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Molecular cloning and characterisation of a putative pectin methylesterase cDNA in *Arabidopsis thaliana* (L.)

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Abstract Pectin methylesterase (PME) is a cell wall enzyme that catalyses the de-esterification of pectins leading to fundamental changes which confer new properties to the micro-environment of each cell. In order to elucidate the meaning of PME-mediated changes of pectin in the time course of cell differentiation, we attempted to study the regulation of PME genes in *Arabidopsis thaliana*. In this report, the first full cDNA sequence showing sequence similarities with other PME genes characterised so far in other plant species has been isolated from an *Arabidopsis* shoot cDNA library. This ATPME1 cDNA is 1,970 bp long and contains an open reading frame encoding a protein of 64,1 kDa and a basic pI of 8.7 as predicted from the nucleotide sequence. Northern blot analyses denoted changes in the expression level of the ATPME1 mRNA according to plant organs. High mRNA levels were found in young developing organs such as cauline leaves while they were significantly lower in rosette leaves, stems and inflorescences, and almost undetectable in roots. Beside this molecular approach, isoelectrofocusing analyses revealed the occurrence of three PME isoforms in *Arabidopsis*. Two PME isoforms with pI values of 4.9 and 9.1 were found throughout the plant, but at a higher level in the root, while an other PME isoform with a pI of 5.7 was essentially detected in the inflorescence. The relationship between our observations and the data reported for other plant species is discussed.

Key words: Pectin methylesterase; Growth; Cell expansion; Cell wall; *Arabidopsis thaliana*

1. Introduction

Pectin methylesterase (PME; EC 3.1.1.11) takes part in the modulation of cell wall structural and functional features during plant development and defence reactions against pathogens [1]. Most studies reported so far regarding plant development mainly focused on the relationships of the PME with fruit maturation [2]. However, little is known about the role of this enzyme in other aspects of plant development particularly in cell expansion and maturation of the cell wall [3].

The enzyme catalyses the de-esterification of the methyl-esterified galacturonic acid residues of the pectins generating free carboxyl groups within the cell wall. It is argued that changes in pectin esterification affect extra-cellular pH and ionic balances. These may influence subsequently the structural interactions between cell wall components [4] and also the activity of a wide range of hydrolytic enzymes involved in cell wall loosening and morphogenesis [3]. The way by which all of these PME-mediated changes are co-ordinated spatially and temporally around each cell allowing coherent growth of the whole plant is not yet elucidated [5].

In most dicotyledonous plants, several PME isoforms occur in tissues which can be distinguished with respect to their molecular weight, pI and biochemical features. However, only a few molecular studies have been undertaken to clone the PME genes. Several PME cDNAs have been isolated from fruits of tomato [6] and french bean [7] thereby confirming the existence of a multigene family in some plant species. In the same way, full-length genomic sequences corresponding to PME-like genes have been isolated from *Brassica* [8] and *Petunia* [9] and both genes are up-regulated in developing pollen. In *Arabidopsis thaliana*, a cDNA sequencing project currently under way [12] allowed to identify two partial sequences similar to plant PME genes. A sequence of 315 bp of a cDNA isolated from cell suspensions [10] and another sequence of 247 bp of a cDNA isolated from flower buds [11] show similarities with PME cDNAs characterised in french bean [7] and *Brassica* [8], respectively.

Since PME may take part in developmental processes by modulating structural and functional cell wall properties around each cell, within tissues and organs, we found the model plant species *A. thaliana* advantageous for investigating thoroughly the regulation of the PME genes during development of normal and transgenic plants. As a first step towards this goal, we report the sequence and the expression of a gene which is similar to other PME genes characterised so far [6,7]. Furthermore, a biochemical approach allowed us to identify several PME isoforms differentially distributed throughout the plant.

2. Materials and methods

2.1. Plant growth conditions

Arabidopsis thaliana (L.) ecotype Columbia was grown on soil in a growth chamber under a 16-h fluorescent illumination ($300\,\mu E \cdot m^{-2} \cdot s^{-1}$)/8-h dark cycle at 24°C. Four- to five-week-old plants were collected for DNA, RNA and cell wall protein isolation.

2.2. Nucleic acid techniques

A cDNA library was prepared in Lambda ZAPII (Stratagene) from 2-week-old shoots of *A. thaliana* ecotype Columbia ($2.5 \cdot 10^9$ pfu/ml). Two synthetic oligonucleotide primers (Pr 2108: 5'-GTGAATGAAG-CGGTGGC-3'; Pr 2109: 5'-AGCCTGATCGGAATGCC-3') complementary to the ends of the sequence reported in [10], were used to amplify a 298 bp PCR fragment (PCR298) directly from the cDNA library. Amplification was performed with 10 μl of the cDNA library as template and achieved after 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 45 s. After ^{32}P-labelling (Prime-it, Amersham), the

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PCR298 fragment was used as a probe for Southern blot hybridisation and library screening. The DNA isolation, fractionation and analysis were performed according to the procedure described [13].

For Northern blot analysis, total RNA was isolated under standard conditions (RNA Quick-Bioprobe) from the root, the rosette leaves, the stem, the cauline leaves and the inflorescence, namely the upper part of the stem bearing the floral branches. Another primer (Pr 2583: 5′-GTTTCCCTGAATCTTGC-3′) complementary to the newly isolated ATPME1 cDNA and the primer Pr 2109 were both used to produce a longer fragment (PCR935: 935 bp) after PCR amplification of the ATPME1 cDNA template. After labelling, this fragment was used as a probe for Northern hybridisation.

All standard molecular biology techniques were performed as described [14]. Nucleotide sequence was determined on both strands using the T7 polymerase sequencing kit (Pharmacia).

2.3. Cell wall protein extraction and IEF

Cell walls from the root, the rosette leaves, the stem comprising also the cauline leaves, and the inflorescence were prepared using a slightly modified procedure than already described [15]. Frozen material was homogenised in 1 mM phosphate buffer, pH 6.0, 3 to 5 times with intermittent filtration through miracloth. Crude cell wall was extracted with 0.1% (v/v) Triton X-100 and abundantly washed with bidistilled water. In order to elute most of the proteins ionically bound to the cell wall components, preparations were treated twice with 1.5 M NaCl under 30 min agitation at 4°C. Cell walls were then removed after filtration through miracloth and the filtrates containing the cell wall proteins were desalted and concentrated by ultrafiltration on PM10 membranes (Amicon).

For IEF, samples were calibrated either in terms of protein content assayed using a standard procedure (Bio-Rad), or in terms of PME activity determined according to the following protocol. Two microliters of protein sample were added to 1 ml substrate solution containing citrus pectin in excess 0.5% (w/v), 0.2 M NaCl and 0.15% (w/v) Methyl red, pH 6.8, in a microcuve. Pectin de-esterification lowers the pH changing the colour from yellow to red. This colour change can be measured kinetically with a spectrophotometer (Shimadzu) at 525 nm and gives a linear slope proportional to the PME activity of the cell wall extract. A calibration curve was obtained by adding 1 to 100 nEq H^+ to 1 ml of the substrate. This curve showed a linear relationship between the OD and the amount of nEq H^+ added, up to an OD of 1.5, and was used to convert the activities into nEq H^+/min. Calibrated samples were loaded onto 0.5 mm polyacrylamide slab gels containing 10% acrylamide, 0.26% bisacrylamide, 10% ampholines pH 3–10 (Pharmacia). IEF was conducted at 30 W, 2000 V, 50 mA, during 1.5 h. Following IEF, PME activity was located on the gel after treating the gels as described [16]. Briefly, gels were soaked in 0.1 M citrate-phosphate buffer, pH 7.2, for 30 min and incubated for 1 h at 37°C on an 1.5% agar gel containing 0.5% (w/v) pectin as substrate. Gels were then soaked 30 min in 0.1 M malic acid. The de-esterification of pectin was visualised on the gels after staining with 0.02% (w/v) Ruthenium red overnight and destaining in water.

3. Results

3.1. Molecular cloning and nucleotide sequencing

Two oligonucleotide primers complementary to the ends of the EMBL sequence reported in [10] were used to generate a fragment of 298 bp (PCR298) after PCR amplification. Southern blot analysis on *Arabidopsis* DNA revealed that the PCR298 fragment when used as a probe hybridised mainly to a single genomic fragment when the DNA was digested by *Eco*RV, *Eco*RI or *Hin*dIII, as shown in Fig. 1. As the hybridisation was performed under high stringency conditions, the occurrence of weak bands also detected for these digests may denote the presence of other specific genomic fragment with less homology. *Bam*HI and *Pst*I digests only gave a positive smear in the region above 12 kb which comprises both newly generated restriction fragments of high molecular weight and also undigested genomic DNA. In this region the large size of the DNA molecules makes them more susceptible to partial degradation which then may appear as a smear after hybridisation. The smear is almost quite unapparent when digestion is achieved with *Eco*RV, *Eco*RI or *Hin*dIII.

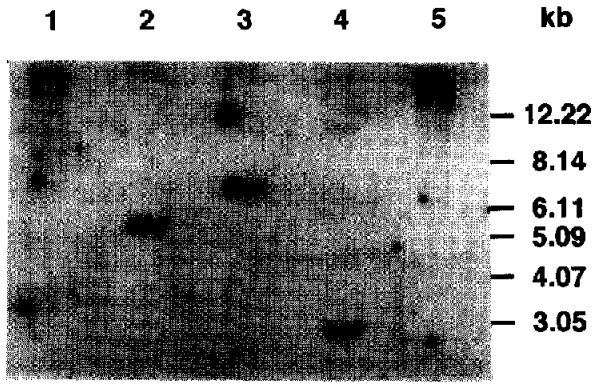

Fig. 1. Genomic DNA gel blot analysis of *A. thaliana*. Plant DNA (10 μg) was digested, separated on 0.75% gel electrophoresis, blotted to positively charged nylon membranes and probed with PCR298. Lanes: 1, *Bam*HI; 2, *Eco*RV; 3, *Eco*RI; 4, *Hin*dIII; 5, *Pst*I. DNA molecular size markers (1 kb ladder, Gibco-BRL) are shown on the right.

The screening of the shoot cDNA library led to the isolation of a clone carrying a cDNA fragment which we named ATPME1. The sequence of ATPME1 is 1,970 bp in length and shows at the 3′ end a short poly(A) tail of 22 residues (Fig. 2). The putative translational start of ATPME1 giving the longest possible ORF (1760 bp) is found at position 58 from the 5′ end. This reading frame encodes a 586 amino acid protein with a calculated molecular mass of 64.1 kDa including the initiating Met^1, and an estimated pI of 8.7. The N-terminal region of the polypeptide includes a basic building block corresponding to a putative signal peptide. The most likely signal peptide cleavage site can be predicted to be at position Ala^{49} in accordance with the rule described in [17]. Moreover, six potential N-linked glycosylation sites, specified by the sequence Asn-X-Ser/Thr, were found mostly in the N-terminal region of the polypeptide.

3.2. ATPME1 mRNA expression analysis throughout organs of Arabidopsis

The PCR935 probe used for Northern blot analysis comprises almost 50% of the complete ATPME1 cDNA sequence. This probe allows us to detect a single mRNA band among total RNAs with a size of 2 kb, similar to the size of the ATPME1 cDNA (Fig. 3). Data denote changes in the expression level of the ATPME1 mRNA according to plant organs. The gene is expressed at a high level in cauline leaves and at a moderate level in stem and rosette leaves. However, ATPME1 transcripts were almost undetectable in the inflorescence and the root. Results reported for cauline and rosette leaves correlated with the juvenility or the growth capacity of the organs as ATPME1 gene expression was significantly higher in the newly formed cauline leaves than in the leaves forming the rosette.

3.3. IEF of the PME isoforms synthesised throughout the plant

In order to examine quantitative changes in the expression of some PME isoforms as a function of plant organs, cell wall protein samples were calibrated with respect to their protein

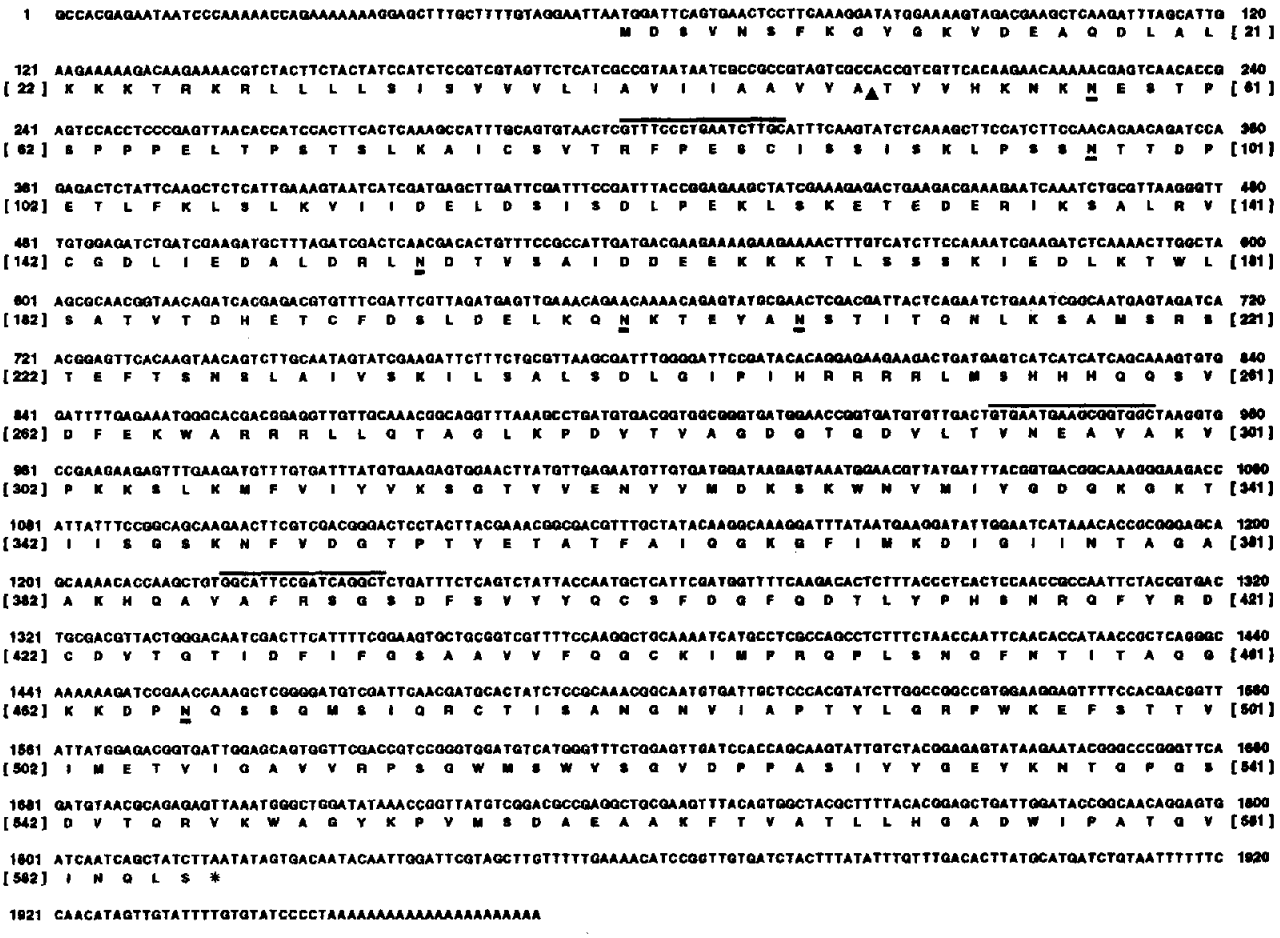

Fig. 2. Nucleotide and deduced amino acid sequences of the *A. thaliana* cDNA ATPME1 (EMBL accession no. X81585). Putative signal cleavage is indicated as (▲). The symbol (*) denotes a stop codon. Potential *N*-glycosylation sites are underlined. Upper lines indicate primers used for PCR amplification.

content and analysed by IEF. As indicated in Fig. 4A, several isoforms with different pIs were detected in the plant. In the region of alkaline pH, it is not clear whether the signal corresponds to two distinct PME isoforms or denotes a partial degradation leading to a modified mobility of a single isoform normally having a pI about 9.1. This basic isoform was abundant in the root and the stem while it occurred at a lower level in the rosette leaves and the inflorescence. A similar ubiquitous pattern was observed for an acidic PME isoform with a pI of 4.9. The difference in intensity between the two isoforms may denote changes in their respective synthesis and/or esterase activity.

In a complementary experiment, protein samples were calibrated according to their PME activity instead of their protein content. Under these conditions, results denoted qualitative changes between organs which concern another PME isoform with a more neutral pI of 5.7. Unlike the other two PME isoforms reported above, the PME with a pI of 5.7 was not synthesised ubiquitously but was essentially detected in the inflorescence. It was not detected elsewhere in the plant except for the root where traces were observed.

4. Discussion

Within the past decade, particular attention has been focused on the role of the most complex cell wall polysaccharides 'pectins' and related enzymes during plant growth and development. The de-esterification of pectins catalysed by the PMEs leads to a complete reorganisation of the cell wall in several ways. For instance, it lowers the pH thereby enhancing the activity of other hydrolases [18]. Moreover, it makes pectins more susceptible to degradation by pectinases [19] and/or to their cross-linkage with other cell wall components [4]. All of these changes are supposed to be intimately related to cell wall loosening during whole plant growth [18], permeation and ex-

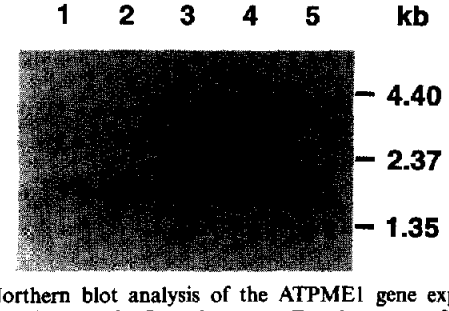

Fig. 3. Northern blot analysis of the ATPME1 gene expression in *Arabidopsis* plants at the flowering stage. Equal amount of total RNA (20 μg) extracted from the inflorescence (1), the stem (2), the cauline leaves (3), the rosette leaves (4) and the root (5) were probed with PCR935. RNA size standards (Gibco-BRL) are indicated on the right.

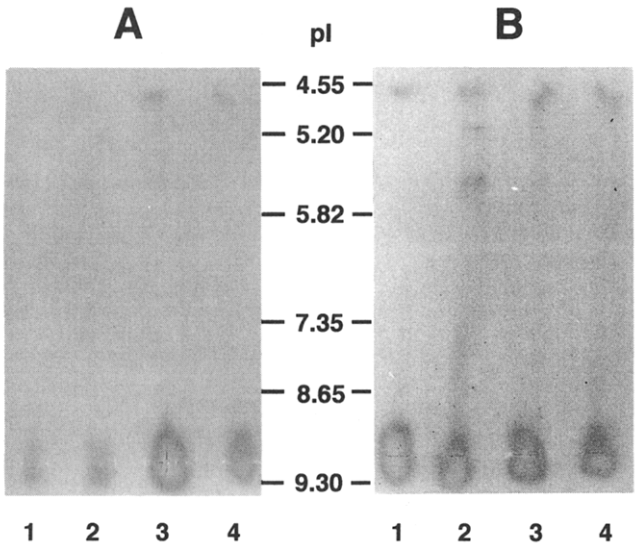

Fig. 4. Isoelectric focusing of cell wall proteins prepared from 4–5 week old *Arabidopsis* plants. Samples were calibrated either in terms of protein content to 1.5 μg (A) or in terms of PME activity to 2 nEq H^+/min (B). Protein extracts isolated from the rosette leaves (1), the inflorescence (2), the root (3) and the stem including cauline leaves (4) were separated throughout a pH gradient and were revealed with respect to their PME activity. A calibration kit (Sigma pH 3–10) used for pI determination is scaled in the middle.

tension of the pollen cell wall [8,9], weakening of the cell wall during fruit ripening [20] and abscission [21].

The present data infer that at least three isoforms of PME are present in *Arabidopsis*. Two of them with pI 4.9 and pI 9.1 are synthesised ubiquitously but at a level significantly higher in developing organs such as the root and stem. The third isoform has a pI of 5.7 closer to the neutrality and was essentially detected in the inflorescence. As in other plant species [22], the presence of multiple PME isoforms in *Arabidopsis* suggests that they have their own specialised function, specifically and temporarily associated with a particular cell type, tissue or organ during plant development. Particularly, the most basic PME isoform in *Arabidopsis* has a pI and a pattern similar to the ubiquitous basic PME isoforms of pI 9.0 and above which are detected in tomato [22]. In this plant species, it is suggested that these basic isoforms might contribute to pectin de-esterification within growing organs subject to cell enlargement.

In an attempt to study the regulation of genes encoding for PME in *Arabidopsis*, we first isolated a clone from a shoot cDNA library using a DNA fragment probe showing sequence similarities with some quite well characterised tomato PME genes [6]. The isolated *Arabidopsis* ATPME1 cDNA encodes a putative protein precursor of 586 amino acids which shows striking similarities with the PME B16 of tomato [6] and the PME-like protein Bp19 of *Brassica* [8]. Each of these three proteins appears to be produced as secretory precursors (Fig. 5). The deduced amino acid sequence of the ATPME1 gene product encloses a putative signal peptide with nearly the same length as for the tomato precursor. Downstream of this signal peptide, an unusually long N-terminal extension with more than 200 residues shows no significant sequence similarity with the N-terminal regions of either the tomato (14%) or the *Brassica* (17%) precursors. However in each case this region seems to be more susceptible to post-translational N-glycosylation than the other parts of the proteins (Fig. 2). As suggested for tomato and *Brassica*, it is likely that this N-terminal leader sequence targets the protein from the endoplasmic reticulum to the apoplasm or makes the enzyme inactive or more stable during export [6,8].

Comparative analyses on the C-terminal regions bring more reliable indications about the putative function of the *Arabidopsis* gene (Fig. 5). In the tomato B16 precursor the N-terminal leader sequence is released from a cleavage site located immediately prior to the C-terminal region which therefore comprises the catalytic domain of the enzyme [6]. Since the C-terminal

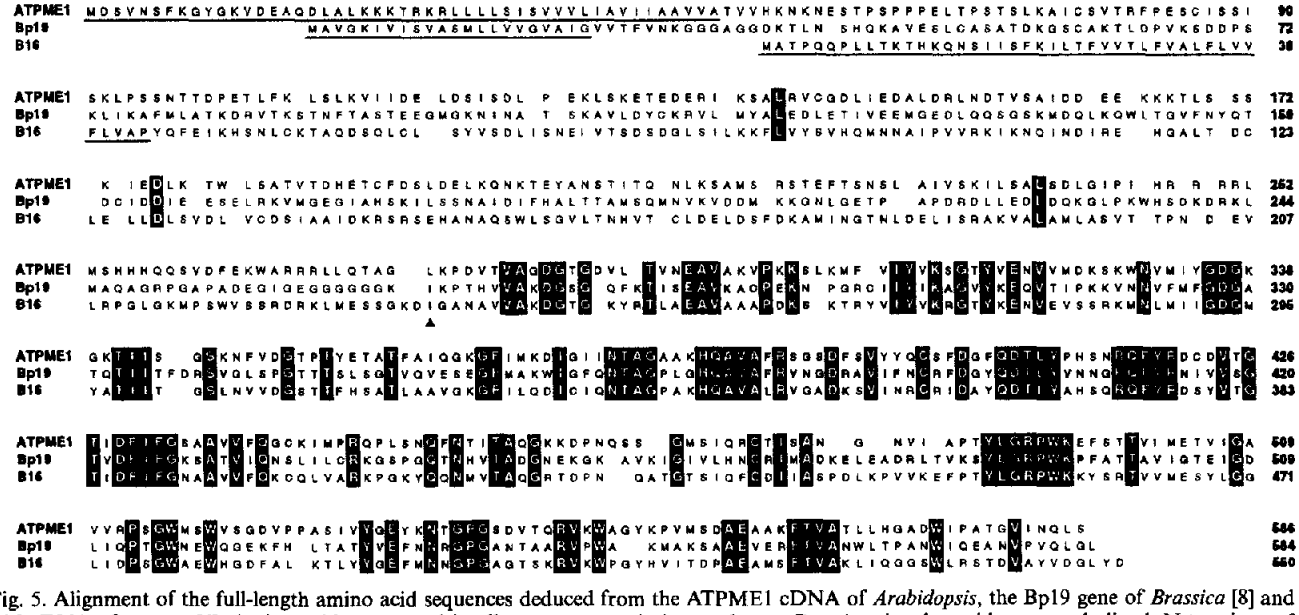

Fig. 5. Alignment of the full-length amino acid sequences deduced from the ATPME1 cDNA of *Arabidopsis*, the Bp19 gene of *Brassica* [8] and the B16 cDNA of tomato [6]. Amino acids conserved in all sequences are in inverted type. Putative signal peptides are underlined. N-terminus of the mature B16 protein is marked with (▲).

region of the ATPME1 precursor shares striking sequence similarity with this particular C-terminal region of the tomato PME (52%) or the PME-like protein of *Brassica* (40%), it is likely that the ATPME1 gene encodes a PME-like protein. Moreover one may expect that the same post-translational cleavage affects the ATPME1 precursor at a similar cleavage site as for tomato (Fig. 5). If so this processing should produce a mature protein with a lower molecular mass of 33.5 kDa and a basic pI about 9.0 close to the pI of the basic PME isoform detected biochemically.

However, the correlation between the ATPME1 gene and the PME isoforms reported here in *Arabidopsis* remains hypothetic until the function of the gene has been elucidated experimentally. Therefore, we are focusing our attention on the expression of the ATPME1 gene in heterologous eukaryotic and prokaryotic organisms. The proteins we expect to produce either under a biologically active or inactive form, will be used for further biochemical and immunological investigations.

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