

Materials and methods

Drosophila culture

Flies were kept on a standard diet at 25 °C under a 12-h light/12-h dark cycle (12L:12D), which consisted of 218.75 g cornmeal, 112.5 g molasses, 75 g yeast, 62.5 g glucose, and 62.5 g sucrose in 3 L of water. *Act5C*-GAL4 (BDSC#4414), *elav*-GAL4 (BDSC#458), and *ppl*-GAL4 (BDSC#58768) flies were obtained from the Bloomington *Drosophila* Stock Center (BDSC). To standardize the genetic background of flies, all were outcrossed with the *iso*³¹ (BDSC#5905) wild-type line for five generations.

Amplification of *mrjp* genes and construction of transgenic *Drosophila*

Total RNA was first extracted from the heads of local *Apis mellifera* using the TRIzol reagent (Vazyme, R401), and then, 1 µg was used for reverse transcription (Vazyme, R312). The *mrjp*1–9 genes were amplified using specific primers (Table 1) and cloned into the pUAST-attB vector after double digestion with *Xho*I and *Xba*I. The constructed plasmids, pUAST-attB-MRJPs, were microinjected into *Drosophila* line P{CaryP}attP2 (BDSC#25710) at the 68A4 site on chromosome III. Positive transgenic offspring were identified and confirmed through red eye marker screening. To standardize the genetic background, all flies were outcrossed with the *iso*³¹ wild-type line for five generations.

Table 1 Primers used for gene amplification

Name	Sequences (5'→3')
<i>mrjp</i> 1-f	ccgCTCGAGATGACAAGATTGTTTATGCTGGTATG
<i>mrjp</i> 1-r	gcTCTAGATTACAAATGGATTGAAATTTTGAAG
<i>mrjp</i> 2-f	ccgCTCGAGATGACAAGGTGGTTGTTTCATG
<i>mrjp</i> 2-r	gcTCTAGATTAATTATCATTCTGATTGTTATTCTTTTG
<i>mrjp</i> 3-f	ccgCTCGAGATGACAAAGTGGTTGTTGCTG
<i>mrjp</i> 3-r	gcTCTAGATTAATGTAATTTGAAGAATGATGAACTT
<i>mrjp</i> 4-f	ccgCTCGAGATGACAAAATGGTTGCTGTTG
<i>mrjp</i> 4-r	gcTCTAGATTAATCGTTATTGTTATGCCGATT
<i>mrjp</i> 5-f	ccgCTCGAGATGACAACCTGGTTGTTGCTG
<i>mrjp</i> 5-r	gcTCTAGATTAATTATTATGCTTATTTGATTGTTATTCTG
<i>mrjp</i> 6-f	ccgCTCGAGATGACAAATTGGTTACTGCTGATAG
<i>mrjp</i> 6-r	gcTCTAGACTAATCTAAATGAGCTTGATTCTTATATTTAT
<i>mrjp</i> 7-f	ccgCTCGAGATGACAAGGTGGTTGTTTATGG
<i>mrjp</i> 7-r	gcTCTAGACTAATTAATGAAGCGTCTATTGTGAT
<i>mrjp</i> 8-f	ccgCTCGAGATGATAAGATGGTTGCTGCTGATG
<i>mrjp</i> 8-r	gcTCTAGATCActtatcgtcgtcatccttgaatcAGGAGATATGCAACGAGTATTCTT
<i>mrjp</i> 9-f	ccgCTCGAGATGTCCTTTCAATATCTGGTGGTTG
<i>mrjp</i> 9-r	gcTCTAGATCAAAGGAAAATTGAGAAAAAATTTG

f: forward; r: reverse.

RNA extraction and sequencing library preparation

Neuron-specific driven *elav*-GAL4 female flies overexpressing MRJPs or the mScarlet-i fluorescent protein as a control were raised at 25 °C for 7 d, after which the heads were collected, with 30 flies per group. Total RNA was extracted from the heads using the TRIzol reagent (Vazyme, R401) following the manufacturer's instructions. RNA quality was assessed using the 5300 Bioanalyzer (Agilent Technologies), and quantification was performed with the NanoDrop™ 2000 spectrophotometer (Thermo Scientific). RNA purification, reverse transcription, library construction, and sequencing were conducted at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai). For transcriptome library preparation, 1 µg of total RNA was processed using Illumina's TruSeq™ RNA Sample Preparation Kit (San Diego, CA). Briefly, mRNA was isolated via polyA selection with oligo(dT) beads, followed by fragmentation using a fragmentation buffer. Double-stranded cDNA was synthesized with the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, CA) using random hexamer primers (Illumina). The synthesized cDNA then underwent end repair, phosphorylation, and the addition of an "A" base while following Illumina's library construction protocol. Library size selection was performed on 2% Low-Range Ultra Agarose to obtain 300-bp cDNA target fragments, followed by 15 cycles of polymerase chain reaction (PCR) amplification with Phusion DNA Polymerase (NEB). Quantified by TBS380, the RNA-seq libraries were loaded onto the Illumina NovaSeq 6000 platform for paired-end sequencing.

Differential expression and functional enrichment analysis

To identify differentially expressed genes (DEGs) between the two groups, gene expression levels were calculated based on transcripts per million reads (TPMs). Gene abundance was quantified using RNA-sequencing (RNA-seq) by Expectation-Maximization (RSEM) (<https://deweylab.biostat.wisc.edu/rsem>), and differential expression analysis was performed with DESeq2 (Love et al., 2014). Gene Set Enrichment Analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) was conducted using R version 4.1.0, with all DEGs between the groups serving as input. The log₂FoldChange value represented the degree of expression change between the groups and was used to create a gene list sorted by gene names and values in a descending order. The *Drosophila* gene sets were retrieved from the Molecular Signatures Database (MSigDB) using the "msigdb" package. Enrichment analysis was then performed using the clusterProfiler package (Wu et al., 2021), a versatile tool for interpreting omics data. Weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) was conducted using an online analysis tool provided by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Biological validation experiments

To verify the effects of MRJP expression in different tissues on individual size variation, transgenic upstream activating sequence (UAS)-MRJP flies were crossed with different GAL4 driver lines: *Act5C*-GAL4 (all cells), *elav*-GAL4 (neuronal cells), and *ppl*-GAL4 (fat body cells). As controls, each GAL4 driver line was separately crossed with *iso*³¹ flies, and different UAS-*mrjp* lines were separately crossed with wild-type *iso*³¹ flies as another control. After eclosion, the female offspring were reared on standard food for seven days before being used for body size measurement.

Flies were anesthetized on a CO₂ pad and placed on their sides to position the head antennae, balancing organ, and tail tip within the field of view. Photos were taken with a microscopic imaging system set to a uniform focal length. A smooth curve was drawn to connect the antennae, balancing the organ and tail tip, allowing for pixel measurement along the curve to determine its length.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software for Windows (GraphPad Software, San Diego, CA, USA). The data was displayed as the mean±standard deviation (SD), and the figure was shown as a scatter dot plot. Normality was assumed using the Shapiro-Wilk test. Differences between groups were analyzed using ordinary one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test or Kruskal-Wallis test. A *P*-value of less than 0.05 was considered statistically significant.

References

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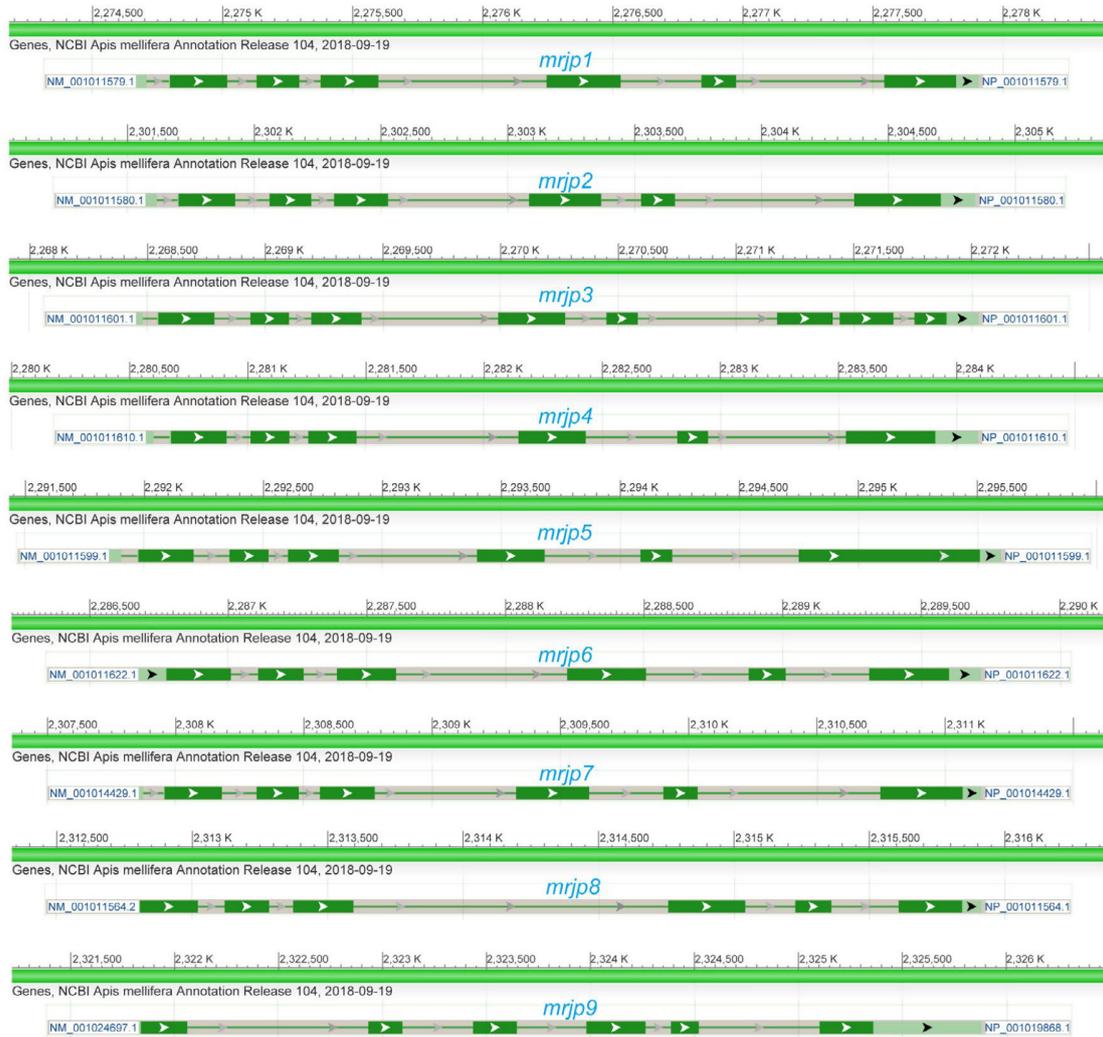


Fig. S1 Comparative analysis of genomic structures for different MRJPs.

The images are sourced from the NCBI database(<https://www.ncbi.nlm.nih.gov/genec/>). For each gene, the upper diagram represents the genomic scale, while the lower diagram shows the gene structure. Rectangular boxes indicate exons, and lines represent introns.

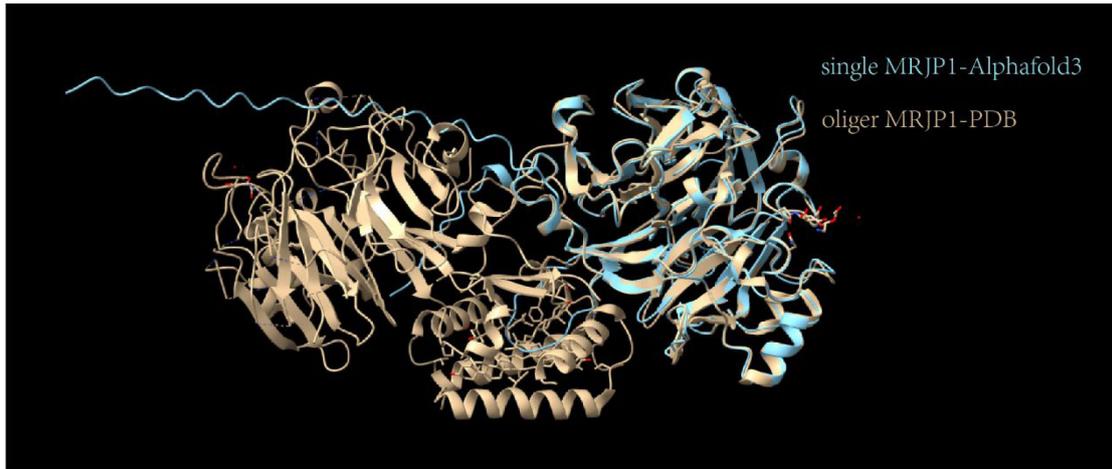


Fig. S2 Comparison of the tertiary structure of monomeric MRJP1 predicted by AlphaFold 3 with the reported tertiary structure of multimeric MRJP1.

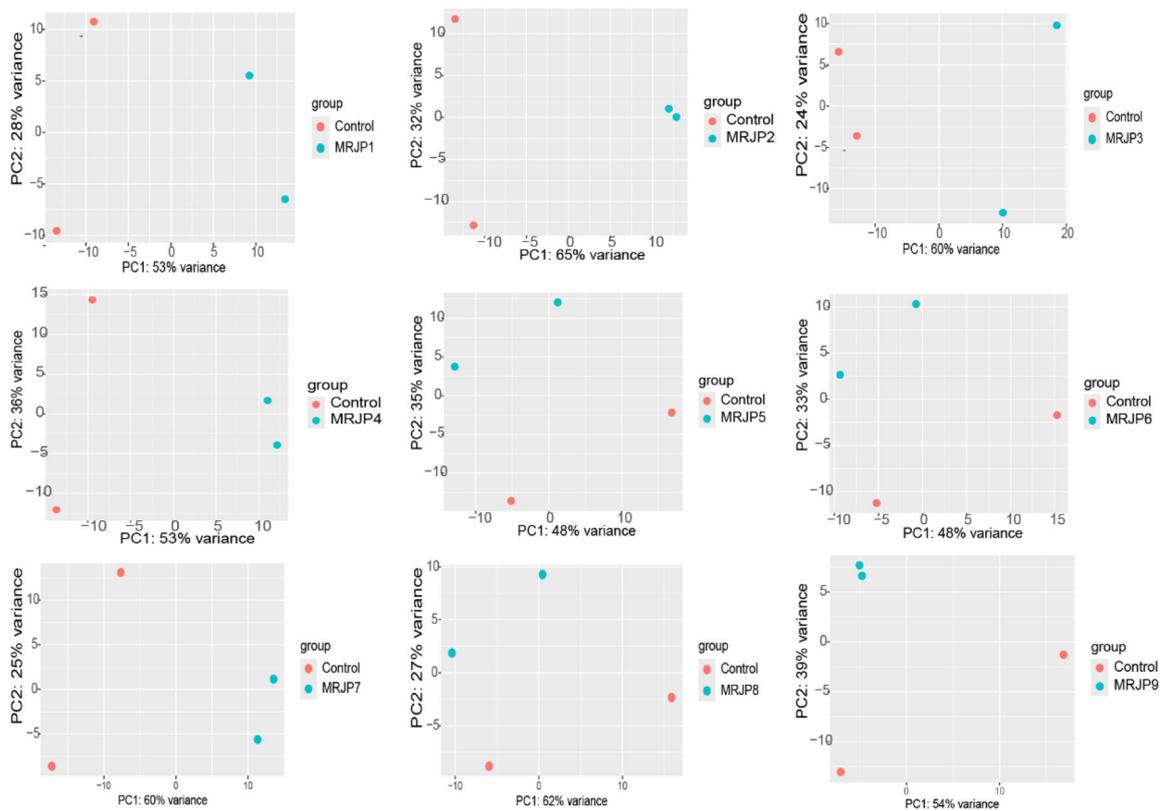


Fig. S3 Principal component analysis of transcriptomic data comparing overexpression of different MRJPs in the brain to the control group.

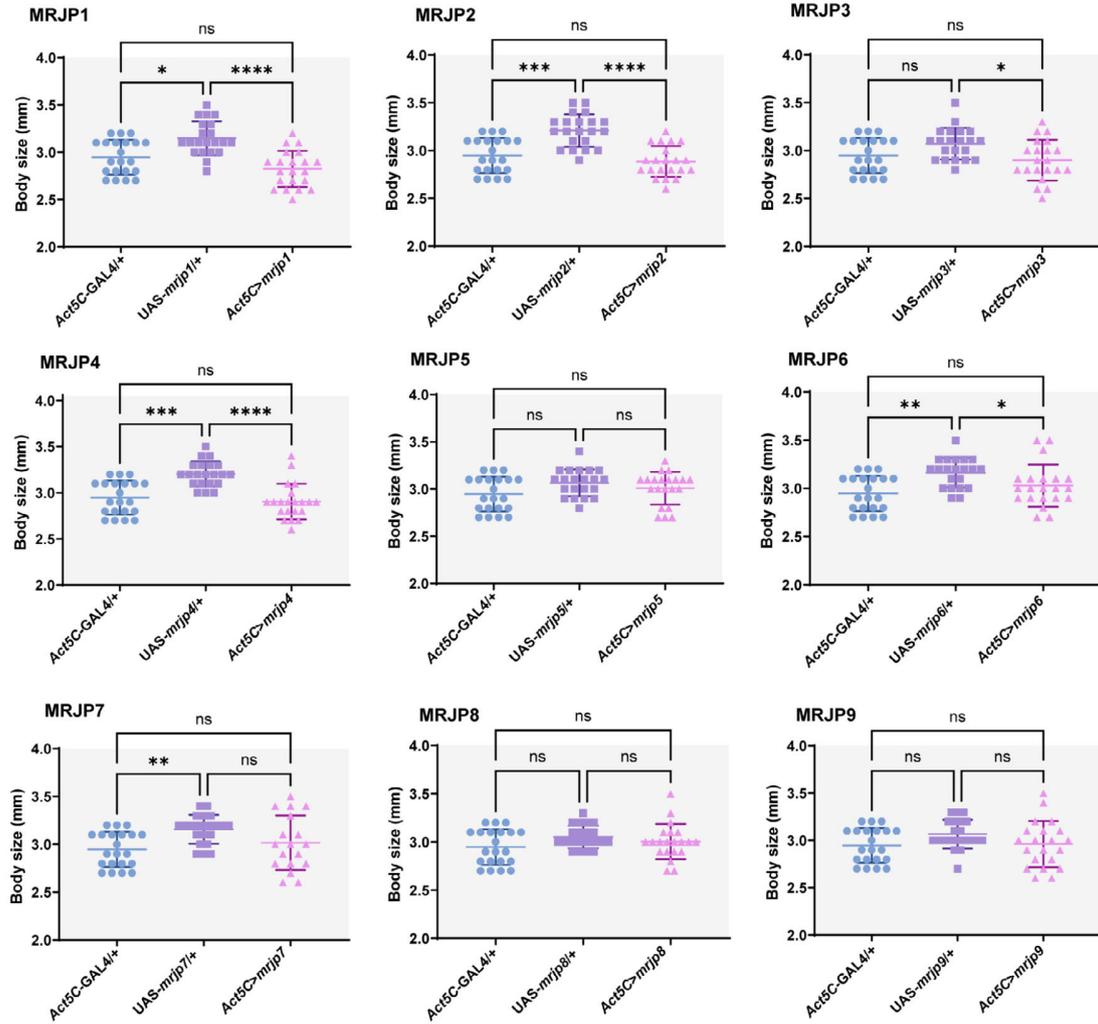


Fig. S4 Changes in body size of *Drosophila* following whole-body overexpression of different MRJPs.

Data are presented as mean±SD, with values displayed in scatter dot plots ($n=18-21$). *Act5c-GAL4/+* represents the heterozygous control, and *Act5C>mrjps* indicates the experimental group in which *UAS-mrjps* expression is driven by the ubiquitous driver *Act5C-GAL4*. ns, no significance; * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$.

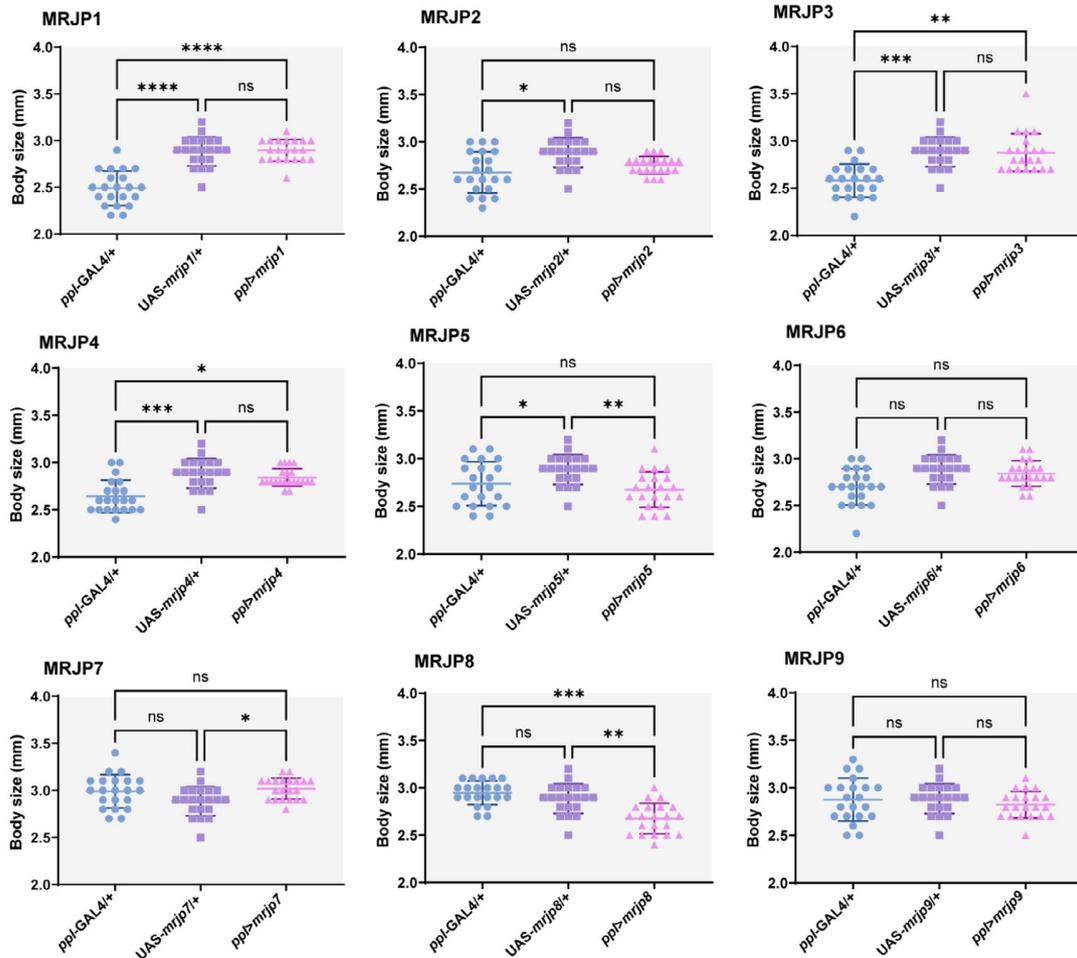


Fig. S5 Changes in body size of *Drosophila* following fat body overexpression of different MRJPs.

Data are presented as mean±SD, with values displayed in scatter dot plots ($n=21$). *ppl-GAL4/+* represents the heterozygous control, and *ppl>mrjps* indicates the experimental group in which *UAS-mrjps* expression is driven by the fat body driver *ppl-GAL4*. ns, no significance; * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$.

Table S1 Gene and protein sequences of MRJPs

Name	Sequences (5'→3')
<i>mrjp1</i>	<p>ATGACAAGATTGTTTATGCTGGTATGCCTTGGCATAGTTTGTCAAGGTACGACAGGCAACA TTCTTCGAGGAGAGTCTTTAAACAAATCATTACCCATCCTTCACGAATGGAATTCCTTGAT TATGATTTCCGGTAGCGATGAAAGAAGACAAGATGCAATTCTATCTGGCGAATACGACTACA AGAATAATTATCCATCCGACATTGACCAATGGCATGATAAGATTTTTGTACCATGCTGAG ATACAATGGCGTACCTTCCTCTTTGAACGTGATATCTAAAAAGGTCGGTGATGGTGGTCCT CTTCTACAACCTTATCCCGATTGGTCGTTTGCTAAATATGACGATTGCTCTGGAATCGTGAG CGCCTCAAACCTTGGCATCGACAAATGCGACAGATTGTGGGTTCTGGACTCAGGTCTTGTC AATAATACTCAACCCATGTGTTCTCCAAAAGTCTCACCTTTGATCTGACTACCTCGCAATT GCTCAAGCAAGTTGAAATACCACATGATGTTGCCGTAATGCCACTACAGGAAAGGGAAG ATTATCATCTCTAGCTGTTCAATCTTTAGATTGCAATACAAATAGCGATACTATGGTGTACA TAGCAGACGAGAAAGGTGAAGGTTAATCGTGATCATAATTCTGATGATTCCTCCATCG ATTGACTTCCAACACTTTCGATTACGATCCTAAATTTACCAAATGACGATCGATGGAGAA AGTTACACAGCCCAAGATGGAATTTCTGGAATGGCTCTTAGTCCCATGACTAACAATCTCT ATTACAGTCCGTAGCTTCCACCAGTTTGTATTATGTTAACACGGAACAATTCAGAACATCC GATTATCAACAGAATGACATACATTACGAAGGAGTCCAAAATATTTTGATACCCAATCGT CCGCTAAAGTAGTATCAAAGAGTGGCGTTCTTCTTTCGGATTGGTGGGCGATTACAGCTCTT GGCTGCTGGAACGAACATCGAACACTTGAAAGACACAATATCCGTACCGTCGCTCAAAGT GATGAGACTCTTCAAATGATCGCTAGCATGAAGATTAAGGAAGCTCTTCCACACGTGCCTA TATTCGATAGGTATATAAACCGTGAATACATATTGGTTTTAAGTAACAAAATGCAAAAAAT GGTGAATAATGACTTCAACTTCGACGATGTTAACTTCAGAAATTATGAACGCGAATGTAAAC GAATTGATATTGAACACTCGTTGCGAAAATCCCGATAATGATCGAACACCTTTCAAATTT CAATCCATTTGTAA</p>
MRJP1	<p>MTRLFMLVCLGIVCQGTGNILRGESLNKSLPILHEWKFFDYDFGSDERRQDAILSGEYDYKNN YPSDIDQWHDKIFVTMLRYNGVPSLNVISKKVGDDGPLLQPYPDWSFAKYDDDCSGIVSASKL AIDKCDRLWVLDLGLVNNTQPMCSPKLLTFDLTTSQLLKQVEIPHDVAVNATTGKGRSLSLAV QSLDCNTNSDTMVYIADEKGEGLIVYHNSDDSFHRLTSNTFDYDPKFTKMTIDGESYTAQDGIS GMALSPMTNLYYSPVASTSLYYVNTEQFRSDYQQNDIHYEGVQNILDTQSSAKVVSKSGVL FFGLVGDSALGCWNEHRTLERNIRTVAQSDLELQMIASMKIKEALPHVPIFDRIYINREYILVLS NKMQKMNNDNFDDVNFNFRIMNANVNELILNTRCENPDNDRTPFKISHL</p>
<i>mrjp2</i>	<p>ATGACAAGGTGGTTGTTTCATGGTGGCATGCCTCGGCATAGCTTGTCAAGGCGCCATTGTTT GAGAAAATTCTCCAAGAAACTTGAAAAATCATTGAACGTAATTCACGAATGGAAGTATTT TGATTATGACTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCTGGCGAATATGA CCATACGAAAAATTATCCCTTCGACGTCGATCAATGGCGTGATAAGACTTTTGTACCATA CTAAGATACGATGGTGTTCCTTCTACTTTGAACGTGATATCTGGTAAAACTGGTAAGGGTG GACGACTTTTAAAACCATATCCTGATTGGTCGTTTGCAGAGTTTAAAGATTGCTCTAAAATT GTGAGCGCTTCAAATTTGCGATTGACAAATTCGACAGATTGTGGGTTTTGGATTCAGGTC TTGTCAATAGAAGTGTACCTGTATGTGCTCCAAAGTTGCACGTCTTTGATCTGAAAACCTCA AATCACCTTAAGCAAATCGAGATACCGCATGATATTGCCGTGAATGCCACCACAGGAAAG GGAGGGCTAGTGTCTTTGGCTGTTCAAGCTATAGATCTTGCAAATACTTTAGTGTACATGGC AGACCATAAAGGTGATGCTTTAATCGTCTACCAAAATGCCGATGATTCCTTCCATCGATTG ACTTCCAACACTTTCGACTACGATCCAGATATGCCAAAATGACGATCGATGGAGAAAGTT TCACACTGAAAAATGGAATTTGTGGAATGGCTCTTAGTCCCGTGACGAACAATCTTTATTA</p>

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MRJP7 MTRWLFMVACLGIACQGAILRENSARNLKNLSKVMHEWKYIDYDFGSEEKRQAAIQSDEYDH
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