Cloning and Functional Analysis of Tobacco Pectin Methylesterase*

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Abstract In order to study the interactions between plant virus movement protein and pectin methylesterase (PME), PME gene from tobacco genome was cloned using RT-PCR method and the sequence was determined (GenBank accession No. AY238968). Sequence analysis showed that there were two conserved domains in PME protein: PMEI and pectinesterase. Multiple copies of PME gene were detected in tobacco genome through Southern blot analysis. Western blot result indicated that two types of PME protein existed in tobacco. But Northern hybridization detected only a full length transcript of PME, which further identified that there was post-translational process. Yeast two-hybrid result demonstrated that PME could not interact with the identified movement protein of rice dwarf virus (RDV), Pns6, but does interact with Pns11, a nucleic acid binding protein of RDV. It implies Pns11 may participate in the movement of RDV.

Key words pectin methylesterase, sequence analysis, yeast two-hybrid system, movement protein

Cell-to-cell movement is an essential step for the successful infection of plant viruses, which requires a number of virus and host encoded proteins to participate[1,2]. Many plant virus cell-to-cell movement proteins (MPs) can interact with host factors, such as plant homeodomain leucine-zipper protein[3], multiprotein bridging factor 1 (MBF1)[4] and other unknown proteins[5]. It is yet to be determined how these host proteins contribute to virus spread.

Pectin methylesterase, a ubiquitous cell wall-associated plant enzyme, catalyzes the demethylesterification of cell wall polygalacturonans, releasing acidic pectin and methanol[6]. Recent studies has shown unequivocally that PME was a host-cell receptor for the tobacco mosaic virus (TMV) movement protein[7]. It was found that PME is associated with plasmodesmata by immunodetection, in addition to its localization to peripheral region[8]. The enzyme plays a central role in all processes requiring remodeling of plant cell wall, being involved in the cell extension, growth and in the fruit ripening process[9,10].

In order to investigate the role of PME in RDV movement, we cloned the *PME* gene from tobacco and analyzed the interaction of PME with Pns6 and Pns11, using yeast two-hybrid system, respectively.

1 Materials

1.1 Plant materials and bacteria

Tobacco (*Nicotiana benthamiana*) was kindly provided by University of California, Berkeley. The *E. coli* DH5α and plasmid pBluescript KS(+) are from our laboratory.

1.2 Enzymes and other reagents

Restriction enzymes, T4 DNA ligase and Goat Anti-Rabbit IgG (Fc) Alkaline Phosphatase-Conjugate were purchased from Promega. Reverse transcription kit was from GIBCO BRL Company. DIG labeling kit and Alkaline Phosphatase-Conjugate anti-digoxigenin antibody were from Roche Company. Yeast two-hybrid system was from Clontech. PME polyclonal antibody from rabbit was a generous gift from Dr. Citovsky (State University of New York, Stony Brook).

2 Methods

2.1 Total RNA isolation and cDNA cloning

Total RNA was extracted from tobacco leaves, as described by Chomczynski *et al.* and Yu *et al.*[11,12] The RNA samples obtained were further purified twice by phenol-chloroform extraction and RNase-free DNase Ⅰ (Promega) treatment. The single-strand cDNA was synthesized by SuperScript Ⅱ RNase H RTase (GIBCO BRL), using the 3′ primer (5′ GTGGATCCGATTTCAGAGACCAAG 3′, the *Bam*H Ⅰ site is underlined), which is complimentary to the 3′ terminal 16 nucleotides of PME ORF. The resulting cDNA was amplified by PCR using LATaq DNA polymerase (Takara) with the 3′ primer and 5′ primer (5′ GTGAATTCATGTTGGATTCCGGCAAG 3′, the *Eco*R Ⅰ site is underlined), which is corresponding to six 5′-terminal amino acids of PME open reading frame (ORF).

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2.2 Southern blot analysis

Total DNA was extracted based on CTAB methods. 15 μg total DNA was completely digested with *Eco*R Ⅰ and *Hind*Ⅲ, separated on 1.0% agarose gel and blotted onto a positively charged nylon membrane (Boehringer Mannheim). Hybridization was carried out overnight at 68℃ by using DIG labeled partial tobacco PME cDNA fragment as a probe (from 964nt to 1 351 nt). Colorimetric detection was performed with NBT and BCIP (Boehringer Mannheim) according to the manufacturer's instructions.

2.3 Northern blot analysis

Total RNA of tobacco, potato and rice were extracted and electrophoresed on denaturing 1.2% formaldehyde-agarose gel and transferred onto a positively charged nylon membrane. RNA was fixed to the membrane by vacuum baking at 80℃ for 2 h. The blots were hybridized overnight in DIG high SDS Hybridization buffer at 50℃ with a DIG-labeled DNA probe (see Southern blot). The detection of blots was performed as described above.

2.4 Western blot analysis

Protein extraction and detection were performed using methods described in Molecular Cloning[13]. The dilution ratio for first and second antibodies is 1∶4 000 and 1∶5 000, respectively.

2.5 Construction of plasmids used in two-hybrid vectors

Open reading frame of S6 and S11 were obtained from cDNA clone of RDV[14,15]. They were released by digestion with *Pst* Ⅰ (blunted by T4 Polymerase), followed by digestion with *Eco*R Ⅰ, respectively. The fragments were inserted into pGBKT7 digested with *Eco*R Ⅰ and *Sma* Ⅰ, to generate recombinant plasmid pGBK-S6 and pGBK-S11, in framed with coding sequence of GAL4 domain. The identified full-length PME cDNA was digested with *Eco*R Ⅰ and *Bam*H Ⅰ, and subcloned into *Eco*R Ⅰ-*Bam*H Ⅰ sites of pGAD, producing PME fusion with GAL4 activation domain. All recombinant constructs were sequenced to verify the correction of open reading frame.

2.6 Yeast two-hybrid analysis

The constructs were transformed into yeast AH109 strain by lithium acetate method as described in the MATCHMAKER library protocol (Clontech). For the *in vivo* assays, fresh transformants were plated onto synthetic dropout (SD) medium lacking L-tryptophan, L-leucine, L-histidine (SD/-Trp/-Leu/-His) at 30℃ for 4 d. Visible colonies were subsequently transferred onto the SD/-Trp/-Leu/-His/-Ade plate (previously plated 100 μl 2 g/L X-α-gal) and incubated for 2 ~ 3 days at 30℃. As negative control, we co-transformed recombinant pGAD-PME construct with the empty pGBK vector, and as positive control, we used a known interacting protein pair, i. e. a DNA-BD/murine p53 protein and an AD/SV40 large T-antigen protein, respectively (Clontech Laboratories, Inc.).

3 Results

3.1 cDNA cloning and characterization of PME

In order to obtain the full length of tobacco PME gene, RT-PCR was carried out with the template of tobacco total RNA and 5′ and 3′ specific primer of tobacco PME cDNA. A specific product of 1 700 bp was amplified. The amplified PCR product was gel-purified and inserted into the pBluescript KS (+) digested with *Eco*R Ⅴ producing plasmid pBS-TPME. Sequence of positive clone was determined by dideoxynucleotide sequencing. Sequence analysis showed that tobacco PME cDNA was 1 732 bp in length, and contained a single ORF which encoded a peptide of 571 amino acids (Figure 1). The deduced molecular mass of PME was 62 ku. The tobacco PME cDNA sequence in this study was released in GenBank with accession number AY238968. The similarity of nucleic acid sequence with the reported sequence in GenBank (*N. tabacum*, AJ401158) was 99.0%, and the amino acid similarity was 99.4%.

3.2 Analysis of deduced amino acid sequence of PME

The deduced amino acid sequence of tobacco PME was analyzed using Conserved Domain search program. We found that a typical leader peptide localized in the N terminal of PME. It was a highly hydrophobic region between 17 ~ 36 amino acids, which formed a transmembrane domain. The likely incised site localized between Gly^{37}-Val^{38}, as predicted by computer program. The leader peptide may play an important role in PME localization and transportation.

Further analysis showed that there was a conserved PME inhibitor domain (PMEI) at the N terminal of PME (Figure 1 and 2). PMEI domain inhibits pectin methylesterases (PMEs) and invertases through the formation of a non-covalent 1∶1 complex. It is also found that both PMEs and their inhibitor were expressed as a single polyprotein and subsequently processed in plants[17]. There are four highly conserved Cys amino acids at the N terminal, which probably play an important role in maintaining PMEI secondary structure.

There was a pectinesterase domain at the C terminal, which is highly conserved in most plant PMEs (Figure 1 and 2). Figure 3 shows the relatively conserved C-terminal domains in various plants PMEs. Pectinesterase catalyzes the pectin de-esterification by forming carboxylates and methanol. Further analysis

showed that PME contained two conserved motif: motif Ⅰ (xGxYxEx, where x stands for any amino acid) and motif Ⅱ (GxxDFIFG)[17]. The conserved tyrosine in motif Ⅰ may play a role in the catalytic mechanism. Motif Ⅱ corresponds to the best conserved region of C terminal, an octapeptide located in the central part of pectinesterase domain.

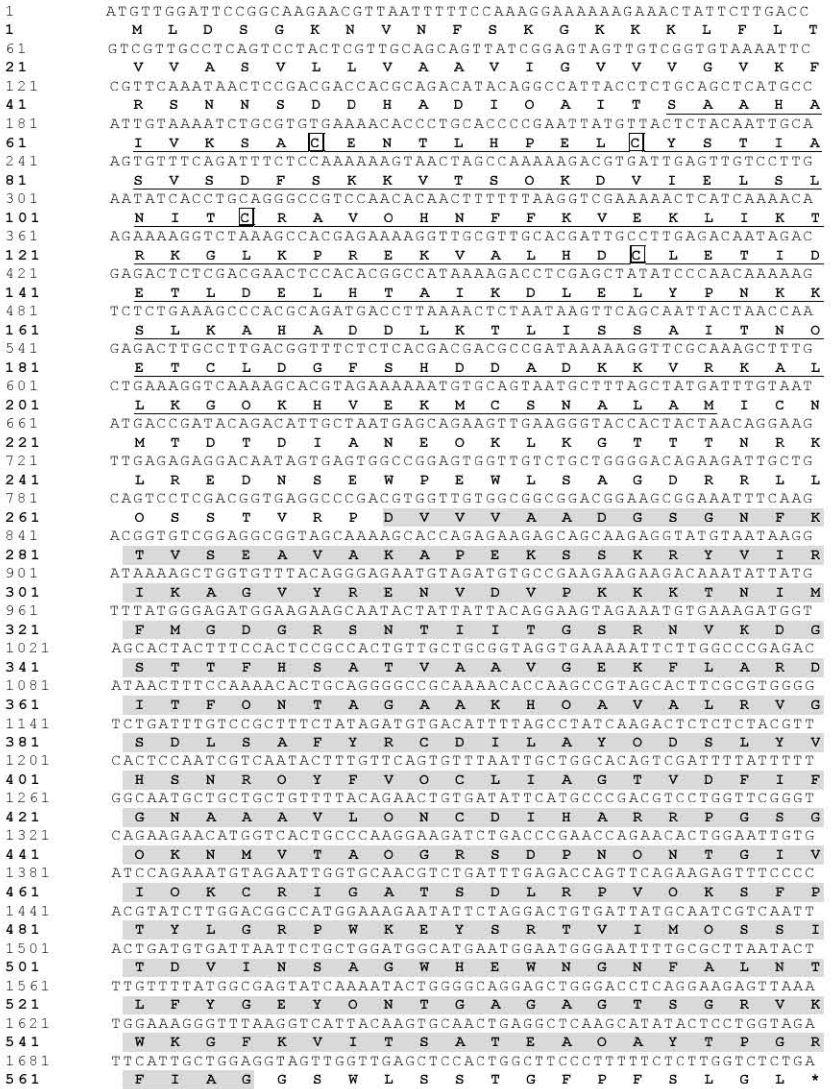

Fig. 1 Sequence of tobacco PME and deduced amino acid sequence

The PMEI domain is underlined, and the shadow region is pectinesterase domain. Conserved Cys was bordered.

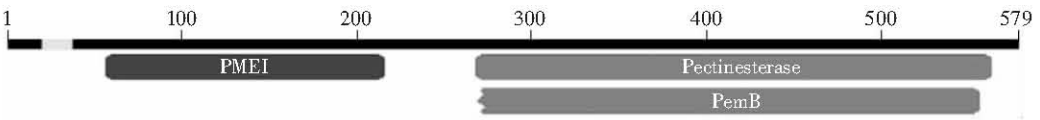

Fig. 2 Conserved domain search result of PME

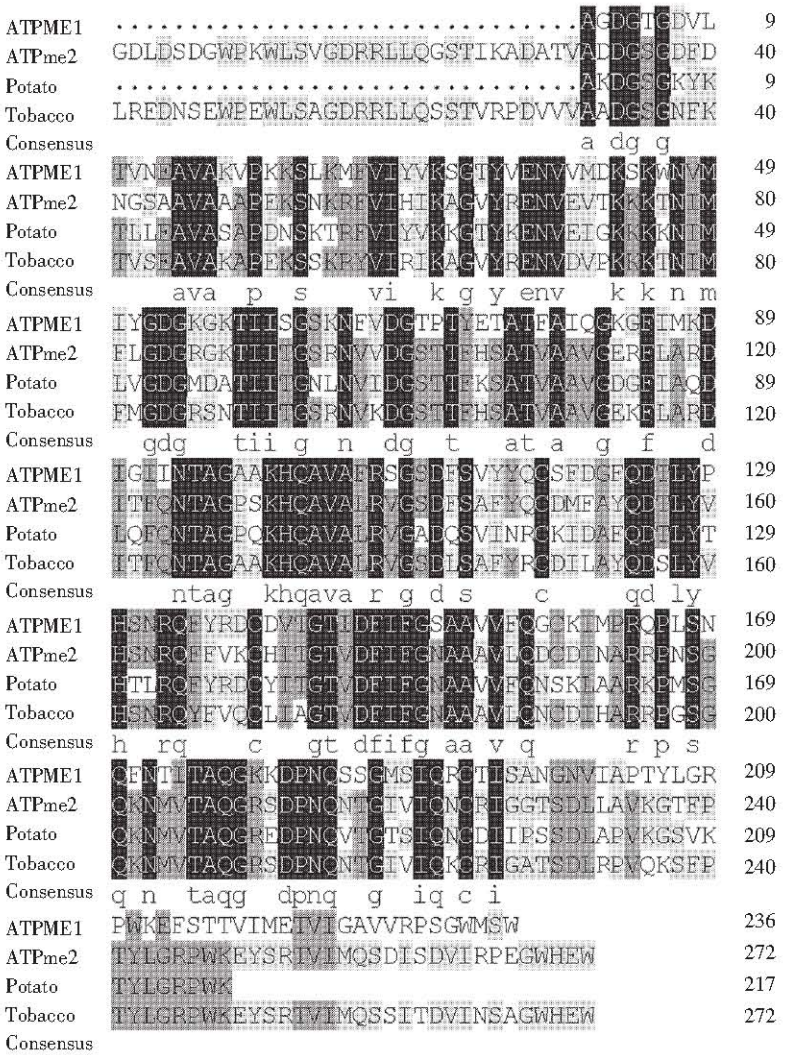

Fig. 3　Alignment of partial deduced tobacco PME amino acid sequence with other plant PME proteins (partial sequences)
ATPME1 is from *Arabidopsis* young tissues (X81585), ATPme2 is from *Arabidopsis* genomic clone (U25649). Potato is from potato plant (U49330), Tobacco is from tobacco plant.

3.3　Southern blot analysis

The copy number of tobacco PME was determined by Southern blot analysis using genomic DNA, digested with *Eco*R Ⅰ, *Hin*d Ⅲ, and *Eco*R Ⅰ + *Hin*d Ⅲ, respectively. Neither of the enzymes cut within the probe sequence. The result indicated that there were five intensive hybridization signals in each lane (Figure 4). It suggests that there may be multi-copies of PME in tobacco genome, which is similar to that in tomato[18].

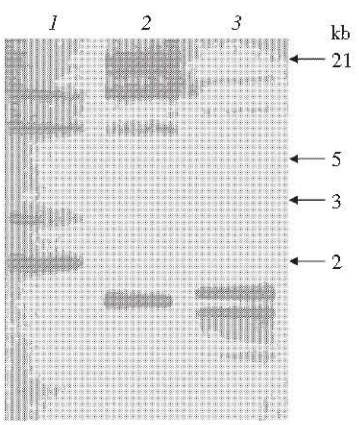

Fig. 4　Southern blot of tobacco genomic DNA
Each lane contains 15 μg tobacco genomic DNA digested with *Hin*d Ⅲ (*1*), *Eco*R Ⅰ (*2*), *Hin*d Ⅲ + *Eco*R Ⅰ (*3*), and hybridized to the PME probe (covers from 964 nt to 1351 nt) labeled with DIG.

3.4 Western blot analysis

Total protein from tobacco, rice and potato were separated on 10% SDS-PAGE and transferred onto the nitrocellulose membrane. PME was detected by a polyclonal antibody against tobacco PME in a standard protocol[13]. The result showed that there were two forms of proteins, of which the molecular masses were about 60 ku and 40 ku, respectively (Figure 5). The molecular mass of deduced tobacco PME was 62 ku, similar to the slower moving protein. The molecular mass of fast moving protein is similar to the pectinesterase domain (molecular mass is 35 ku), i. e. the mature PME. It implied that PME had been processed during the maturing process.

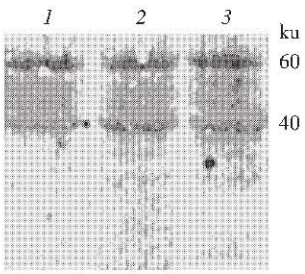

Fig. 5 Western blot analysis of PME protein

Immunodetection of PME proteins in different plants with anti-PME antibodies. Arrowheads indicate the position of the PME protein. *1*: tobacco; *2*: rice; *3*: potato.

3.5 Northern blot analysis

Northern blot hybridization with the partial tobacco PME gene probe was carried out to determine whether PME gene was processed in RNA level. The result showed that RNA from tobacco contained a transcript corresponding to the appropriate size of PME coding sequence (Figure 6). No small fragment was detected. It suggested that PME was processed after translation. There were no detectable hybridization signals in rice and potato. One possible reason is that the probe sequence could not match the PME gene sequence in these plants. We have analyzed the fragment which we use for hybridization and found that there are less than 40% similarity among tobacco, rice and potato.

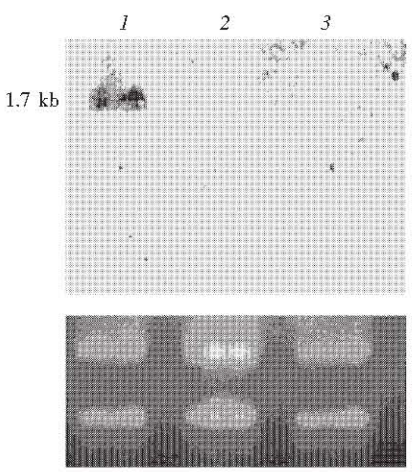

Fig. 6 RNA blot analysis of PME

An RNA gel blot containing 12 μg of total RNA in each lane was hybridized with the DIG-labeled partial *PME* gene DNA probe (from 964 nt to 1 351 nt). As a control of equal RNA loading, a picture stained with ethidium bromide is shown under the blots. *1*: tobacco, *2*: rice, *3*: potato.

3.6 Yeast two-hybrid assay

The pGAD-PME and pGBK-S6 constructs were co-transformed into the yeast strain AH109. Figure 7 showed that PME interacted with RDV Pns11, but did not interact with RDV Pns6, although Pns6 could complement the cell-to-cell movement in a movement defective PVX mutant.

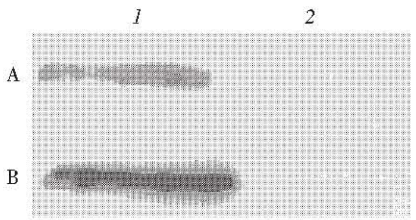

Fig. 7 Identification of the interaction of Pns6, Pns11 with PME in *S. cerevisiae* AH109

Yeast cells expressing the combination of interacting proteins indicated were analyzed for β-galactosidase activity in SD/-Trp/-Leu/-His/-Ade media. The plasmids used for the transformation are indicated bellow. A*1*: pGBK-S11 + pGAD-PME; A2: pGBK-S6 + pGAD-PME; B*1*: pGBK-p53 + pGAD-SV40; B2: pGBK + pGAD-PME.

4 Discussion

In the past few years, it has been shown that PME was encoded by a multigene family[19]. The systematic sequencing of *Arabidopsis* genome has identified 67 PME-related genes[20]. PME plays important regulation roles in fruit development process. Tiemean *et al.*[21] found that PME enzyme activity was inhibited in transgenic plants expressing high levels of anti-sense PME RNA, which had a marked influence on fruit pectin metabolism and increased the soluble solids content of fruits. PME is also produced during microorganism infection, which can degrade cell wall and destroy plant defense system[22].

Southern blotting result showed there were multi-copies PME in tobacco genome. To avoid the possibility of incomplete digestion of total DNA, we digested the genomic DNA with *Hind* Ⅲ and *Eco*R Ⅰ. The copy numbers of PME gene was the same as the result in the single enzyme digestions. It showed total DNA were completely digested.

The molecular mass of most plant mature PMEs were between 32 ~ 42 ku. However, the PME cDNAs reported to date, encode polypeptides with calculated

molecular masses ranging from 57 ku to 65 ku[17]. Western blot result showed that there were two forms of PME in tobacco cells, thus might imply the existence of a post-translational processing[23]. We cloned a full-length tobacco PME cDNA through RT-PCR methods, and the result of *in vitro* translation of tomato PME[18], which all identified that PME was processed after translation. Taken together, the results further identified that most plant PME genes undergo extensive processing procedure to produce the mature form of PME. The PME polyclonal antibody used in this study was made from mature PME purified from tobacco cells[8], which can only recognize the C terminal domain of PME. Therefore, the unprocessed and mature PME were detected in tobacco.

Using functional complementation analysis, Li *et al.*[24] have found that Pns6 was the movement protein of rice dwarf virus (RDV). In our yeast two-hybrid analysis, no interaction occurred between PME and RDV Pns6. It was also found that tomato bushy stunt virus (TBSV) movement protein P22 did not interact with PME[3], although P22 belongs to the TMV MP-like class of MPs[25]. But TBSV P22 can interact with a new plant homeodomain leucine-zipper protein[3]. Therefore, we suggested that Pns6 may not directly interact with PME, but interact with other unknown host factors. We also found that Pns11 did interact with Pns6 and cytoskeleton protein, actin, in addition to interact with PME. It implied that Pns11 may involve in the cell-to-cell movement, together with Pns6. RDV is a symmetrical double-shelled icosahedral particle, whereas TMV is rod-shaped virus[26]. Possibly, there were different movement mechanisms between them.

In order to understand the movement mechanism of RDV and find some host-encoded factors, we are now screening the rice cDNA library using Pns6 as bait protein.

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References

1 Carrington J C, Kasschau K D, Mahajan S K, *et al.* Cell-to-cell and long-distance transport of viruses in plants. Plant Cell, 1996, **8** (10): 1669 ~ 1681

2 Itaya A, Ma F, Qi Y, *et al.* Plasmodesma-mediated selective protein traffic between "symplasmically isolated" cells probed by a viral movement protein. Plant Cell, 2002, **14** (9): 2071 ~ 2083

3 Desvoyes B, Faure-Rabasse S, Chen M H, *et al.* A novel plant homeodomain protein interacts in a functionally relevant manner with a virus movement protein. Plant Physiol, 2002, **129** (4): 1521 ~ 1532

4 Matsushita Y, Miyakawa O, Deguchi M, *et al.* Cloning of a tobacco cDNA coding for a putative transcriptional coactivator MBF1 that interacts with the tomato mosaic virus movement protein. J Exp Bot, 2002, **53** (373): 1531 ~ 1532

5 Huang Z, Andrianov V M, Han Y, *et al.* Identification of arabidopsis proteins that interact with the cauliflower mosaic virus (CaMV) movement protein. Plant Mol Biol, 2001, **47** (5): 663 ~ 675

6 Willats W G, Orfila C, Limberg G, *et al.* Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. J Biol Chem, 2001, **276** (22): 19404 ~ 19413

7 Dorokhov Y L, Makinen K, Frolova O Y, *et al.* A novel function for a ubiquitous plant enzyme pectin methylesterase: the host-cell receptor for the tobacco mosaic virus movement protein. FEBS Lett, 1999, **461** (3): 223 ~ 228

8 Chen M H, Sheng J, Hind G, *et al.* Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. EMBO J, 2000, **19** (5): 913 ~ 920

9 Nari J, Noat G, Diamantidis G, *et al.* Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. Interplay between limited cell-wall autolysis, pectin methyl esterase activity and electrostatic effects in soybean cell walls. Eur J Biochem, 1986, **155** (1): 199 ~ 202

10 Richard J, Noat G. Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. A theory of the ionic control of a complex multi-enzyme system. Eur J Biochem, 1986, **155** (1): 183 ~ 190

11 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem, 1987, **162** (1): 156 ~ 159

12 Yu L, Li Y, Gu G, *et al.* A fast, sensitive and specific method for rice dwarf virus detection by northern blot hybridization. Acta Virol, 1999, **43** (6): 361 ~ 365

13 Sambrook J, FritschE F, Maniatis T. Molecular Cloning. 2nd. New York: Cold Spring Harbor Laboratory Press, 1989. 888 ~ 897

14 刘一飞, 李 毅, 陈章良, 等. 水稻矮缩病毒基因组第六号片编码区的 cDNA 克隆及序列分析, 病毒学报, 1994, **36** (5): 246 ~ 250
Li Y F, Li Y, Chen Z L, *et al.* Chinese J Virol, 1994, **36** (5): 246 ~ 250

15 肖 锦, 李 毅, 陈章良等. 水稻矮缩病毒基因组第十一号组分基因序列和编码蛋白的功能分析, 生物工程学报, 1996, **12** (3): 361 ~ 366
Xiao J, Li Y, Quan S, *et al.* Chin J Biotech, 1996, **12** (3): 361 ~ 366

16 Giovane A, Balestrieri C, Quagliuolo L, *et al.* A glycoprotein inhibitor of pectin methylesterase in kiwi fruit. Purification by affinity chromatography and evidence of a ripening-related precursor. Eur J Biochem, 1995, **233** (3): 926 ~ 929

17 Markovic O, Jornvall H. Disulfide bridges in tomato pectinesterase: variations from pectinesterases of other species; conservation of possible active site segments. Protein Sci, 1992, **1** (10): 1288 ~ 1292

18 Gaffe J, Tiznado M E, Handa A K. Characterization and functional expression of a ubiquitously expressed tomato pectin methylesterase. Plant Physiol, 1997, **114** (4): 1547 ~ 1556

19 Micheli F, Holliger C, Goldberg R, *et al.* Characterization of the pectin methylesterase-like gene AtPME3: a new member of a gene family comprising at least 12 genes in *Arabidopsis thaliana.* Gene, 1998, **220** (1 ~ 2): 13 ~ 20

20 Kaul S, Koo L H, Jenkins J, *et al.* Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana.* Nature, 2000, **408** (6814): 796 ~ 815

21 Tieman D M, Harriman R W, Ramamohan G, *et al.* An antisense pectin methylesterase gene alters pectin chemistry and soluble solids

in tomato fruit. Plant Cell, 1992, **4**（6）: 667 ~679
22 高增贵，陈 捷，高洪敏，等. 玉米茎腐病菌产生的细胞壁降解酶种类及其活性分析. 植物病理学报，2000，**30**（2）：148 ~152
Gao Z G, Chen J, Gao H M, *et al*. Acta Phytopathologyica Sin, 2000, **30**（2）: 148 ~152
23 Nari J, Noat G, Ricard J. Pectin methylesterase, metal ions and plant cell-wall extension. Hydrolysis of pectin by plant cell-wall pectin methylesterase. Biochem J, 1991, **279**（Pt2）: 343 ~350
24 Li Y, Bao Y M, Wei C H, *et al*. Identification of a nonstructural protein encoded by a plant double-stranded RNA virus with cell-to-cell movement function. J Virol, 2004, **78**（10）: 5382 ~5389
25 Melcher U. The "30K" superfamily of viral movement proteins. J Gen Virol, 2000, **81**（Pt1）: 257 ~266
26 McLean B G, Zupan J, Zambryski P C. Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco cells. Plant Cell, 1995, **7**（12）: 2101 ~2114

烟草果胶甲基酯酶（PME）基因的克隆及功能分析*

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摘要 为了研究果胶甲基酯酶（pectin methyl-esterase，PME）（EC 3.1.1.11）与植物病毒运动蛋白之间的相互作用，应用RT-PCR方法从烟草（*Nicotiana benthamiana*）中克隆了PME基因，并测定了全序列（GenBank登录号AY238968）. 序列分析显示该基因由两个保守的结构域组成（PMEI和pectinesterase）. DNA印迹结果表明，该基因在基因组中存在多个拷贝，蛋白质印迹表明，植物总蛋白中存在两种形式的PME蛋白，但RNA印迹结果显示，在烟草细胞中只检测到全长的PME转录产物. 酵母双杂交结果表明，PME与水稻矮缩病毒Pns11（具有非特异的核酸结合活性）之间存在相互作用，而没有检测到PME与已知的运动蛋白Pns6之间的相互作用，推测Pns11蛋白可能参与了水稻矮缩病毒粒子的运动.

关键词 果胶甲基酯酶，序列分析，酵母双杂交，运动蛋白
学科分类号 Q

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