A novel function for a ubiquitous plant enzyme pectin methylesterase: the host-cell receptor for the tobacco mosaic virus movement protein

Yu.L. Dorokhov[b], K. Mäkinen[c], O.Yu. Frolova[a], A. Merits[c], J. Saarinen[c], N. Kalkkinen[c], J.G. Atabekov[a,*], M. Saarma[c]

[a]*Department of Virology, Moscow State University, Moscow 119899, Russia*
[b]*A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia*
[c]*Institute of Biotechnology, Viikki Biocenter, P.O. Box 56 (Viikinkaari 9), Fin-00014 University of Helsinki, Helsinki, Finland*

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Abstract Plant virus-encoded movement proteins promote viral spread between plant cells via plasmodesmata. The movement is assumed to require a plasmodesmata targeting signal to interact with still unidentified host factors presumably located on plasmodesmata and cell walls. The present work indicates that a ubiquitous cell wall-associated plant enzyme pectin methylesterase of *Nicotiana tabacum* L. specifically binds to the movement protein encoded by tobacco mosaic virus. We also show that pectin methylesterase is an RNA binding protein. These data suggest that pectin methylesterase is a host cell receptor involved in cell-to-cell movement of tobacco mosaic virus.

Key words: Tobamovirus; Movement protein; Cell-to-cell movement; Tobacco; Pectin methylesterase; Receptor

1. Introduction

During the past decade evidence has accumulated that plant virus-encoded movement proteins (MPs) are required to mediate virus spread between plant cells via plasmodesmata (PD) (reviewed in [1–3]). The cell-to-cell movement of plant viruses is a function of both the viral and host genomes, in which virus-encoded MPs and host-encoded factors (HFs) are involved. Several viral MPs have been localized to PD in virus-infected plants as well as in transgenic plants expressing MP genes [4–9]. The best studied MP is the 30-kDa protein of tobacco mosaic virus common strain U1 (TMV U1). Expression of TMV MP in plants results in a significant increase of the plasmodesmata size exclusion limit (SEL) [10]. Recent results [11,12] show that TMV MP itself is able to move between cells suggesting that it contains a unique signal required for PD targeting, increase of PD permeability and translocation. It is widely accepted that the ability of TMV MP to bind single-stranded nucleic acids [13,14] is important for intercellular translocation of the viral genome. It has been proposed [15] that TMV MP and RNA form an extended linear ribonucleoprotein (RNP) complex that is targeted to and translocated through PD. The molecular mechanisms involved in MP cell wall (CW) localization, PD SEL increase and intercellular translocation of the TMV genome are still poorly understood. Once the MP-RNA complexes reach the PD, they are expected to interact with the HFs that bind the MP. It has been reported [2] that the TMV MP is able to interact with tobacco CW-associated 38K protein (p38) suggesting that p38 is a specific CW receptor for TMV MP. p38 was found to function as a protein kinase (PK), phosphorylating TMV MP at its carboxy-terminus [2]. However, it is not known whether different viruses utilize a common receptor and how many proteins are involved in TMV MP binding. Here we show that a ubiquitous CW-associated plant enzyme, pectin methylesterase (PME), of *Nicotiana tabacum* specifically binds the MP encoded by TMV. Our results suggest that the PME serves as the host cell receptor involved in the cell-to-cell translocation of the TMV infection.

2. Materials and methods

2.1. Isolation of CW-associated proteins

Fully expanded leaves (2 g) were homogenized in 2 ml of PBS (7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl; pH 7.5) plus 1 mM PMSF. The homogenate was filtered through Miracloth (Calbiochem) to obtain the CW fraction which was washed by PBS plus 1 mM PMSF three times to remove cytoplasmic contaminants. The CW fraction was homogenized in 10 volumes of PBS buffer plus 1 mM PMSF and 0.1% Triton X-100 and centrifuged again. This procedure was repeated five times followed by five washes ($1000\times g$ for 5 min at 4°C) in PBS buffer plus 1 mM PMSF. The resulting pellet was resuspended in LiCl of different molar concentrations, incubated by rotating (120 rpm, 4 h) at room temperature and centrifuged ($10\,000\times g$ for 10 min at 4°C). The pellet was discarded and the supernatant proteins were precipitated with 10% TCA plus 0.001% BSA after dialysis.

2.2. Examination of the TMV MP binding ability to the CW-associated proteins by blot overlay binding assay (BOBA)

2.2.1. Expression and purification of MPs. The $(His)_6$ fusions of MPs of two tobamoviruses (TMV U1 and crTMV) [16], potato virus X (PVX) 25K MP [17] and *Escherichia coli* DHFR were expressed in *E. coli* and purified using Ni-NTA-agarose (Qiagen) as described in the manufacturer's protocol (Qiagen). The purity and concentration of all the proteins isolated from *E. coli* were verified by SDS-PAGE.

2.2.2. Radioiodination of proteins by chloramine T. The mixture (35 μl) of MP (0.1 μg/μl), 0.5 M phosphate buffer, pH 7.5, 3 μl of Na-^{125}I (1 mCi/10 μl), 15 μl of chloramine T) was incubated at room temperature for 20 min, then 50 μl $Na_2S_2O_5$ (2.4 mg $Na_2S_2O_5$ in 1 ml 0.05 M phosphate buffer, pH 7.5, dissolved ex tempore) was added and the mixture was incubated for 30 min. In order to stop the reaction, 15 μl of 0.1 M NaI and 50 μl of 1% BSA/PBS were added. Iodinated proteins were purified by G-25 Sephadex PD-10 column chromatography. The specific radioactivity of proteins was $3–5\times10^7$ cpm per 1 μg of protein.

2.2.3. Electrophoresis and electroblotting. Samples were boiled in electrophoretic sample buffer (SB) for 5 min and insoluble material removed by centrifugation at $5000\times g$ in a microfuge. Polypeptides were separated by SDS-PAGE. The gels were either stained with

*Corresponding author. Fax: (7) (095) 938-06-01.
E-mail: atabekov@genebee.msu.su

Abbreviations: BOBA, blot overlay binding assay; CW, cell wall; MP, movement protein; PME, pectin methylesterase; PD, plasmodesmata; TMV, tobacco mosaic virus

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Coomassie brilliant blue (CBB) R250 or electrotransferred to Immobilon-P (Millipore, pore size 0.45 μm).

2.2.4. BOBA experiments. Immobilon-P filters with transferred proteins were incubated with blocking solution (BS) (3% BSA in PBS, 0.1% Tween 20, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) at room temperature for 2 h. Then the Immobilon-P filters were placed into BS containing 1% BSA and $2–3\times10^6$ cpm of iodinated proteins and incubated with gentle agitation for 2 h at room temperature. A 500-fold excess of unlabeled MP was used in the competition assay. Washing was done in two steps: (1) gentle agitation in PBS, containing 0.1% Tween 20, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ for 15 min; (ii) gentle shaking in PBS containing 0.1% Tween 20, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.5 M NaCl; this step was repeated three times. The filters were analyzed using phosphoimager (Fujifilm BAS-1500).

2.3. MP affinity column (MPAC) purification of tobacco CW proteins

After washing with 0.5 M NaCl (160 ml) and twice with distilled water (50 ml), 3 ml of EAH-Sepharose (Amersham-Pharmacia) was mixed with 5 mg of bacterially expressed $(His)_6$-tagged TMV MP (4.3 ml) and 1 M carbodiimide (0.7 ml), pH 4.5, and incubated with gentle mixing at 4°C for 18 h, pH 5.0. The MP Sepharose column was washed with 1 M acetic acid (5 ml) and incubated with it for 4 h at 4°C. Then MPAC was washed six times with 10 ml of 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl. The washing step was repeated six more times with 10 ml of 0.1 M Tris-HCl, pH 8.3, containing 0.5 M NaCl. Finally, the MPAC was washed and stored with equilibration buffer (EB) (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM LiCl) at 4°C. The CW proteins were extracted with 1.0 or 2.0 M LiCl, dialysed and subsequently loaded into MPAC and incubated for 4 h at room temperature. The MPAC was washed five times with 2 ml EB and stepwise elution of proteins was performed with EB containing 0.1 M, 0.25 M, 0.5 M, 1.0 M, and 2.0 M LiCl.

2.4. Examination of the TMV MP binding to the CW-associated proteins by BOBA-Western approach

Immunodetection of TMV MP was done with the rabbit antiserum raised against the $(His)_6$ MP. Immobilon-P filters after electrotransfer of CW proteins were incubated for 4 h at room temperature with renaturation solution containing 3% BSA in TL buffer (25 mM Tris-HCl, pH 8.0, 50 mM LiCl) with gentle mixing. Filters were washed with TL buffer three times and incubated in 20 ml of sterile TL buffer, containing TMV MP (20 μg), 1% BSA, for 2 h at room temperature. Filter washing with TLT buffer (25 mM Tris-HCl, pH 8.0, 50 mM LiCl, 0.1% Tween 20) was done three times for 20 min each. Then antiserum (diluted to 1/4000) in PBS containing 0.1% Tween 20 (PBST) for 45 min was added. After washing (2×20 min) with PBST blots were incubated for 45 min with an alkaline phosphatase-conjugated goat anti-rabbit antiserum (Sigma) (diluted to 1/8000) in PBST for visualization.

2.5. Isolation of the 33-kDa protein and its partial sequence analysis

MP binding, CW-associated proteins with M_rs of 33 and 34 kDa were separated by 12.5% SDS-PAGE. After staining with CBB R250 the two bands were cut out and the proteins digested in-gel with modified trypsin (Promega V5111, USA). The resulting peptides were extracted and analyzed by mass-mapping using MALDI-TOF mass spectrometry. The obtained mass maps from the two proteins were very similar suggesting that the two bands represent different forms of the same protein. Search with the obtained mass profile among the existing databases could not give any significant data to identity the proteins. To further investigate their identity, the peptides from the two proteins were separated by micro-reversed phase chromatography on a 0.3×150 mm PepMap C18 column (LC-Packings, The Netherlands) and collected. The obtained peptide chromatograms from the two bands were very similar confirming the suggestion from mass mapping that the two bands represent different forms of the same protein. Selected peptides were subjected to sequence analysis using a Procise 494 HT sequencer (Applied Biosystems, CA, USA).

2.6. Cloning of the 3′-terminal part of the tobacco PME gene

cDNA encoding the 3′-terminal part of tobacco PME was synthesized from tobacco poly(A)$^+$ mRNA with AMV reverse transcriptase (Promega). The primer designated 33K-*Sal*I-M (5′-ATGGTCGACTTACCTGCCAGGAGTATAAGCTTG-3′; the *Sal*I site is underlined), corresponding to seven C-terminal amino acids of peptide IX (see Fig. 3A), was heated at 70°C with mRNA, and extended at 42°C for 1 h in 35 μl reaction mixture containing 4 mM $MgCl_2$, 1 mM deoxyribonucleoside triphosphates, 25 units of RNasin, 4 mM dithiothreitol, and 100 units enzyme. The cDNA was purified by phenol extraction and ethanol precipitation. The 3′-end of the PME gene was amplified by PCR with primers 33K-*Sal*I-M and 33K-*Nco*I-P. The latter primer (5′-ATGCCCATGGGCAAGAGGTATGTGATTAG-G-3′; the *Nco*I site is underlined) corresponds to peptide I amino acids sequence (Fig. 3A) and the remaining eight nucleotides correspond to nucleotides 971–978 of the tomato PME gene. The PCR generated a major product of 815 bp which was gel-purified using Qiagen's protocol and analyzed by sequencing.

3. Results

3.1. Binding of the TMV MP to CW-associated proteins

Two experimental approaches were used to address the question of to which host CW-associated proteins TMV MP

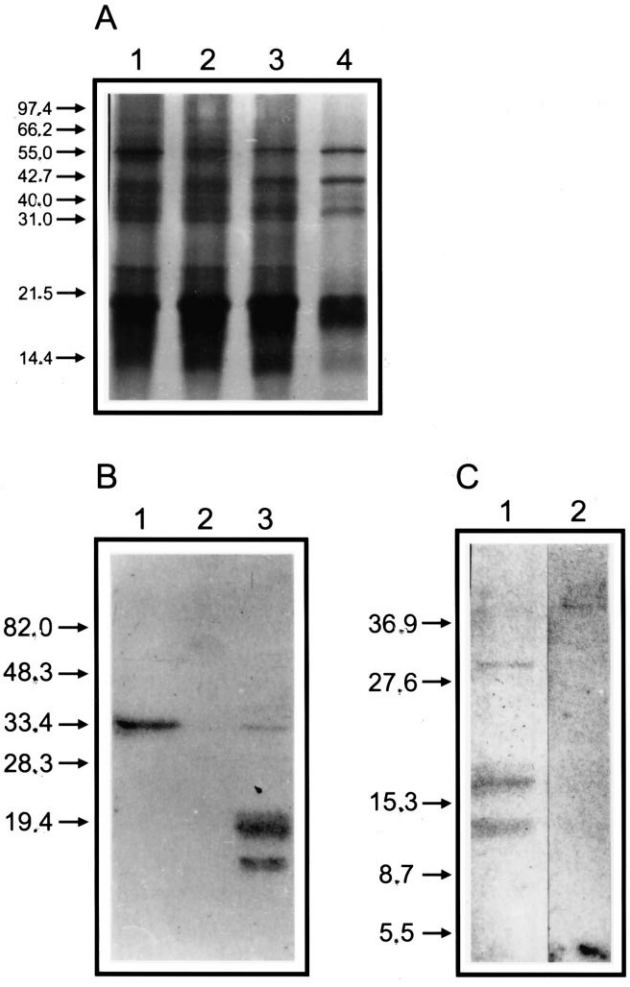

Fig. 1. Detection of interaction between TMV U1 ^{125}I-MP and tobacco CW-associated proteins by BOBA. A: SDS-PAGE analysis of tobacco CW-associated proteins extracted with different molar concentrations of LiCl: 1.0 M (lane 1), 2.0 M (lane 2), 4.0 M (lane 3) and 8.0 M (lane 4). The gel was stained with CBB R250. Molecular weight marker is on the left. B: BOBA for CW-associated proteins using iodinated TMV U1 MP as a probe: (lane 1) TMV U1 MP used as a positive control; (lane 2) protein markers of different M_r indicated on the left; (lane 3) tobacco CW-associated proteins. C: BOBA competition assay: (lane 1) tobacco CW-associated proteins; (lane 2) BOBA of CW-associated proteins after addition of an excess (1:500) of non-labeled TMV MP to the ^{125}I-MP probe.

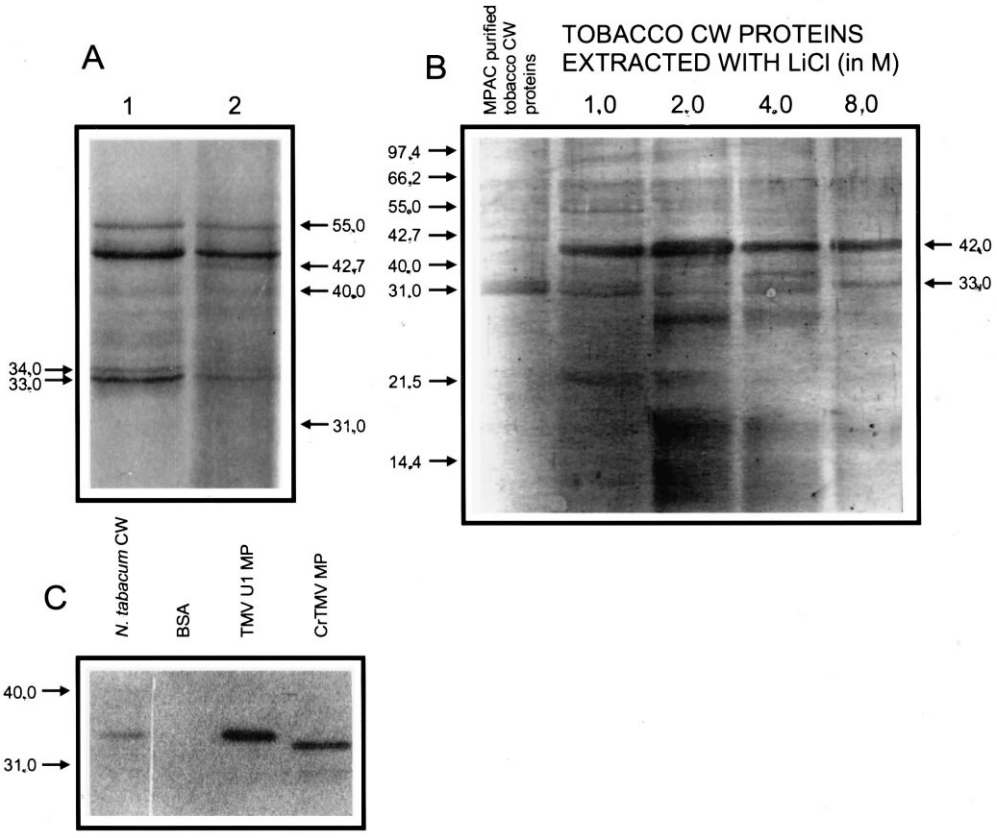

Fig. 2. BOBA-Western and Northwestern analyses of MPAC-purified CW proteins of *N. tabacum*. A: SDS-PAGE analysis of MPAC-purified CW proteins of *N. tabacum*. MPAC-fractionated proteins eluted with: (lane 1) 0.25 M LiCl; (lane 2) 0.5 M LiCl. B: BOBA-Western analysis of MP binding to CW-associated proteins of *N. tabacum* which were extracted by different LiCl consentrations. C: Analysis of the RNA binding ability of the CW-associated 33-kDa protein by Northwestern blotting as described [18]. (Lane 1) the 33–34-kDA protein; (lane 2) BSA as a negative control; (lanes 3 and 4) MPs of TMV U1 and crTMV, respectively, as positive controls.

binds. Firstly, the bacterially expressed, ^{125}I-labeled MPs of two tobamoviruses differing in their host ranges were used as specific probes to identify the MP binding CW polypeptides. In addition to TMV U1 MP, a MP preparation of the second tobamovirus systemically infecting cruciferous plants (crTMV) [18] was used. The CW proteins of *N. tabacum* L., *Brassica napus* L., *Hordeum vulgare* L, hosts and non-hosts for TMV and crTMV were used. The CW proteins were fractionated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with ^{125}I-labeled $(His)_6$-tagged MPs. Complexes formed by MPs and CW proteins were detected by autoradiography. Secondly, a similar series of experiments was carried out using a BOBA-Western approach. The CW proteins were again separated by SDS-PAGE, transferred to nitrocellulose membrane and renatured. Then the membrane was incubated with non-labeled $(His)_6$-MP and the CW-protein-MP complexes were detected by antibodies to the MP. SDS-PAGE analysis of tobacco CW-associated proteins extracted with different molar concentrations of LiCl revealed several major proteins including 33-kDa, 42-kDa, and 55-kDA proteins and smaller products in the range of 14–20 kDa (Fig. 1A). Subsequent BOBA experiments showed that the ^{125}I-labeled MP of TMV U1 (Fig. 1B, lane 3; Fig. 1C, lane 1) binds to the 33-kDa protein and to two small proteins of 14 kDa and 19 kDa. Washing of filters in solutions containing 2.0 M NaCl did not remove these radioactive bands showing that the MP binding of these proteins is tight. ^{125}I-MP did not bind to either of the marker proteins used (Fig. 1B, lane 2), whereas it was efficiently bound to non-labeled TMV MP preparation (Fig. 1B, lane 1). A 500-fold excess of unlabeled TMV MP completely blocked the binding of ^{125}I-MP in BOBA (Fig. 1C, lane 2). In a series of BOBA experiments it was found that the ^{125}I-labeled MPs of TMV U1 and crTMV revealed similar MP binding proteins (33 kDa, 14 kDa, 19 kDa) in CW fractions from different plant species including *N. tabacum, B. napus* and *H. vulgare* (data not shown). On the other hand, no interaction between ^{125}I-labeled 25-kDa MP of PVX or DHFR with proteins of 33 kDa, 14 kDa, and 19 kDa could be detected (data not shown). It is noteworthy that non-labeled $(His)_6$-fused TMV MP associated efficiently with the ^{125}I-MP probe (Fig. 1B, lane 1), showing that TMV MP can form oligomers.

Since TMV MP was able to interact with certain CW proteins, the TMV MPAC chromatography approach was used to purify these proteins. Fig. 2A shows that at LiCl concentrations of 0.25 and 0.5 M 33-kDa and 34-kDa proteins were eluted from MPAC. At least two additional TMV MP binding proteins of about 55 kDa and 42–45 kDa were detected (Fig. 2A). A similar 33- and 34-kDa double band was revealed by BOBA-Western in a fraction of MPAC-purified CW proteins (Fig. 2B). It can been seen from Fig. 2B that, in addition to the 33- and 34-kDa double band, BOBA-Western readily revealed the 42-kDa protein as a major band in the fractions of CW proteins extracted by 1.0–8.0 M LiCl. Therefore, the

42-kDa protein can be regarded as a second major MP binding CW-associated protein.

3.2. Examination of the RNA binding ability of the CW-associated proteins

Fig. 2C presents the results of Northwestern analysis of RNA binding to the MP-binding 33-kDa protein of *N. tabacum*. The 30-kDa MPs of TMV U1 and crTMV were used as positive RNA binding controls, whereas BSA, which lacks RNA binding ability, was taken as a negative control. Fig. 2C reveals that labeled TMV RNA transcripts clearly bind to the 33-kDa CW protein of *N. tabacum*.

3.3. Isolation and amino acid sequence analysis of the TMV MP binding proteins

In the first series of experiments the 14-kDa and 19-kDa proteins detected by BOBA and BOBA-Western were isolated and sequenced (see Section 2). The sequences of two peptides derived from the 19-kDa protein (RIIPRHVLLAVRNDEEL and LAQESSRLARYNL) and one peptide from the 14-kDa protein (RQGRTLYGFG) were screened in SwissProt/Protein Data Base databanks. Examination of the amino acid sequence databanks allowed us to identify the 14-kDa protein as H4.2 and the 19-kDa protein as H2a. This observation indicated that the CW extracts were contaminated with nuclear proteins. Next we focused on the analysis of the 33- and 34-kDa proteins. These MPAC-purified 33- and 34-kDa proteins were shown by microsequencing to represent two isoforms of the same protein. The MPAC-purified 33-kDa protein was digested in gel by trypsin. Nine tryptic peptides of the

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MTRVEDFFSK QIDFCKRKKK IYLAIVASVL LVAAVIGVVA GVKSHSKNSD  50

DHADIMAISS SAHAIVKSAC SNTLHPELCY SAIVNVSDFS KKVTSQKDVI  100

ELSLNITVKA VRRNYYAVKE LIKTRKGLTP REKVALHDCL ETMDETLDEL  150

HTAVEDLELY PNKKSLKEHV EDLKTLISSA ITNQETCLDG FSHDEADKKV  200

RKVLLKGQKH VEKMCSNALA MICNMTDTDI ANEMKLSAPA NNRKLVEDNG  250

EWPEWLSAGD RRLLQSSTVT PDVVVAADGS GDYKTVSEAV RKAPEKSSKR  300
(I)          (II)                (III)
YVIR         ENVCVPK             SNTIITGSR
YVIRIKAGVY RENVDVPKKK TNIMFMGDGK SNTIITASRN VQDGSTTFHS  350
                  (IV)              (V)
               WITFQNTAGAK         VGSDLSAFYR
ATVVRVAGKV LARDITFQNTAGASKHQAVA LCVGSDLSAFYRCDMLAYQD    400

TLYVHSNRQF FVQCLVAGTV DFIFGNGAAV FQDCDIHARR PGSGQKNMVT  450

          (VI)          (VII)       (VIII)
    TDPNQNTGIVIQNCR  IGATSDLRPVQK SFPTYLGRPWK
AQGRTDPNQNTGIVIQKCR  IGATSDLRPVQK SFPTYLGRPWKEYSRTVIM   500

                                                      V
QSSITDVIQP AGWHEWNGNF ALDTLFYGEY ANTGAGAPTS GRVKWKGHKV  550
      (IX)
ITSATEAQAYTPGR
ITSSTEAQAYTPGRFIAGGS WLSSTGFPFS LGL                    583
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Fig. 3. Peptide analysis of CW-associated tobacco PME and nucleotide sequence of its gene. A: Amino acid sequence comparison of the tobacco CW-associated 33-kDa protein-derived peptides and tomato PME. The peptides derived from the 33-kDa CW-associated and MP binding protein are underlined, marked by Roman numerals and aligned on the tomato PME amino acid sequence. The numbers of the last tomato PME residue in each line are indicated on the right. Bold letters indicate homologous amino acids of 33-kDa peptides and tomato PME. B: Nucleic acid sequence comparison of the 3′-terminal part of tobacco PME (33-kDa protein) (lower line) and tomato PME (upper line). Numbering on the right corresponds to the tomato PME gene [19]. The tomato PME start codon AUG, and the *Nco*I and *Sal*I sites derived from primers 33K-*Nco*I-P and 33K-*Sal*I-M, respectively, are underlined. Bold letters indicate non-identical nucleotides. The nucleotide sequence of the 3′-terminal part of tobacco PME has been submitted to the EMBL Nucleotide Sequence Database and has accession number AJ249786.

B

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GACCAATGTC ACGGATATAA AACCCCCACC AATCGATCCA ATTTCTCCAC   50
AACTCTCCCT TAAATTTCTT CATCCAAAAT GACACGTGTT GAAGATTTTT  100
TCAGCAAACA AATCGATTTT TGTAAAAGGA AGAAAAAAAT CTACTTGGCC  150
ATTGTTGCCT CAGTCCTGCT GGTTGCTGCA GTAATCGGAG TAGTCGCCGG  200
AGTAAAATCT CATTCGAAAA ACTCCGACGA TCATGCAGAC ATAATGGCCA  250
TTTCGTCTTC AGCCCATGCT ATTGTAAAAT CTGCGTGTAG CAACACTCTA  300
CACCCCGAAC TGTGTTACTC TGCGATTGTC AATGTTTCTG ATTTCTCAAA  350
AAAAGTAACA AGCCAAAAAG ATGTGATTGA ATTGTCCTTG AATATCACTG  400
TCAAAGCCGT TCGACGCAAC TACTATGCAG TCAAGGAACT CATCAAAACT  450
AGAAAAAGGTT TAACCCCACG AGAAAAGGTT GCGCTGCATG ACTGCCTGGA  500
GACGATGGAC GAGACACTCG ACGAGCTCCA CACTGCTGTA GAAGATCTGG  550
AGCTATATCC CAACAAAAAA TCATTGAAAG AACACGTCGA AGACCTGAAA  600
ACTCTAATAA GTTCCGCAAT TACAAACCAG GAAACTTGCC TCGACGGTTT  650
CTCTCACGAT GAGGCCGATA AAAAGGTACG CAAGGTTTTG TTGAAAGGCC  700
AAAAGCACGT GGAAAAAATG TGCAGCAATG CTTTAGCTAT GATCTGTAAC  750
ATGACCGATA CCGACATTGC AAATGAGATG AAATTATCGG CCCCGCCAA   800
TAATAGGAAG TTAGTAGAGG ATAACGGCGA GTGGCCGGAG TGGTTGTCCG  850
CCGGCGACAG GAGGTTATTG CAGTCGTCGA CGGTGACGCC AGATGTGGTT  900
GTGGCGGCCG ACGGAAGCGG AGATTACAAA ACGGTGTCAG AGGCGGTACG  950

AAAAGCGCCA GAGAAGAGTA GCAAGAGGTA TGTGATTAGG ATAAAAGCTG 1000
          ccatgg gcaagagata tgtgattagg ATAAAGGCTG

GTGTTTACAG GGAAAACGTG GATGTGCCAA AGAAGAAGAC GAATATTATG 1050
GTGTTTACAG GGAGAATGTA GATGTTCCGA AGAAGAAGAC AAATATTATG

TTTATGGGAG ATGGCAAAAG CAATACAATA ATCACAGCAA GTAGGAATGT 1100
TTTATGGGAG ATGGCAGGAG CAATACTATT ATTACAGGAA GTAGAAATGT

GCAAGATGGT AGCACTACCT TCCACTCTGC TACAGTTGTC CGCGTGGCAG 1150
GAAAGATGGT AGCACTACTT TCAACTCCGC TACAGTTG-C CGCGGTAGGG

GAAAAGTTCT TG-CCCGGGA TATAACCTTC CAAAACACAG CAGGAGCCTC 1200
GAAAAATTCT TGGCCCGAGA CATAACTTTC CAAAACACAG CAGGGCCGC

GAAGCATCAA GCCGTGGCAC TCTGCGTGGG CTCTGATTTG TCCGCATTTT 1250
AAAACACCAA GCCGTAGCAC TTCGCGTGGG ATCTGATTTG TCCGCTTTCT

ATAGATGTGA CATGTTGGCT TATCAGGACA CCCTCTACGT CCACTCTAAT 1300
ATAGACGTGA CATTTTAGCC TATCAGGACA GTCTCTACGT CCACTCCAAT

CGTCAATTCT TTGTACAATG TTTAGTCGCT GGTACCGTTG ATTTTATATT 1350
CGTCAATACT TTGTTCAGTG TTTAATTGCT GGCACAGTCG ATTTTATTTT

CGGGAATGGT GCTGCTGTTT TTCAAGA-CT GTGATATTCA CGCCAGGCGT 1400
TGGCAATGCT GCTGCTGTTT T-ACAGAACT GTGATATTCA TGCCCGACGT

CCTGGTTCGG GCCAGAAGAA CATGGTTACT GCTCAAGGAA GAACTGACCC 1450
CCTGGTTCGG GTCAGAAGAA CATGGTCACT GCCCAAGGAA GAACTGACCC

TAACCAGAAT ACTGGTATTG TTATCCAGAA ATGTAGAATT GGTGCAACGT 1500
GAATCAGAAC ACTGGTGTTG TGATCCAAAA TTGTAGAATT GGTGCAACGT

CTGATTTGAG ACCGGTGCAG AAGAGTTTCC CTACATATCT AGGAAGGCCG 1550
CTGATTTGAG ACCAGTTCAG AAGAGTTTTC CCACGTATCT AGGAAGGCCG

TGGAAAGAAT ATTCGAGGAC AGTGATCATG CAATCGTCGA TTACTGATGT 1600
TGGAAAGAAT ATTCCAGAAC TGTGATTATG CAATCGTCAA TTACTGATGT

GATCCAGCCT GCTGGATGGC ATGAATGGAA TGGGAATTTT GCATTGGATA 1650
TATTAATTCT GCTGGATGGC ATGAATGGAA TGGGAATTTT GCGCTTAATA

CTCTGTTTTA TGGGGAGTAT GCAAATACAG GGGCAGGAGC ACCGACGTCA 1700
CTTTGTTCTA TGGCGAGTAT CAAAATACTG GAGCAGGAGC TGGGACCTCA

GGAAGAGTTA AATGGAAAGG GCACAAGGTA ATTACAAGTT CAACTGAGGC 1750
GGAAGAGTTA AATGGAGAGG ATTTAAGGTC ATTACAAGTG CAACTGAGGC

CCAAGCTTAT ACTCCTGGCA GGTTCATTGC TGGAGGTAGT TGGTTGAGCT 1800
Ccaagcttat actcctggca ggtaagtcga c

ACAGGCTTCC CTTTTTCTCT TGGTCTTTAA AACTAAAAAT AAACACGAGC 1850
GAGTTTCAAT TATTTGGAGA CCTAAGTGTG CAGGGCCTTG GCATTTAAAT 1900
AACAAGATCC TTTCACGAAT TTCCCTGTCT TGTCAACTTC TATTCGTGCT 1950
GAGGAATTTT AAGTAATGTA CATGTTAATA TTAACTATTG TGTGC       1955
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33-kDa protein were microsequenced. The amino acid sequences of these peptides were screened in the GenBank/EMBL data banks. Fig. 3A shows that amino acid sequences of peptides derived from the 33-kDa MP binding protein are highly homologous to the reported tomato PME sequence [19].

3.4. Isolation and cloning of the PME gene of N. tabacum

The results presented above indicate that the 33-kDa MP binding protein represents the C-terminal part of tobacco PME. In order to confirm this conclusion the 3′-terminal part of PME mRNA was amplified by RT-PCR, isolated, cloned and sequenced (Fig. 3B). Sequence identity between the tomato PME gene and that encoding the tobacco CW-associated TMV MP binding 33-kDa protein is 85.9% in an 803-nucleotide overlap providing further evidence to conclude that the 33-kDa protein represents the tobacco PME.

4. Discussion

BOBA, BOBA-Western and MPAC were used to identify in the CW fraction of *N. tabacum* proteins that were able to bind the bacterially expressed $(His)_6$-tagged MPs of TMV U1 (Fig. 1B,C and 2A,B) and of crTMV (data not shown). MP binding proteins of 33 and 34 kDa (Fig. 2A,B) were shown by amino acid sequencing to represent the same protein. Formation of double bands by one protein in SDS-PAGE in not an unusual phenomenon (e.g. see [20,21]). This type of heterogeneity could be due to the aberrant mobility of the protein isoform conformomers. This protein was one of the major TMV MP binding CW-associated proteins of *N. tabacum* isolated by MPAC (Fig. 2A). BOBA-Western experiments of MPAC-purified CW proteins (Fig. 2B, left lane) provided additional evidence for the MP binding ability of the 33-kDa protein. However, even without the MPAC purification procedure this protein was readily identified by BOBA-Western in the total mixture of the proteins extracted from the CW fraction by LiCl (Fig. 2B). In a separate Northwestern experiment it was found that the 33-kDa CW-associated protein exhibited RNA binding activity in addition to its MP binding properties. However, the RNA binding efficiency of the 33-kDa protein was considerably lower than that of a tobamovirus MP (Fig. 2C). The functional significance of RNA binding ability of the 33-kDa protein is obscure. One can speculate that both the MP binding and RNA binding abilities of this protein may be involved in targeting the TMV MP-RNA complexes to PD.

Microsequence analysis of the 33-kDa protein-derived tryptic peptides indicated that this protein is highly homologous to tomato PME (Fig. 3A). In order to confirm this conclusion the 3′-terminal end of the PME gene, encoding the amino acid sequenced part of tobacco PME, was isolated using RT-PCR, cloned and sequenced. It was found that the sequence homology between the tomato PME gene and that encoding the tobacco 33-kDa TMV MP binding protein was extremely high (Fig. 3B). These results confirms the identity of this protein to be tobacco PME. It is apparent that CW targeting of a natural PME in the cell is mediated by the signal sequence that is present at the N-terminus of non-processed PME. This conclusion follows from the nucleotide sequence reported for tomato PME ([19], see also Fig. 3B of this paper).

PME is known to be a ubiquitous enzyme in the plant kingdom (for review, see [22]). It is noteworthy that the 33-kDa protein could be detected by BOBA in CW fractions from plants species belonging to different families including *N. tabacum, B. napus* and *H. vulgare*. PME catalyzes the demethoxylation of pectins and is considered to be responsible for chemical modifications of pectin embedded in the plant CW. PME has been suggested to be involved in CW growth and regeneration [19,23]. The ability of the CW-associated PME to specifically bind the virus-encoded MP is a novel functional property of this protein suggesting that PME has a role as the virus receptor in virus cell-to-cell movement. It is obvious that the biological function of this tobacco PME is not limited to its role in virus infection.

At least two additional CW-associated MP binding proteins, about 42 kDa and 55 kDa in size, could be isolated by MPAC (Fig. 2A). It could be noted that when the total mixture of CW-associated tobacco proteins extracted by LiCl was examined by BOBA-Western, the 42-kDa protein produced a major band (Fig. 2B), whereas the MPAC-purified CW proteins contained a very small amount of the 42-kDa protein (Fig. 2B, left line). It could be hypothesized that the 33-kDa and 42-kDA proteins competed for the TMV MP immobilized on MPAC suggesting that the affinity of the 33-kDa protein for the MP is considerably higher than that of the 42-kDa protein.

As mentioned above, it has been reported [2] that the tobacco CW-associated 38-kDa protein kinase (PK) serves as the CW receptor for TMV MP. It is obvious that the 38-kDa PK and 33-kDa PME are two distinct proteins. However, the CW fractions may contain several different MP binding proteins including 38-kDa PK not detected by the methods used in this study and the proteins of 33-kDa PME, 42 kDa, and 55 kDa revealed in this study. It is also not ruled out that the 38-kDa PK studied in [2] actually corresponds to the MP binding protein identified in our work as the 42-kDa protein.

While this article was in preparation we became aware that V. Citovsky and colleagues had experimentally shown that TMV MP is able to bind tobacco PME.

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