Characterization of the pectin methylesterase-like gene AtPME3: a new member of a gene family comprising at least 12 genes in Arabidopsis thaliana

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Abstract

Pectin demethylesterification appears to be catalysed by a number of pectin methylesterase (PME) isoenzymes in higher plant species. In order to better define the biological role of these isoenzymes in plant cell growth and differentiation, we undertook molecular studies on the PME-encoding genes in Arabidopsis thaliana. In this paper, we report the characterization of AtPME3, a new PME-related gene of 4 kb in length that we have mapped on Chromosome III. AtPME3 encodes a putative mature PME-related isoenzyme of 34 kDa with a basic isoelectric point. Since the extent of the gene family encoding PME in higher plant species is still unknown, we resorted to the use of degenerate primers designed from several well-known consensus regions to identify new PME-related genes in the genome of Arabidopsis. Our results, in combination with several known expressed sequences tags (ESTs), indicate that the Arabidopsis genome contains at least 12 PME-related genes. Consequently, a method of systematic gene expression analysis has been applied in order to discern the expression pattern of these 12 genes throughout the plant at the floral stage. Whereas most of these genes appeared to be more or less ubiquitously expressed throughout the plant, several genes are distinguishable by their strikingly specific expression in certain organs. The present data bring a new insight into the role of specific PME-related genes in flower and root development. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Cell wall; Growth; Differentiation; Gene expression profile

1. Introduction

Plant cell wall components and architecture are not irreversibly fixed once cell division is complete, but continue to undergo later changes during growth and differentiation of the cell. Among the various changes that confer to the cell wall its dynamic properties, the demethylesterification of the pectins, catalysed by the pectin methylesterases, has many effects. It is established that it influences the interactions between cell wall components (Liners and van Custem, 1994), modulates the activity of pH-dependent cell wall hydrolases (Mountacut et al., 1991) and makes pectins more susceptible to their degradation by pectinolytic enzymes (Koch and Nevins, 1989). Moreover, it has been shown that this event may interfere with signalling processes since it influences the elicitor activity of pectin-derived oligosaccharides (Jim and West, 1984). In muro, the demethylesterification of pectins is spatially and temporally regulated according to the cell-type and the developmental stage of organs (Knox et al., 1990; Li et al., 1995; Steele et al., 1997).

During the past 5 years, particular attention has been devoted to molecular studies of the PMEs, leading to the characterization of several related genes in various higher plant species. Some of these PMEs were shown to be ubiquitously expressed (Gaffa et al., 1997), whereas others are specifically expressed during fruit ripening (Harriman et al., 1991), macrosporogenesis and germination of the pollen grain (Alban et al., 1991; Mu et al., 1994), or stem elongation (Bordenave et al., 1996). These data suggest that PMEs are encoded by a family of genes that are differentially regulated in cell type in...
response to specific developmental or environmental cues. In other respects, only PME of phytopathogenic bacteria and fungi have been shown to be expressed in heterologous prokaryotic (Laurent et al., 1993) and lower-eukaryotic (Christgau et al., 1996) systems. Concerning higher plants, the lack of data in this respect denotes that, during exportation towards the apoplast, plant PME isoenzymes probably undergo organism-specific post-translational processing that is necessary for their structural and functional integrity. As a consequence, the functional characterization of the PME-related genes identified so far is generally difficult, and requires alternative methods based, for instance, on the over-expression of the genes in heterologous plant systems (Gaff et al., 1997). However, such genes can be predicted to be functional genes by the fact that their sequence contains consensus regions shared by all the PMEs that have been functionally characterized in bacteria, fungi or higher plant species. Two of these consensus regions encode the aa motifs GTXDFIFG and YLGRPWK that have been recognized as signatures for PME products (Markovic and Jornvall, 1992).

We report here the characterization of a new member of the PME-related gene family, which we named AtPME3. Considering that this whole related family is probably not restricted to the three AtPMEs that we have characterized so far (Richard et al., 1994, 1996), we took advantage of the consensus sequences of PMEs in order to identify new PME-related genes in Arabidopsis, and to obtain estimates of the size of this gene family in one species representative of higher plants. The method was based on the use of degenerate oligo primers targeted against sequences encoding the consensus motifs GTXDFIFG and YLGRPWK. The results indicate that the Arabidopsis genome contains at least 12 PME-related genes. We employed a method of large-scale gene expression analysis to investigate the expression of these 12 genes in Arabidopsis at the floral stage.

2. Materials and methods

2.1. Screening of Arabidopsis cDNA and genomic DNA libraries

A cDNA library, prepared in λ-ZAPII (Stratagene, La Jolla, CA) from 2-week-old shoots of Arabidopsis thaliana ecotype Columbia was screened using the previously characterized AtPME2 as probe (Richard et al., 1996). This probe was obtained by partial PCR amplification of a genomic DNA fragment containing AtPME1 and AtPME2, and cloned into pBluescript (clone pB5). Oligo primers, Pr2435 (5′-ACTATACCCGGAG-CTCTTCCAHGGMCGACCAARATAC-3′) and Pr2565 (5′-CATTATCA-CGGTCCGTG-3′), designed from the sequence of AtPME2, were used in a PCR reaction performed on 10 pg of pB5. A hot-start procedure and the following PCR program were used: 94°C for 5 min; pause at 94°C to add enzyme to the tube; 30 cycles of 94°C for 1 min, 53°C for 1 min 15 s, 72°C for 1 min 30 s, 72°C for 5 min. The PCR product obtained (PCR1725) was labelled with (α-32P)dCTP (T7 QuickPrime Kit, Pharmacia, Piscataway, NJ) and used to screen the cDNA library under low-stringency conditions (55°C) in order to improve cross-hybridization of the probe to some heterologous PME-like cDNA sequences. A positive pBluescript clone, named pB16, was isolated, sequenced on both strands using the Thermosequenase sequencing kit (Amersham, Buckinghamshire, UK) and identified as a PME-related cDNA, which we named AtPME3. The full-length sequence of the AtPME3 cDNA was further used as a probe to isolate the AtPME3 gene from a λ-ZAPII Arabidopsis ecotype Columbia genomic DNA library (Stratagene). This subsequent cloning was done under high-stringency conditions (65°C) and allowed the isolation of a pBluescript phagemid, named pB6, containing AtPME3.

2.2. Southern blot analysis and genetic mapping

Arabidopsis genomic DNA was isolated by standard methods (Watson and Thompson, 1986). DNA was digested by restriction nucleases and analysed by Southern blot following standard procedures (Sambrook et al., 1989). Hybridization under high-stringency conditions (65°C) was performed using the full-length AtPME3 cDNA. The CIC-YAC library (Creusot et al., 1995) was screened using as a probe a fragment (PE800) corresponding to the promoter region of AtPME3, to minimize cross-hybridization of the probe to heterologous PME-related genomic sequences that may be present within the YAC collection. Labelling and hybridization procedures were similar as described in Section 2.1.

2.3. PCR amplification of the genomic DNA

Two degenerate primers (Pr1.1: 5′-AMTGGAACARTCGATTTCATYTTCGG-3′; Pr2.2: 5′-GAATATTCCTTCCAHGGMCAGCAAAATAC-3′), designed from consensus sequences of genes encoding PMEs, were used in PCR reaction to amplify genomic DNA. The reaction was carried out under the conditions described in Section 2.1, in 100 μl, containing 62.5 ng of DNA, 2.5 μl of Taq DNA polymerase, 1 μM of each primer and 200 μM dNTPs in 1× buffer. The 200-bp PCR product obtained (PCR200) was purified using the Wizard resin as described by the manufacturers (Promega, Madison, WI), cloned into pBluescript...
and further identified by sequencing using the Thermosequenase.

2.4. Large-scale gene expression analysis

The method used for the analysis of the 12 PME-related genes listed in Table 1 (see below) was adapted from the method described by Zhao et al. (1995). The seven PCR200 products AtPMEpcrA to F, cloned into pBluescript, were amplified by PCR using the UP (5’-GTTAACAAGCCGGCACCAGT-3’) and the RP (5’-GGGCACCTGAATTTCATGGTC-3’) primers, under the conditions described in Section 2.1. For AtPME3 as well as for the four EST sequences that we could not amplify by PCR using the Pr1.1 and Pr2.2 degenerate primers, an alternative method of PCR amplification was used to obtain the corresponding cDNA or genomic DNA fragments. For AtPME3, two oligo primers (PrX8: 5’-ATATGCCGACATGTAGCT-3’; PrX9: 5’-GGTCTGCAGCTCTGGATGACGAAGCAG-3’) allowed amplification of a 356-bp fragment containing the full-length sequence of AtPME3 from pBl. This fragment was subcloned into pBluescript. For the ESTs numbered as E9, E10, E11 and E12, oligo primers were designed to amplify, from Arabidopsis genomic DNA, different fragments of 433 bp (PrX23: 5’-CGAACCAGCCGGCTAGTCTCTAAAGATCC-3’; PrX23: 5’-CCAGGCCAGGATGATCAATCAAACTATC-3’), 307 bp (PrX15: 5’-AGAAACATCAGGGTGCAGCCTCTCC-GAT-3’; PrX16: 5’-CCGTCGAGCTCTGGATGACGAAGCAG-3’), 402 bp (PrX42: 5’-CGGATCTAGAC-3’), and 210 bp to make the radioactive concentration per filter constant (PrX42: 5’-GTGCGGATCAG-3’). The ss cDNA probes were further purified on Sephadex G50 (Nick columns, Pharmacia). The probes were added to the Church and Gilbert hybridization buffer (Church and Gilbert, 1984) and incubated for 20 min at 37 °C. Reverse transcription was performed at 42 °C for 50 min. After a denaturation of 5 min at 90 °C, 2 µl of RNAse H (Promega) were added to the reaction mixture, and incubated for 20 min at 37 °C. The ss cDNA probes were further purified on Sephadex G50 (Nick columns, Pharmacia). The RNA fragments were hybridized overnight at 65 °C and then washed successively twice for 15 min in 2× SSC (150 mM NaCl, 15 mM Na₃citrate, pH 7.0), 0.1% SDS at room temperature, 

Table 1  
PME-related gene family of Arabidopsis thaliana: PME-related sequences identical or homologous to the PCR200 products

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>PCR200 product</th>
<th>Number of clones</th>
<th>Library screening</th>
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<tr>
<td>EMBI-GenBank</td>
<td>Name</td>
<td>Number of clones</td>
<td>ESTs</td>
</tr>
<tr>
<td>1</td>
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<td>0</td>
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</tr>
</tbody>
</table>

*EMBL-GenBank Accession Nos of the PCR200 products or loci marked with an asterisk.
15 min in 1× SSC 0.1% SDS at 65°C and twice for 15 min in 0.1× SSC, 0.1% SDS at 65°C.

3. Results

3.1. Molecular cloning and nucleotide analysis

Screening of the Arabidopsis cDNA library using an amplified AtPME2 fragment (PCR1725) as a probe allowed us to isolate the heterologous AtPME3 PME-like cDNA. This was further used as a probe for the isolation of the AtPME3 full-length sequence from an Arabidopsis genomic DNA library (Fig. 1).

The gene AtPME3 is about 4 kb in length and contains two introns, I1 (430 bp) and I2 (690 bp), both bordered by the 5′GT and 3′AG consensus sequences (Csank et al., 1990). The locations of these introns were deduced by comparing the AtPME3 genomic and cDNA sequences. The 1779-bp ORF of AtPME3 encodes a preprotein of 64 kDa sharing a high structural similarity with most plant PME precursors identified so far. It carries a signal peptide cleavage site predicted at position Ala40 (Von Heijne, 1986). Taking into account previous reports on higher plant PMEs (Hall et al., 1994; Bordenave et al., 1996), it is likely that the pro-AtPME3 undergoes an additional post-translational proteolytic cleavage in the vicinity of Gly277, releasing a mature protein of 34 kDa with an IEP of 9.8, which mainly corresponds to the carboxy-terminal region of the precursor (Fig. 1).

High-stringency Southern blot hybridization, using the full-length AtPME3 cDNA as a probe, revealed one major hybridizing band when the genomic DNA was digested by EcoRV, HindIII, PstI or SalI (Fig. 2). These data provide evidence that AtPME3 is present as a single copy within the genome of Arabidopsis. The faint additional band observed for SalI and PstI digestion may reflect the presence of PME-related sequences with a weaker similarity to AtPME3 within the genome.

The PE800 probe used for the hybridization of the CIC-YAC library comprised the complete AtPME3 promoter region. Two YAC clones within the library could be shown to belong to a single contig bordered by markers nga162 and m228 on Chromosome III (Fig. 3). Moreover, a probe designed from the promoter region of AtPME1 hybridized to a single YAC clone, which has not yet been physically mapped to any chromosome markers of Arabidopsis (data not shown).
3.2. Extent of the PME gene family of Arabidopsis

An aa sequence alignment of various plant prepro-PMEs reveals a strong similarity, particularly in the carboxy-terminal region, which corresponds mainly to the catalytic domain of the PME precursors (Hall et al., 1994). Within this region, two short consensus motifs, GTXDFIFG and YLGRPWK, have been recognized as a signature for prokaryote, as well as eukaryote, PMEs. The occurrence of these motifs prompted us to use degenerate oligo primers designed from these specific consensus regions in order to amplify sequences from possibly unknown PME-related genes of the Arabidopsis genome.

These primers, named Pr1.1 and Pr2.2, were designed based on a nt alignment of PME-related sequences partially or fully identified in Arabidopsis. Their combination in PCR reaction amplified a single DNA product of 200 bp, named PCR200, from Arabidopsis genomic DNA. Subsequent cloning of the PCR200 products led to the isolation of approximately 200 PCR200-recombinant clones from which 90 clones were further analysed. As a preliminary step, partial sequencing of the PCR200 inserts using ddATP allowed us to classify the 90 clones into seven distinct groups (AtPMEpcrA–G) within which all members were identical according to their respective ddATP termination profile. The whole sequence of the PCR200 insert was then determined for each group.

Based on their deduced aa sequences, all of the seven PCR200 inserts thus identified display a remarkable sequence similarity to PME-related genes already characterized in higher plant species. As shown in Table 1, four of the PCR200 products (AtPMEpcrD–G) are identical to some genomic or EST sequences already identified in Arabidopsis. By contrast, the PCR200 products AtPMEpcrA, AtPMEpcrB and AtPMEpcrC do not correspond to any sequences in the databanks. They represent, therefore, newly identified PME-related sequences detected within the genome of Arabidopsis.

The frequency of the PCR200-recombinant clones varies from one group to another, suggesting that the two degenerate oligo primers do not amplify each of the PME-related genes at the same efficiency. This is particularly evident for the genomic sequences corresponding to AtPMEs, as well as for some of the ESTs, for which amplification was not detected (Table 1). The data raise from nine to 12 the number of genes so far identified within the PME-related gene family in Arabidopsis. The aa sequences deduced from the newly identified PCR200 products, the AtPMEs, the Arabidopsis ESTs, and all known PME-related sequences of other higher plant species are given in Fig. 4. All sequences are bordered by the GTXDFIFG and YLGRPWK consensus regions, except for the EST T88066 whose sequence is still unknown between these regions.

3.3. Expression pattern of the Arabidopsis PME-related genes

The expression of the 12 PME-related genes has been analysed throughout organs of Arabidopsis at the floral stage, using a method based on the hybridization of
organ-specific ss cDNA probes to the corresponding genomic fragments. The sizes of these genomic fragments and their position with regard to the consensus regions encoding the aa motifs GTXDFIFG and YLGRPWK, are indicated in Fig. 5. The profile expression of the 

PME-related genes is shown in Fig. 6. 

AtPME3 shows a ubiquitous signal throughout the plant at the floral stage that seems notably more intense in the rosette leaves and, at a slightly lower level, in the floral branch. The intensity of the AtPME3 signal is similarly low in the siliques, flowers and roots. The same profile can be observed for AtPME1, which, however, shows an equally high level of signal in both the rosette leaves and the floral branch. Unlike AtPME1 and AtPME3, the signal obtained for AtPME2 is essentially confined to the rosette leaves and is almost undetectable in other plant organs. 

Among genes that have been identified via the analysis of the PCR200 products as well as via ESTs, some of them show a more or less basal ubiquitous profile throughout the plant, whereas others produce a signal in specific organs. This is easily discernible for the gene corresponding to the EST 118G2T7, which shows a strikingly high signal in the flowers and to a slightly lower level in flower-derived organs, namely in the siliques. Moreover, the signal corresponding to the PCR200 product AtPMEpcrD also appears preferentially in the siliques. Another sign of organ-specific profile of the PMEIs is reflected by the gene corresponding to the PCR200 product AtPMEpcrA. This gene differs from all other PME-related genes described in this study by its singularly high signal observed within the roots, which gradually decreases from the roots towards the upper parts of the plant. As observed for AtPME2, the gene related to the EST 155G8T7 shows a more remarkable signal within the rosette leaves, whereas only a trace was observed in the other four organs studied. 

The genes corresponding to the PCR200 products AtPMEpcrF and AtPMEpcrB display a very similar profile among the plant organs to that observed for AtPME1. The signals observed for these genes are notably more intense in the rosette and in the floral branch. Only minor differences between them occur in the flowers and siliques. The signal obtained for the genes related to the PCR200 product AtPMEpcrC and the ESTs ATTS2941 and ATTS5512 is low in the rosette, flowers (AtPMEpcrC) and siliques (ATTS5512). Compared to the control, the signal corresponding to the EST ATTS2941 is scarcely detectable throughout the plant.
4. Discussion

The characterization of the whole gene family in Arabidopsis should, therefore, bring more information on the discrete control of pectin demethylsterification during growth and cell-type differentiation. Towards this aim, we report the characterization of a new PME-related gene in Arabidopsis, named AtPME3, which should encode a putative prepro-AtPME3 precursor structurally very similar to other higher plant PMEs or related proteins identified so far. Unlike the previously identified AtPME1 and AtPME2 that have been shown to be tandemly clustered within the genome (Richard et al., 1996), AtPME3 does not seem to form a cluster with any known PME-related genes. Screening of the CIC-YAC library using promoter-specific probes of AtPME3 and AtPME1 (data not shown) revealed that AtPME3 and the tandem AtPME2–AtPME1 are either separated by several hundred kilobases on Chromosome III, or located on different chromosomes. Moreover, public databanks indicate that the gene corresponding to the EST F15264 is located on Chromosome I, whereas those corresponding to the ESTs T42859 and Z35842 are located on Chromosome II. From these data, it is likely that the AtPMEs are fairly scattered within the genome.

In the past 5 years, systematic sequencing of ESTs has greatly contributed to the identification of numerous PME-related sequences in Arabidopsis in addition to those characterized in other higher plant species (Delseny et al., 1997). The overall data obtained via this approach, together with those provided through DNA library hybridization (Richard et al., 1994, 1996), suggested the existence of up to nine PME-related genes in this species. As an alternative to both of these approaches, the use of a couple of degenerate primers designed from two highly conserved regions of the PMEs allowed here the identification of three new genomic sequences (AtPMEpcrA–C), raising to 12 the number of PME-related genes found within the genome of Arabidopsis.

Systematic expression analysis of the PME-related genes has been undertaken in Arabidopsis in order to reach a more in-depth understanding of the possible relationship between the expression of these genes and the development or the function of certain organs in higher plant species. This analysis allowed us to discern three classes of PME-related genes in accordance with their expression pattern throughout the plant at the floral stage. The first class includes genes that are ubiquitously expressed in the plant (AtPME1, AtPME3, AtPMEpcrA, AtPMEpcrB, AtPMEpcrF), the second class comprises genes whose expression is localized in specific organs (AtPME2, AtPMEpcrD, 118B2T7, 155G8T7), and the third class represents genes whose expression has been hardly detectable within the plant at the floral stage (AtPMEpcrC, ATTS2941, ATTS5512). Although it cannot be excluded that some signals may be attributable to cross-hybridization between genes of the PME family already or not yet identified, the high intensity of the signal observed for AtPME3, AtPMEpcrA and the EST 118B2T7 accounts for a more specific hybridization and arouses a particular interest.

The expression profile of genes of the class I, particularly AtPME3, suggests the existence of housekeeping PME-related genes involved in the maintenance of the wall integrity throughout the plant. A similar ubiquitous expression of some PMEs found in tomato (Galbe et al., 1997) and in Arabidopsis (Richard et al., 1994) denotes their widespread distribution in higher plant species. A more detailed investigation of these genes should allow confirmation of their ubiquitous expression and then discrimination of the genes involved in cell-to-cell cohesion and regulation of intercellular space throughout the plant, from genes related to developmental processes such as cell growth or senescence.

The reliability of this expression analysis is supported by the specific expression of the EST 118B2T7 within the flowers and flower-derived organs. This result agrees with the high sequence similarity that we have found between this peculiar gene of Arabidopsis and the pollen-expressed PME-related gene, Bp19, identified in Brassica napus (Albani et al., 1991). Throughout the 166 aa deduced from the 617-bp sequence known of the EST 118B2T7, we found 81% sequence identity with the gene of Brassica.

To our knowledge, these results are the first evidence of a flower-specific PME-related gene in Arabidopsis. The high sequence similarity that the EST 118B2T7 shares with Bp19 of Brassica raises questions about its possible role in pollen development. Bp19 was shown to be specifically expressed during pollen maturation and, unlike the EST 118B2T7, inactive in mature flowers. Bp19 as well as the EST 118B2T7 share only 40% identity at the level of the deduced aa sequences with another pollen-specific PME-related gene, PPE1, identified in Petunia hybrida (Mu et al., 1994). Unlike Bp19, the activity of PPE1 is associated with pollen germination and/or pollen tube growth. Whereas the biological role of these genes is thought to be restricted to pollen development, the sustained high expression within the siliques observed for the EST 118B2T7 suggests another role for this Arabidopsis gene in flower development. In-situ hybridization actually in progress will bring precise clues in this respect.

The possible existence of root-specific PME isoenzymes in higher plant species is supported by previous evidence concerning changes in pectin esterification within root tissues of oat seedlings (Knox et al., 1990). The very high expression of AtPMEpcrA within the root raises questions about its biological function in the development or the function of this particular organ,
i.e. the growth of the root under physical environmental constraints, or the absorption of mineral nutrients from the soil.

Some of very low-expressed or inactive PME-related genes of the class III may correspond to pseudogenes or genes whose expression may be relevant to the developmental stage of the plant. Systematic analysis of plants at different stages, from seedlings up to mature floral plants, will provide more information on the regulation of these particular genes.

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