

# Transformation of tobacco plants with cDNA encoding honeybee royal jelly MRJP1

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## Abstract

For expression of MRJP1 - the most abundant protein of honeybee royal jelly - in plants, plasmid carrying the expression cassette composed of CaMV 35S RNA promoter, cDNA encoding MRJP1 with its native signal peptide, and nos<sup>3'</sup> as transcription terminator in binary vector pBin19 was prepared. The plasmid was introduced into tobacco (*Nicotiana tabacum* L. cv. Wi38) plants by *Agrobacterium tumefaciens*-mediated transformation. Transgenic F<sub>1</sub> and F<sub>2</sub> generation was grown from the seeds of the primary obtained transgenic tobacco plants. Immunoblot analyses of protein leaf extracts from transgenic plants showed expression of MRJP1.

*Additional key words:* *Agrobacterium tumefaciens*, major royal jelly protein, *Nicotiana tabacum*, recombinant protein, transgenic plant.

## Introduction

There are many examples of plants that produce transgenic proteins serving a wide spectrum of purposes including their nutrient values enrichment (Schroeder *et al.* 1991, Tabe and Higgins 1998). In recent years, many cereals and legumes have been transformed, *e.g.* by increasing of free lysine content in rice seeds (Christou *et al.* 1991, Lee *et al.* 2001). Transformed wheat (Becker *et al.* 1994) and birdsfoot trefoil can accumulate the maize seed storage proteins rich in amino acids containing sulphur, gamma zein and beta zein (Bellucci *et al.* 2002). In transgenic potato high-methionine or high-lysine proteins have been expressed (Tu *et al.* 1998).

The proteins of honeybee royal jelly (RJ) represent a new potential for enrichment of crop plants in nutritive and physiologically active proteins. Recent research concerning characterization of individual RJ proteins (Hanes and Šimúth 1992, Klaudiny *et al.* 1994, Albert *et al.* 1996, 1999a, Ohashi *et al.* 1997) showed that major RJ proteins belong to one protein family (Schmitzová

*et al.* 1998, Albert *et al.* 1999b, Malecová *et al.* 2003). Nine members of the MRJPs (49 - 87 kDa) family have been identified until now. Proteins MRJP1, MRJP2, MRJP3s, MRJP4, MRJP5s represent about 82 % of total protein content of RJ (Schmitzová *et al.* 1998). The most abundant is MRJP1 and represents about 48 % of RJ water-soluble proteins. It has some structural properties causing its self oligomerization in RJ (Šimúth 2001) and forms stable complex with 54 amino acids peptide apisimin (Bíliková *et al.* 2002). MRJP1 has relatively high content of essential amino acids - 48 % (Schmitzová *et al.* 1998). Therefore, it could be a potential ingredient of functional foods. It seems that the MRJP1 has more functions than only nutritional role in the larval development of honeybee. Recently it was found that it is expressed in mushroom bodies of adult honeybees brain (Kucharski and Maleszka 1998). The 350 kDa protein with N-terminal sequence identical with MRJP1 isolated from RJ has shown a proliferation stimulating activity

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**Abbreviations:** BAP - 6-benzylaminopurine; cDNA - DNA complementary to RNA; MRJP - major royal jelly protein; NAA -  $\alpha$ -naphthaleneacetic acid; PCR - polymerase chain reaction; RJ - royal jelly; SDS-PAGE - sodium dodecylsulphate polyacrylamide gel electrophoresis.

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for human monocytic cell lines (Kimura *et al.* 1995).

We have started with heterologous expression of MRJP1 in *E. coli* using pQE 32 system (Júdová *et al.*

## Materials and methods

**Plasmid constructs:** Standard methods in recombinant DNA were used according to Ausubel *et al.* (1996). The MRJP1 cDNA from pBluescript SK(-) (Klaudiny *et al.* 1994, Júdová *et al.* 1998, Schmitzová *et al.* 1998) was retrieved as a *PstI-XbaI* fragment. The *XbaI* overhang was made blunt through treatment with Klenow fragment, and inserted into the *PstI-SphI* sites of pMM23 (Morgan and Ow 1995) where the *SphI* overhang was made blunt with the Klenow fragment. The resulting plasmid, pMM-MRJP1, contains the MRJP1 cDNA with its native signal peptide between the CaMV 35S RNA promoter and the nos3' terminator. Plasmid pMM-MRJP1 was linearized with *HindIII* and ligated to *HindIII* treated *Agrobacterium* binary vector pBin19 (Bevan 1984). The selection of the recombinant plasmid (pBin19-MRJP1) was made in the *E. coli* polA-deficient strain JZ294 (*argH, strA, polA:Tn10*), which fails to support ColE1 replication (Dale and Ow 1991) (Fig. 1).

**Transgenic plants:** pBin19-MRJP1 was transferred by bacterial conjugation from *E. coli* to the *Agrobacterium* strain GV3111(pTiB6S3SE) by the triparental mating procedure (Ditta *et al.* 1980). The resulting *Agrobacterium* strain was used for the transformation of tobacco leaf explants using the modified procedure of Horsch *et al.* (1985). *Nicotiana tabacum* L. (cv. Wisconsin 38) seeds after sterilization (treatment with 70 % ethanol for 1 min, 15 % bleach and 0.2 % Triton for 15 min and with sterile H<sub>2</sub>O 5 × for 10 min) were placed onto Murashige and Skoog (1962; MS) agar medium (4.3 g dm<sup>-3</sup> of MS salts, 30 g dm<sup>-3</sup> of sucrose, 10 g dm<sup>-3</sup> agar, pH 5.8) and grown in 16-h photoperiod with irradiance of 40 - 65 μmol m<sup>-2</sup> s<sup>-1</sup>. Small leaf pieces (1 cm<sup>2</sup>) of 4- to 6-week-old tobacco plants were used for transformation. Their facial sides were incubated with appropriate *Agrobacterium* strain suspension at 26 °C in sterile conditions in 16-h photoperiod for 2 - 3 d. Leaf explants were then placed onto MS shoot-inducing agar medium [MS agar medium, 0.3 mg dm<sup>-3</sup> NAA, 0.3 mg dm<sup>-3</sup> BAP, 2 cm<sup>-3</sup> dm<sup>-3</sup> of B 5 vitamin stock solution (100 mg cm<sup>-3</sup> myo-inositol, 10 mg cm<sup>-3</sup> thiamine-HCl, 1 mg cm<sup>-3</sup> nicotine acid, 1 mg cm<sup>-3</sup> pyridoxine - HCl)] supplemented with 100 μg cm<sup>-3</sup> kanamycin to select transformants and 500 μg cm<sup>-3</sup> cefotaxime to prevent *Agrobacterium* growth. These explants were incubated for several weeks (every two weeks subcultured onto fresh MS shoot-inducing agar medium) in sterile condition at 23 °C. Induced callus tissue was transferred onto MS shoot-inducing agar medium without NAA and

1998). The aim of this work was to clone MRJP1 cDNA into tobacco and to analyze its expression in transgenic plants.

cultured until shoots induction. Shoot regenerants (1 - 2 cm) were then excised and cultured on MS root-inducing agar medium [MS agar medium, 20 cm<sup>-3</sup> dm<sup>-3</sup> of B5 vitamin stock solution] in Magenta boxes. Small plants of about 5 cm were, transferred to the soil. Seeds were collected from the adult primary transformants (parental generation) after self pollination. They were sterilized and then germinated on MS agar medium with 100 μg cm<sup>-3</sup> kanamycin. The seeds from F<sub>1</sub> generation of transgenic plants were rooted directly in the soil.

**PCR analyses:** Plant DNA was isolated from leaves (~ 0.3 cm<sup>2</sup>) of young plants by the procedure described by Dellaporta *et al.* (1983) and dissolved in 0.02 cm<sup>3</sup> of TE buffer.

PCR with isolated DNAs was carried out in 0.02 cm<sup>3</sup> volume containing 1 × Taq polymerase buffer (10 mM Tris - HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 200 μM dNTP, 0.5 μM of each primer, 0.5 units of AmpliTaq polymerase and 0.0004 cm<sup>3</sup> of genomic DNA. Two sets of primers were used for PCR identification of the MRJP1 cDNA in plants. First set were oligonucleotides derived from the CaMV 35S promoter and nos3' terminator sequences:

35S primer: 5' - GTTCATTCATTGGAGAGG - 3',

nos3' primer: 5' - GTATAATTGCGGGACTCTAA - 3'.

Second set were oligonucleotides derived from MRJP1 cDNA sequence:

forward primer: 5' - CTGGCGAATACGACTACAAG - 3',

reverse primer: 5' - CGCAACGAGTGTCAATATC - 3'.

The amplification was initiated by denaturation for 5 min at 95 °C and 35 cycles was performed as follows: 40 s at 94 °C, 40 s at 56 °C and 1.5 min at 72 °C. The final extension was continued at 72 °C for 10 min.

**Immunoblot analyses:** Tobacco leaves removed from the plants reaching 40 cm were reduced to powder in a mortar in the presence of liquid nitrogen. Soluble proteins were obtained by extraction of 200 mg of leaf powder. For extraction 0.6 cm<sup>3</sup> buffer composed of 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM EDTA, 100 mM NaCl, adjusted to pH 7.0, supplemented freshly with one tablet of serine and cysteine protease inhibitors (Complete mini, without EDTA, Roche Applied Science, Mannheim, Germany) was used. Powders were homogenized with the buffer in microtubes which were kept in an ice-water bath for 30 min. During this period the extraction was performed by repeated short vortexing every 2 min. The homogenates were centrifuged at 15 000 g for 15 min at

4 °C. Supernatants (0.3 cm<sup>3</sup>) were transferred into new microtubes and kept on at -70 °C. Protein concentration in supernatants were estimated using the bicinchoninic acid protein assay (Smith *et al.* 1985). The proteins in 0.01 cm<sup>3</sup> of supernatants were separated by SDS-PAGE (Laemmli 1970) on two parallel 9 % gels employing *Mini-Protean II* electrophoresis cell (*Bio-Rad*, München, Germany). One gel was stained with Coomassie Brilliant Blue R 250. Proteins on the second gel were transferred by wet-electroblotting to nitrocellulose membrane. The membrane was blocked with 5 % non fat dry milk in

TBST buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl and 0.05 % *Tween 20*) for 1 h. Then it was incubated with a rabbit antiserum against the recombinant MRJP1 (Júdová *et al.* 1998) diluted 1:2 000 in the same buffer for 1 h, extensively washed and incubated with anti rabbit HRP-linked swain antibodies diluted 1:150 000 in milk-TBST buffer again for 1 h. After washing the position of the plant recombinant MRJP1 was detected by chemiluminiscent procedure using *SuperSignal West Pico* chemiluminiscent substrate (*Pierce*, Wien, Austria) and X-OMAT AR film (*Kodak*) according to *Pierce* manual.

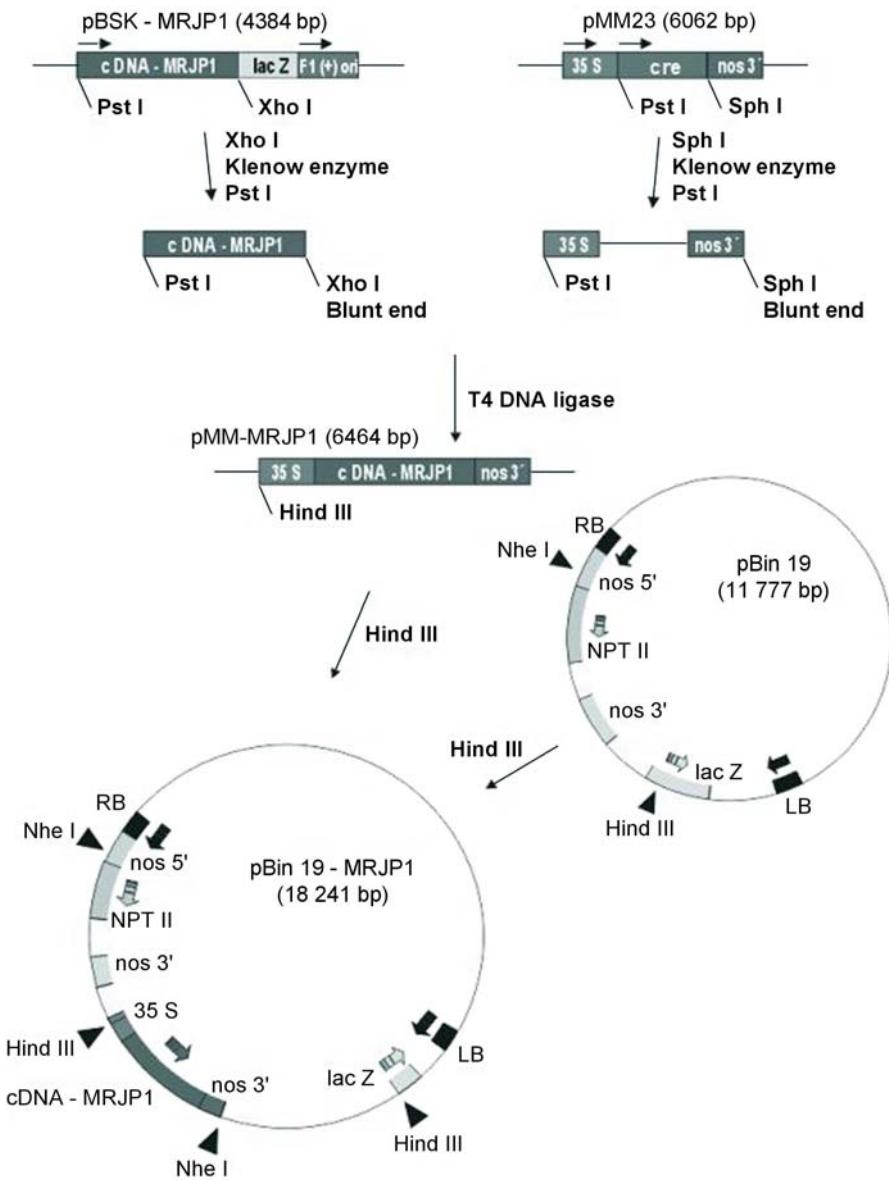


Fig. 1. Construction of plasmid pBin19 - MRJP1. More detailed description is in Materials and methods.

## Results

**Generation of transgenic plants and their PCR analysis:** The MRJP1 cDNA insert has been placed into pBin 19 vector between the CaMV 35S promoter and the NOS terminator in the orientation to permit expression in plants (Fig. 1). *Agrobacterium* carrying the pBin19-MRJP1 construct was used for infection of tobacco leaves. Twenty kanamycin resistant plants were regenerated from leaf explants. The presence of MRJP1 cDNA in the genomic DNA isolated from the regenerated plants were confirmed by PCR amplification using specific MRJP1 cDNA primers, as well as the primers that corresponded to the 35S promoter and the nos3' terminator sequences. The expected PCR products of 1.1 kb employing cDNA specific primers were detected in all regenerated plants (Fig. 2a). The product of 1.7 kb corresponding to 35S and nos3' primers was not detected in all plants (Fig. 2b) suggesting a loss or modification at least of one primer region during the transformation process.

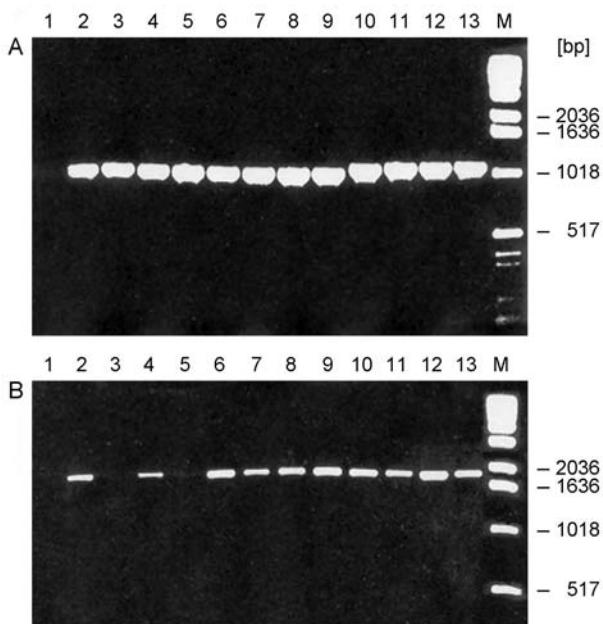


Fig. 2. PCR detection of honeybee MRJP1 cDNA in DNA isolated from leaves of regenerated tobacco plants. Primers specific for MRJP1 cDNA (A) and primers flanking the MRJP1 cDNA, derived from CaMV 35S RNA promoter and nos3' terminator sequences (B) were used for amplification. PCR products were separated in 1.2 % agarose gel. Lane 1 - untransformed tobacco cv. Wisconsin 38; lanes 2 - 13 - transgenic plants; lane M - 1 kb marker (Gibco).

**Transgenic plants of F<sub>1</sub> and F<sub>2</sub> generation:** The seeds of four maternal transgenic plants yielding expected PCR products with both sets of primers were grown to produce F<sub>1</sub> generation of transgenic tobacco plants. The genomic DNAs of twelve offsprings of each plant were

characterized by PCR analysis using 35S and nos3' primers. According to electrophoresis based results only two maternal plants, P<sub>1</sub> and P<sub>2</sub> gave the rise to transgenic offsprings. F<sub>1</sub> generation of tobacco P<sub>1</sub> and P<sub>2</sub> consisted of 77 and 58 % transgenic plants, respectively. Five P<sub>1</sub> tobacco transgenic plants of F<sub>1</sub> generation, named T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub>, T<sub>4</sub>, T<sub>5</sub> giving seeds after self fertilization were used to obtain F<sub>2</sub> progeny. Sets of 13 - 15 young plants were analysed by PCR, similarly as in the case of F<sub>1</sub> generation. The percentual portion of the transgenes in the F<sub>2</sub> offsprings from T<sub>1</sub> - T<sub>5</sub> plants were 70, 93, 73, 92, and 100 %, respectively.

**Expression of honeybee MRJP1 in transgenic plants:** Transgenic plants of F<sub>1</sub> generation and transgenic plants of F<sub>2</sub> generation - 5 to 7 progenies from each of the plants T<sub>2</sub>, T<sub>4</sub>, T<sub>5</sub> were selected for immunoblot analysis. For the negative control 7 nontransgenic plants of tobacco cv. Wisconsin 38 were used. The leaf extracts prepared from plants differed in protein concentration. The concentration varied in extracts of transgenic plants in broader range, between 0.7 - 7.0 g dm<sup>-3</sup>, then it was in nontransgenic plants (3.2 - 6.3 g dm<sup>-3</sup>). The chemiluminescent Western blotting procedure was used for detection of MRJP1 in the leaf extracts and for quantification of its expression based on visual approximal comparison of individual bands intensity with standards. The expression of MRJP1 in the all analysed transgenic plants was identified. Five immunopositive MRJP1 bands were detected on blots from plants expressing MRJP1 in higher levels (Fig. 3). Their M<sub>r</sub> were: tM1 - 54 kDa, tM2 - 51 kDa, tM3 - 46.5 kDa, tM4 - 45 kDa, tM5 - 43 kDa. The band tM3 corresponded with its M<sub>r</sub> 46.5 kDa to theoretical M<sub>r</sub> of complete mature secreted honey bee MRJP1, which was calculated from its amino acid sequence to be 46.8 kDa (Schmitzová *et al.* 1998). No corresponding immunopositive bands were identified in several untransformed tobacco plants (Fig. 3, lane 6 - sample of one of the plants). A strong unspecific immunoreactive diffuse band(s) occurred on immunoblots in all lanes in position close to 60 kDa, also in lanes containing only sample loading buffer. Testing of different chemicals, neither filtration of solutions through 0.45 µm nitrocellulose filters, nor purification of IgG (Harlow and Lane 1988) led to the band(s) elimination.

The level of the MRJP1 expression in individual transgenic plants of the F<sub>1</sub> and F<sub>2</sub> generation was estimated from immunoblot analyses. Overall amount of MRJP1 (summary of amounts of distinct forms) varied from 0.06 to 0.3 µg per 1 g of leaf material. MRJP1 comprised from 0.0012 to 0.0125 % of total soluble protein. Results of expression analysis of several tobacco progenies of T<sub>2</sub> plant (among that the plants with the highest levels of MRJP1 expression were found) are shown in Table 1 and Fig. 3.

Table 1. MRJP1 expression levels in some transgenic tobacco plants. Immunoblots were used for estimation of MRJP1 amount in protein extracts from leaves. The quantification was performed on the base of visual comparison of the intensity of immunopositive signals from different MRJP1 forms (tM1 - tM5 bands) in one gel lane with the intensity of signals that yielded different amounts of isolated honeybee MRJP1 on blots. Overall amount of MRJP1 in all immunopositive bands were estimated for each plant. The data concerning plants 1 to 5 were derived on the base of immunoblot analysis showed in Fig. 3A.

Progenies of plant T2	Protein concentration in leaf extract [g dm <sup>-3</sup> ]	Proteins loaded on gel [μg]	MRJP1 in all immunopos. bands [ng]	MRJP1 in leaf [μg g <sup>-1</sup> (d.m.)]	MRJP1 in total soluble protein [%]
1	1.1	11	0.7	0.21	0.0064
2	2.0	20	0.3	0.09	0.0015
3	1.6	16	0.5	0.15	0.0031
4	0.8	8	1.0	0.30	0.0125
5	0.9	9	0.8	0.24	0.0089
6	1.2	12	0.9	0.27	0.0075
7	1.3	13	0.3	0.09	0.0023

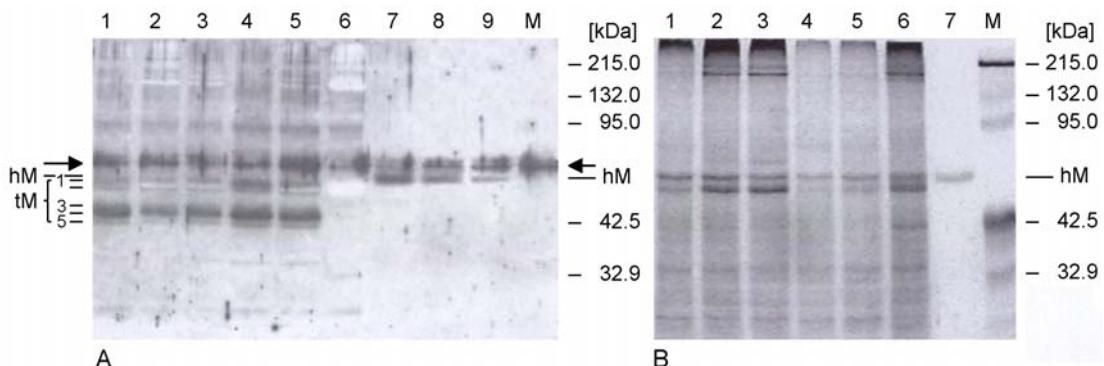


Fig. 3. Immunodetection of MRJP1 in leaf extracts of the transgenic tobacco plants. Soluble protein extracts from leaves of tobacco plants were prepared and proteins in 0.01 cm<sup>3</sup> samples were separated on 9 % SDS-PAGE, electroblotted on nitrocellulose membrane and probed with antiserum against MRJP1 (A), or stained with Coomassie Brilliant Blue R 250 (B). Sensitive *SuperSignal West Pico* chemiluminescent substrate was used for detection of MRJP1 on immunoblot. Lanes 1 - 5 - extracts from leaves of progenies 1 - 5 of T2 transgenic plant containing 11, 20, 16, 8, 9 μg proteins, respectively; lane 6 - extract of untransformed tobacco containing 30 μg of proteins; lanes 7, 8, 9 (A) and 7 (B) - chromatographically purified MRJP1 from honeybee royal jelly, 500, 250, 125 pg and 1 μg, respectively; lane M - kaleidoscope prestained standards (Bio-Rad, München, Germany). The honeybee MRJP1 is marked as hM and the tobacco MRJP1 protein bands are marked as tM with numbers from 1 to 5. The arrows point to the bands with strong unspecific reaction with antiserum.

## Discussion

Transgenic tobacco plants expressing honeybee MRJP1 were prepared. Several bands of the recombinant protein were detected by sensitive chemiluminescent immunoblot analyses in leaf extracts prepared from transgenic plants of F<sub>1</sub> and F<sub>2</sub> generation. The number and molecular mass profile of proteins in these bands suggest post-translational processing of expressed plant MRJP1. We suppose that upper detected bands tM1 and tM2 (Fig. 3) represent the glycosylated forms of plant MRJP1. They moved faster on SDS-PAGE than the honeybee MRJP1 (hM). Honeybee MRJP1 (M<sub>r</sub> 55 kDa) is N-glycosylated protein. Its glycan belongs to high-manose type (Kimura *et al.* 1995). The observed small distinction in M<sub>r</sub> between glycosylated forms of the plant and honeybee MRJP1 could be connected with differences in their

glycan composition and structure caused by some variations in the glycosylation procedure in the plant and in insect cells (Chrispeels *et al.* 1996). Three lower located immuno-positive bands represent presumably non glycosylated forms of MRJP1. Upper band of them (band tM3) corresponded with its M<sub>r</sub> 46.5 kDa to secreted form of MRJP1 without signal peptide. This form of protein was present in tobacco in lower amount as the MRJP1 with M<sub>r</sub> 45 kDa (band tM4), which represented the most intensive band on immuno-blots. Because of small differences in the M<sub>r</sub> of these two forms (1.5 kDa) and the fact that the mobility of proteins on SDS-PAGE corresponds not always accurately with its size, it is not possible to say which of the bands represents really the secreted non glycosylated MRJP1 located in the

endoplasmatic reticulum. For such study further characterization of proteins in the bands is inevitable. Purification of the MRJP1 forms expressed in tobacco plants and determining of their N-terminal sequences, at first of all the putative non-glycosylated forms, will be necessary for their correct characterization. This could also help to resolve the form of MRJP1 protein in the band 5 with estimated  $M_r$  43 kDa.

All analyzed transgenic plants of  $F_1$  and  $F_2$  generation, offsprings of one selected promising parental transgenic plant expressed MRJP1. However, differences in the expression levels between individual plants were observed. It indicates that more copies of MRJP1 cDNA were incorporated into the genome of the parental transgenic plant. There were then inherited into seeds of two generations in different number after self pollination of parental plant as well as their  $F_1$  progenies. The expression of MRJP1 protein in tobacco reached values comparable with expression of other proteins under cauliflower mosaic virus 35S promotor (Schroeder *et al.* 1991, Keinonen-Mettälä *et al.* 1998, Lee *et al.* 2001, Krasnyanski *et al.* 2001).

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The evidence of honeybee protein expression in the experimental model of tobacco plant in general suggests that it is possible to express the honeybee protein in plants. However, the utilization of this protein to influence the nutritional value of some crop plants would require increasing of its expression level in plant cells. Limitations of nuclear genome transformation methods might be overcome by the site - specific introduction of genes into plastid (e.g. chloroplast) chromosomes (Bogorad 2000, Daniell *et al.* 2001, He *et al.* 2001).

Data on physiological properties of RJ proteins, such as suppression of allergic reactions by RJ (Oka *et al.* 2001), or antihypertensive activity of bioactive RJ peptides (Matsui *et al.* 2002) broaden their potential application in pharmacy and indicate their natural function in honeybee evolution, where they could play role of inductor of defense mechanisms during larval development. This recent discovery, that RJ proteins may have important physiological functions as ingredients of functional foods showed potential of transgenic plants for their heterologous expression.

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