AcMRJP1 gene was analyzed using primer derived from the 5' and 3' ends of AcMRJP1 coding sequence. The transformants containing the transgene were identified by the presence of an amplified PCR product of 1.3 kb. The result revealed that the specific 1.3 kb band of AcMRJP1 gene was detected in the putative transformants. For transformed potatos, 8 of 9 plants transformed with pCAMBIA2301-GBSS- AcMRJP1-Nos, 8 of 10 potato transformed with pCAMBIA2301-B33-AcMRJP1-Nos, and 4 of 6 plants transformed with pCAMBIA2301-35S-AcMRJP1-Nos showed positive result of 1.3 kb as the positive control (Figure 3.53-3.55). No band was detected in case of negative control DNA. In case of rice trnasformation, all 6 plants transformed with pCAMBIA1301-35S-AcMRJP1-Nos gave specific bands of 1.3 kb of AcMRJP1 genes. No band was detected in case of negative control DNA (Figure 3.56). The presence of the AcMRJP1 gene in the transformants demonstrated integration of the T-DNA in the transformants.

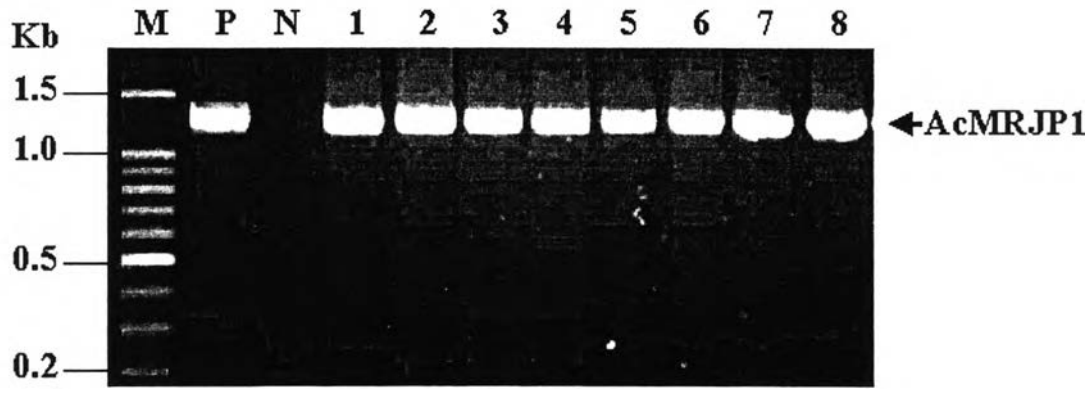

Figure 3.53 PCR analysis of AcMRJP1 gene insertion in the genome of transformed potato plant transformed with pCAMBIA2301-GBSS-AcMRJP1-Nos. PCR product was analyzed on 1.2% agarose gel electrophoresis with ethidium bromide staining. Amplification of a 1.3 kb AcMRJP1 fragment from only the transgenic plants confirms the integration of the T-DNA.

Lane M = 100 bp DNA ladder

Lane P = The amplified products of pCAMBIA2301-35S-AcMRJP1-Nos positive control plasmid

Lane N = The amplified products of non-transfomed plant DNA (negative control)

Lane 1-8 = The amplified product of transformed potato line 2301G-A1, 2301G-B2, 2301G-C3, 2301G-D4, 2301G-H6, 2301G-I7, 2301G-J8, 2301G-N9, respectively.

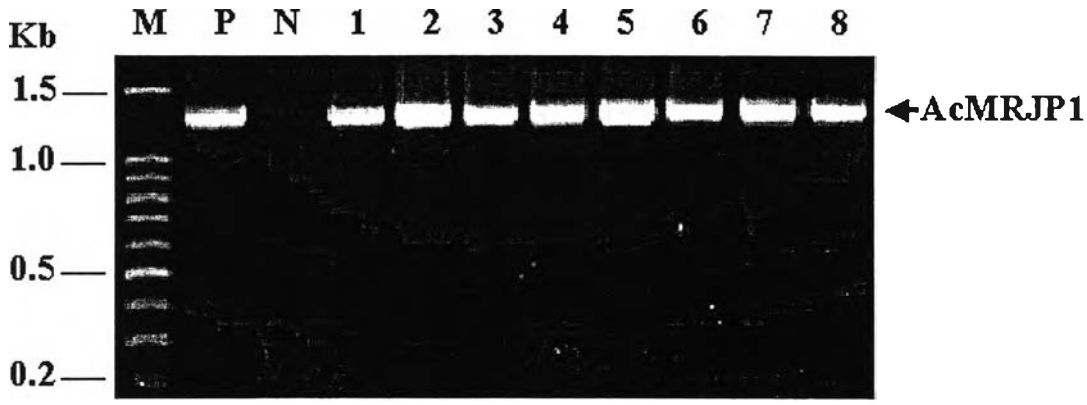

Figure 3.54 PCR analysis of AcMRJP1 gene insertion in the genome of transformed potato plant transformed with pCAMBIA2301-B33-AcMRJP1-Nos. PCR product was analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining. Amplification of a 1.3 kb AcMRJP1 fragment from only the transgenic plants confirms the integration of the T-DNA.

Lane M = 100 bp DNA ladder

Lane P = The amplified products of pCAMBIA2301-35S-AcMRJP1-Nos positive control plasmid

Lane N = The amplified products of non-transfomed plant DNA (negative control)

Lane 1-8 = The amplified product of transformed potato line 2301B-E3, 2301B-F4, 2301B-G5, 2301B-H6, 2301B-I7, 2301B-L8, 2301B-M9, 2301B-N10, respectively.

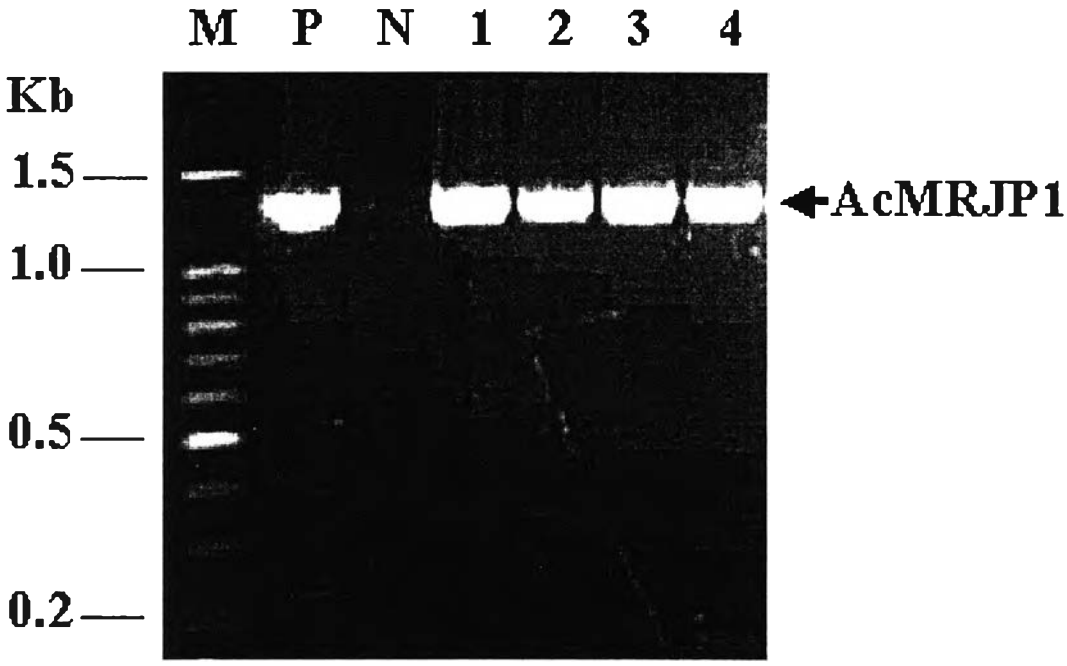

Figure 3.55 PCR analysis of AcMRJP1 gene insertion in the genome of transformed potato plant transformed with pCAMBIA2301-35S-AcMRJP1-Nos. PCR product was analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining. Amplification of a 1.3 kb AcMRJP1 fragment from only the transgenic plants confirms the integration of the T-DNA.

Lane M = 100 bp DNA ladder

Lane P = The amplified products of pCAMBIA2301-35S-AcMRJP1-Nos positive control plasmid

Lane N = The amplified products of non-transfomed plant DNA (negative control)

Lane 1-4 = The amplified product of transformed potato line 2301C-A1, 2301C-B2, 2301C-C3, 2301C-D4, 2301C-E5, 2301C-F6, respectively.

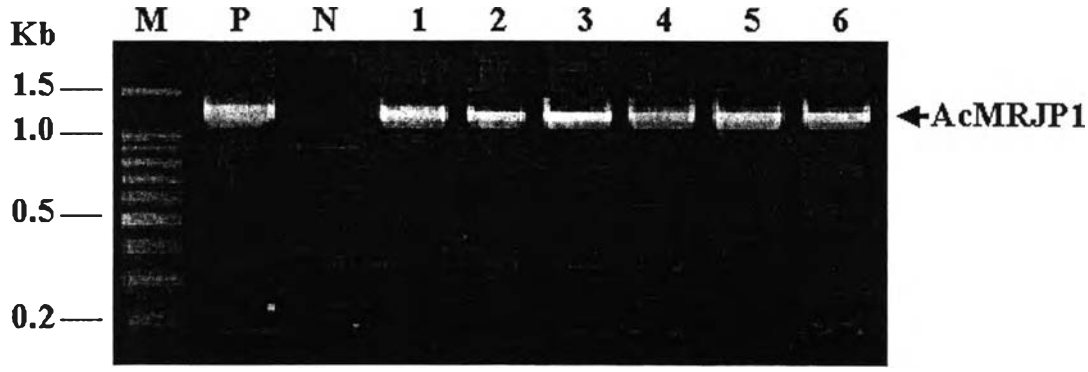

Figure 3.56 PCR analysis of AcMRJP1 gene insertion in the genome of transformed rice plant transformed with pCAMBIA1301-35S-AcMRJP1-Nos. PCR product was analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining. Amplification of a 1.3 kb AcMRJP1 fragment from only the transgenic plants confirms the integration of the T-DNA.

Lane M = 100 bp DNA ladder

Lane P = The amplified products of pCAMBIA2301-35S-AcMRJP1-Nos positive control plasmid

Lane N = The amplified products of non-transfomed plant DNA (negative control)

Lane 1-6 = The amplified product of transformed potato line 1301C-A1, 1301C-B2, 1301C-C3, 1301C-D4, 1301C-E5, 1301C-F6, respectively.

3.4.3.3 Detection of the AcMRJP1 mRNA in transformed plant by RT-PCR

3.4.3.3.1 Plant total RNA extraction

The expression of AcMRJP1 gene at RNA level was examined by the use of reverse transcription PCR (RT-PCR) using primers specific for AcMRJP1 cDNA. Total RNA was isolated from *in vitro* grown microtuber of putative transformed potato and *in vitro* grown leaves of putative rice transformants using TRI REAGENT® (Molecular Research Center, Inc., USA). To eliminate the possibility of DNA contamination in the plant total RNA preparation, the total RNA was incubated with DNase. The total RNA from non-transformed plants was used as a negative control. The concentration of total RNA was determined by measuring the optical density at 260 nm. An amount of extracted RNA was approximately 5-6 μg per 100 mg of plant tissue. An OD_{260} / OD_{280} ratio in the range of 1.8 to 2.0 indicated pure prepared RNA.

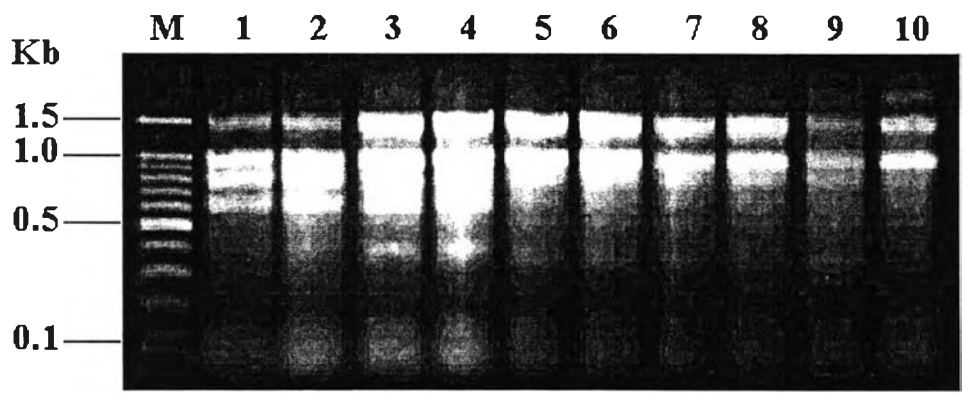

Figure 3.57 A 1.0% agarose gel electrophoresis of total RNA extracted from leaf of rice (lanes 1-2) and tuber of potato (lanes 4-10).

Lane M = 100 bp DNA marker

3.4.3.3.2 Detection of the AcMRJP1 mRNA in transformed potatos by RT-PCR

The transformed potatos containing the AcMRJP1 gene insertion as confirmed by PCR was selected to examine the transcription of the AcMRJP1 transgene by RT-PCR analysis. The non-transformed potato was used as a negative control. The total RNA extracted from microtubers were reverse transcribed. The first strand cDNA was used as a template for amplification of AcMRJP1 transcript. Amplifications of 18S and GBSS transcripts were performed in parallel. The presence of AcMRJP1 gene was analyzed using primer derived from the 5' and 3' ends of AcMRJP1 coding sequence. The transformant containing the transgene were identified by the presence of an amplified RT-PCR product of 1.3 kb. To compare the level of AcMRJP1 RT-PCR products of separate plants, the PCR products were compared on the basis of 18S rRNA (356 bp) and GBSS (173 bp) RT-PCR product levels.

The result showed that AcMRJP1 transcript was found in five lines of transformed potato (2301G-D4, 2301G-H6, 2301G-I7, 2301G-J8, 2301G-N9) transformed with pCAMBIA2301-GBSS-AcMRJP1-Nos, six lines of transformed potato (2301B-E3-5, 2301B-E4-6, 2301B-I7, 2301B-L8, 2301B-M9, 2301B-N10) transformed with with pCAMBIA2301-B33-AcMRJP1-Nos, and three lines of transformed potato (2301C-B2, 2301C-D4, 2301C-F6) transformed with pCAMBIA2301-35S-AcMRJP1-Nos (Figure 3.58-3.60). No AcMRJP1 mRNA was detected in non-transformed negative control potato. The transformants gave DNA bands of the expected sized of AcMRJP1 cDNA, demonstraing that they produced mRNA from the corresponding gene. However, the AcMRJP1 mRNA level in individual transformed lines were low. Most of the transformed line under the GBSS and B33 promoter showing the higher AcMRJP1 transcript level than those under the 35S promoter.

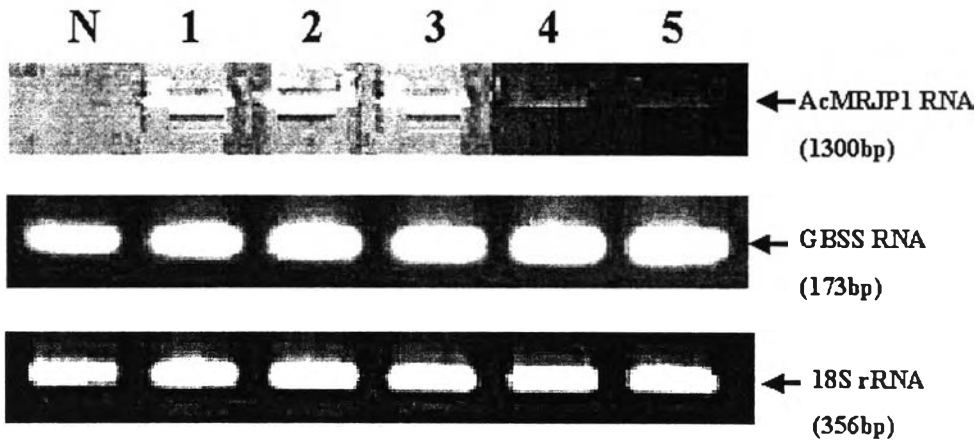

Figure 3.58 RT-PCR analysis of AcMRJP1 mRNA in tubers of potato transformed with pCAMBIA2301-GBSS-AcMRJP1-Nos. Amplifications of 18S and GBSS transcripts were performed in parallel. RT-PCR reaction was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The arrow indicates the expected RT-PCR prodcut of the AcMRJP1 mRNA (1.3 kb).

Lane M = 100 bp DNA ladder

Lane N = The RT-PCR products of non-transfomed plant RNA (negative control)

Lane 1-5 = The RT-PCR product of transformed potato line 2301G-D4, 2301G-H6, 2301G-I7, 2301G-J8, 2301G-N9, respectively.

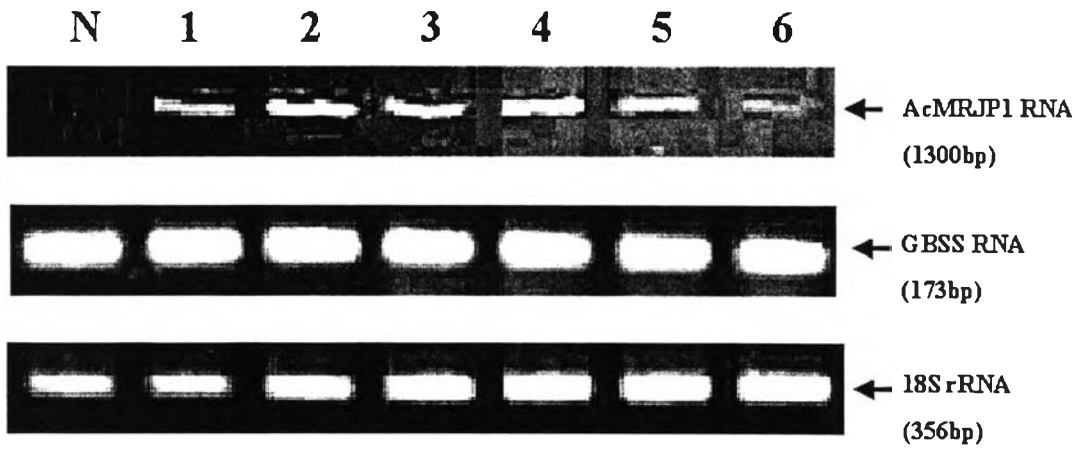

Figure 3.59 RT-PCR analysis of AcMRJP1 mRNA in tubers of potato transformed with pCAMBIA2301-B33-AcMRJP1-Nos. Amplifications of 18S and GBSS transcripts were performed in parallel. RT-PCR reaction was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The arrow indicates the expected RT-PCR prodcut of the AcMRJP1 mRNA (1.3 kb).

Lane M = 100 bp DNA ladder

Lane N = The RT-PCR products of non-transfomed plant RNA (negative control)

Lane 1-6 = The RT-PCR product of transformed potato line 2301B-G5, 2301B-H6, 2301B-I7, 2301B-L8, 2301B-M9, 2301B-N10, respectively.

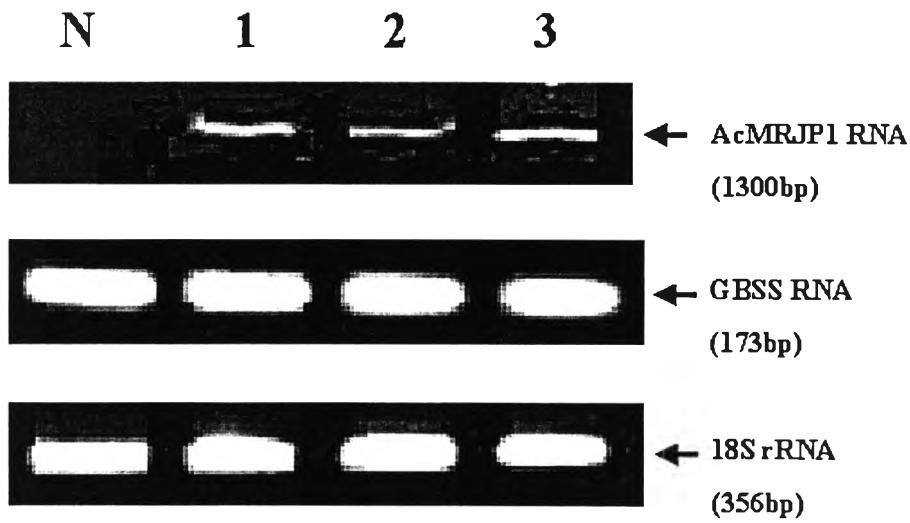

Figure 3.60 RT-PCR analysis of AcMRJP1 mRNA in tubers of potato transformed with pCAMBIA2301-35S-AcMRJP1-Nos. Amplifications of 18S and GBSS transcripts were performed in parallel. RT-PCR reaction was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The arrow indicates the expected RT-PCR prodcut of the AcMRJP1 mRNA (1.3 kb).

Lane M = 100 bp DNA ladder

Lane N = The RT-PCR products of non-transfomed plant RNA (negative control)

Lane 1-3 = The RT-PCR product of transformed potato line 2301C-B2, 2301C-D4, 2301C-F6, rspectively.

3.4.3.3.3 Detection of the AcMRJP1 mRNA in transformed rices

The transformed rice containing the AcMRJP1 gene insertion as confirmed by PCR was selected to examine the transcription of the AcMRJP1 transgene by RT-PCR analysis. The non-transformed potato was used as a negative control. The total RNA extracted from microtubers were reverse transcribed. The first strand cDNA was used as a template for amplification of AcMRJP1 transcript. Amplifications of actin transcripts were performed in parallel. The presence of AcMRJP1 gene was analyzed using primer derived from the 5' and 3' ends of AcMRJP1 coding sequence. The transformants were identified by the presence of 1.3 kb RT-PCR product corresponding to a portion of the AcMRJP1 cDNA amplified from cDNA prepared from leaves of transformed rice plants. To compare the level of AcMRJP1 RT-PCR products of separate plants, the PCR products were compared on the basis of actin (276 bp) RT-PCR product levels.

In transformed rice, six transformed rice plants were chosen to verify the transcription of AcMRJP1 gene. The result showed that AcMRJP1 transcript was found in all six lines of transformed rice plants (1301C-A1, 1301C-B2, 1301C-C3, 1301C-D4, 1301C-E5 and 1301C-F6) transformed with pCAMBIA1301-35S-AcMRJP1-Nos (Figure 3.61). The specificity of the RT-PCR reaction for the AcMRJP1 gene was confirmed by the absence of an amplificaton product form cDNA prepared from leaves of non-transformed negative control rice plant. The transformants gave bands of the expected sized of AcMRJP1, demonstraing that they produced mRNA transcripts of the corresponding gene. However, the AcMRJP1 mRNA levels in individual transformed lines were variable among different transformants. A high level of AcMRJP1 transcript was found in the 1301C-D4 and 1301C-E5 transformed lines.

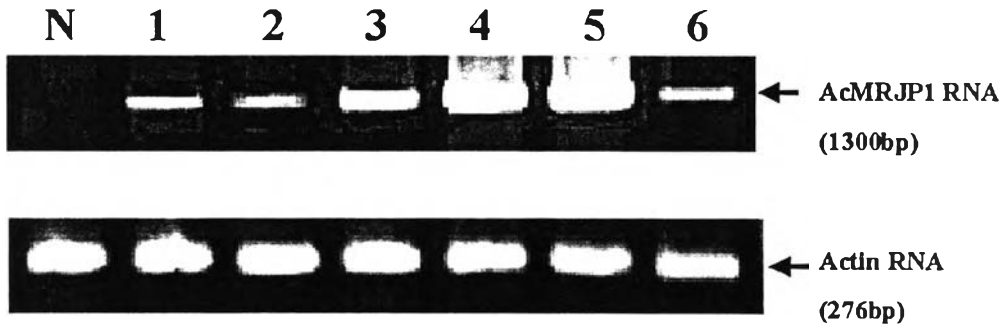

Figure 3.61 RT-PCR analysis of AcMRJP1 mRNA in leaves of rice transformed with pCAMBIA1301-35S-AcMRJP1-Nos. Amplifications of actin transcripts were performed in parallel. RT-PCR reaction was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The arrow indicates the expected RT-PCR prodcut of the AcMRJP1 mRNA (1.3 kb).

Lane M = 100 bp DNA ladder

Lane N = The RT-PCR products of non-transfomed plant RNA (negative control)

Lane 1-6 = The RT-PCR product of transformed rice line plants 1301C-A1, 1301C-B2, 1301-C3, 1301-D4, 1301-E5, 1301-F6, respectively.

3.4.3.4 Detection of AcMRJP1 protein in transformed plants by western blot analysis

3.4.3.4.1 Detection of AcMRJP1 protein in transformed potato

To examine AcMRJP1 expression in transformed potato, protein extracts were prepared from each transformed potato tuber and analyzed by SDS-PAGE following by Western blot analysis. The assay of AcMRJP1 gene expression at the protein level was focused on one of each plant line which expressed high transcript levels. Transformed potato line of 2301G-H6, 2301B-H6 and 2301C-B2 were selected for further analysis by immunoblot. Approximately 100 μg total protein extract from tuber of these transformants were analyzed on 8% SDS-PAGE (Figure 3.62) followed by transfer to PVDF membrane and incubation with anti-His antibody or polyclonal rabbit antiserum against WSPs (water soluble proteins) of RJ of *A. mellifera* (Schmitzova *et al.*, 1998). Recombinant AcMRJP1 from *E. coli* was used as a positive control in all blots.

Western blot analysis with an anti-His antibody showed that transformed potato plants contained tagged proteins that migrated with an apparent molecular mass of approximately 70 kDa and 50 kDa corresponding to the predicted size of the GUS and AcMRJP1, respectively (Figure 3.63A). No proteins of this size were detected in extracts from nontransformed plants. The results indicate that pCAMBIA2301-GBSS-AcMRJP1-Nos, pCAMBIA2301-B33-Nos and pCAMBIA2301-35S-Nos plants synthesized and accumulated AcMRJP1 and GUS protein in their tubers. No bands were detected with total protein of non-transformed potato. In the second immunoblot assay using polyclonal rabbit antiserum against WSPs of RJ, only the 50 kDa protein was positively detected but not the 70 kDa peptide of GUS (Figure 3.63 B). The results indicated that pCAMBIA1301-35S-AcMRJP1-Nos plants synthesized and accumulated AcMRJP1 and GUS protein in tubers. These bands were absent from proteins extracted from non-transgenic control plants.

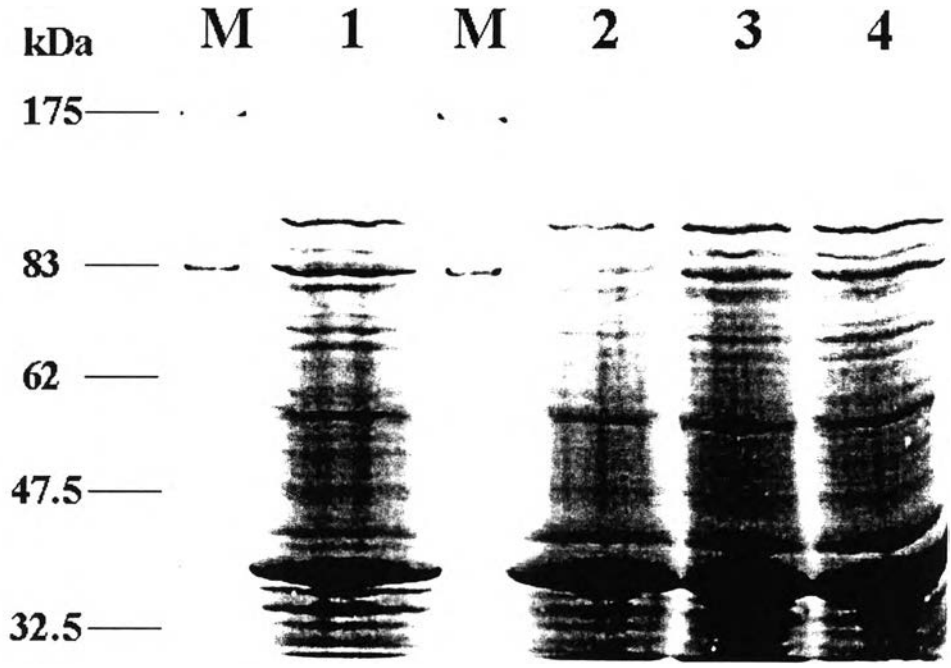

Figure 3.62 SDS-PAGE analysis of protein from tuber extract of the transformed potato tuber. Total protein extract (100 μg) was fractionated by SDS-PAGE. Molecular masses of the protein marker are indicated at the left.

Lane M = Protein marker

Lane 1 = Protein extract from tubers of nontransformed plant

Lanes 2-4= Protein extracts from tubers of transformed plant lines 2301C-B2, 2301G-H6 and 2301B-H6, respectively

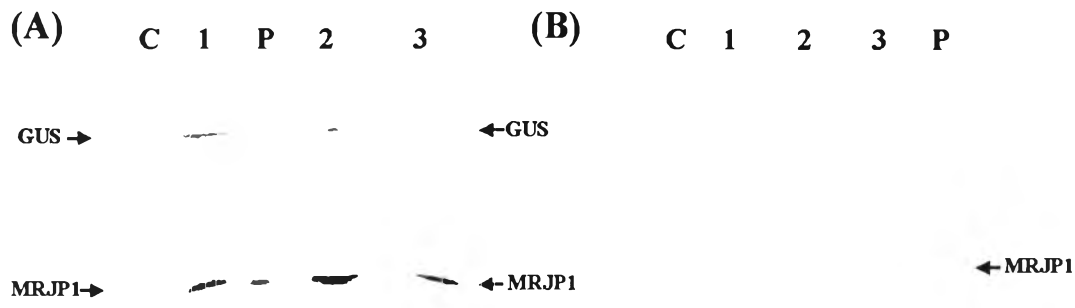

Figure 3.63 Western analysis of protein from tuber extract of the transformed potato plants. Total protein extract (100 µg) was fractionated by SDS-PAGE, blotted onto a PVDF membrane, and probed with polyclonal rabbit antiserum against WSPs of RJ of *A. mellifera* or anti-His antibody. Molecular masses of the protein marker are indicated at the left. The AcMRJP1 and GUS band is indicated by an arrow.

(A) Western blot using the anti-His antibody at a dilution of 1:1,000

Lane M = Protein marker

Lane P = Purified rAcMRJP1 protein from *E. coli* (100 ng, positve control, 50,kDa)

Lane C = Protein extract from tuber tissue of nontransformed plant

Lanes 1 = Protein extracts from tuber of transformed potato line 2301C-B2

Lanes 2 = Protein extracts from tuber of transformed potato line 2301G-H6

Lanes 3 = Protein extracts from tuber of transformed potato line 2301B-H6

(B) Western blot using the polyclonal rabbit antiserum against WSPs of RJ of *A. mellifera* at a dilution of 1:2,000

Lane M = Protein marker

Lane P = Purified rAcMRJP1 protein from *E. coli* (100 ng, positve control, 50,kDa)

Lane C = Protein extract from tuber tissue of nontransformed plant

Lanes 1 = Protein extracts from tuber of transformed potato line 2301G- H6

Lanes 2 = Protein extracts from tuber of transformed potato line 2301C-B2

Lanes 3 = Protein extracts from tuber of transformed potato line 2301B-H6

3.4.3.4.2 Detection of AcMRJP1 protein in transformed rice by Western blot analysis

In order to confirm the presence of AcMRJP1 protein in transformed rice plants, Western blot analysis of total protein from leaves was performed. The 1301-C3, 1301-D4 and 1301-E5 transformants which accumulated high levels of the AcMRJP1 mRNA were selected for further analysis by immunoblot. Approximately 100 μg total protein extract of 1301C-E5 transformants were analyzed on 8% SDS-PAGE (Figure 3.64) followed by transfer to PVDF membrane and incubation with anti-His antibody or polyclonal rabbit antiserum against WSPs of RJ of *A. mellifera* (Schmitzova *et al.*, 1998). Recombinant AcMRJP1 from *E. coli* was used as a positive control in all blots.

Proteins migrating as 70 and 50 kDa polypeptides were detected in rice plants containing the AcMRJP1 transgene when the blots were probed with anti-His antibody (Figure 3.65A). These bands corresponded to the expected sized of GUS and AcMRJP1 protein, respectively. In the second immunoblot assay using polyclonal rabbit antiserum against WSPs of RJ, only the 50 kDa protein band was positively detected (Figure 3.65B). The results indicate that these transformants synthesized and accumulated AcMRJP1 and GUS protein in leaves. These bands were absent from proteins extracted from non-transgenic control plants.

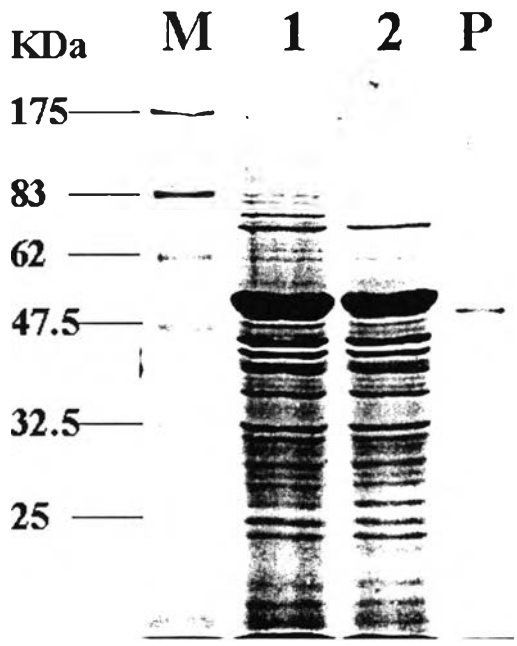

Figure 3.64 SDS-PAGE analysis of protein extract from leaves of representative of the transformed rice plant. Total soluble protein extract (100 μg) was fractionated by SDS-PAGE. Molecular masses of the protein marker are indicated at the left.

Lane M = Protein marker

Lane P = Purified rAcMRJP1 protein from *E. coli* (100 ng, positve control, 50,kDa)

Lane 1 = Protein extract from leaves of transformed plant line 1301-E5

Lane 2 = Protein extract from leaves tissue of nontransformed plant

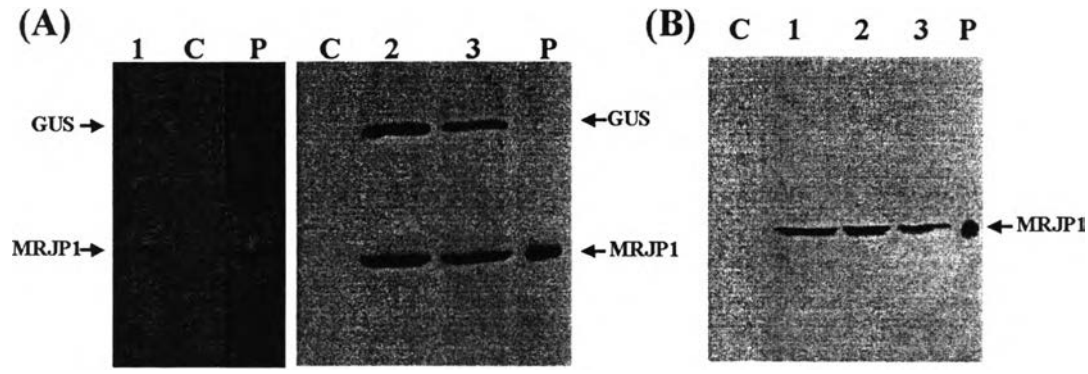

Figure 3.65 Western analysis of AcMRJP1 protein from leaves extract of the transformed rice plants. Total protein extract (100 μg) was fractionated by SDS-PAGE and blotted onto a PVDF membrane. Molecular masses of protein marker are indicated at the left. The AcMRJP1 band is indicated by an arrow.

(A) Western blot using the anti-His antibody at a dilution of 1:1,000

Lane M = Protein marker

Lane P = Purified rAcMRJP1 protein from *E. coli* (100 ng, positve control, 50,kDa)

Lane C = Protein extract from leaves tissue of nontransformed plant

Lanes 1 = Protein extracts from leaves of transformed potato line 1301-C3

Lanes 2 = Protein extracts from leaves of transformed potato line 1301-D4

Lanes 3 = Protein extracts from leaves of transformed potato line 1301-E5

(B) Western blot using the polyclonal rabbit antiserum against WSPs of RJ of *A. mellifera* at a dilution of 1:2,000

Lane M = Protein marker

Lane P = Purified rAcMRJP1 protein from *E. coli* (100 ng, positve control, 50,kDa)

Lane C = Protein extract from tuber tissue of nontransformed plant

Lanes 1 = Protein extracts from leaves of transformed potato line 1301-C3

Lanes 2 = Protein extracts from leaves of transformed potato line 1301-D4

Lanes 3 = Protein extracts from leaves of transformed potato line 1301-E5