PCR cloning and expression analysis of a cDNA encoding a pectinacetylesterase from *Vigna radiata* L.

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Abstract A cDNA clone encoding a pectinacetylesterase (PAE) was isolated from 3-day-old mung bean seedlings using PCR-based techniques. Degenerate oligonucleotide primers were designed according to the N-terminus and internal peptides from the purified PAE. The full-length clone of 1453 bp codes for a signal peptide of 24 amino acids and a mature protein of 375 amino acids. The M_r and the pI of the cDNA-deduced amino acid sequence agree with the values estimated for the purified enzyme. No significant sequence identity between the PAE and any known protein could be found in the databases. Northern analysis revealed developmentally regulated expression of the mRNA in mung been seedlings.

Key words: Pectinacetylesterase; Cell wall; Pectin; cDNA sequence; *Vigna radiata*

1. Introduction

Acetylated carbohydrate polymers, mainly xylan and pectins, have been characterized from a number of plant species [1–4]. In pectins, acetyl groups can esterify galacturonic units of rhamnogalacturonan I (RGI) at C2 or C3. The presence of acetyl is known to modify the properties of the pectin molecules since partial hydrolysis of the acetyl esters in beet pectin leads to an improvement of the gelation propertied [5]. Moreover, deacetylation was shown to affect pectin solubility, making it more soluble in water by decreasing the pectin backbone hydrophobicity [6]. In vivo, the degree of acetylation might then be a structural factor to be taken into account for the development of cell wall properties. Acetylation has been shown to occur between the Golgi and the cell wall during the exocytosis process, the exported acetylated pectins being then incorporated into the cell walls [7]. Acetylesterases specific for deacetylation of pectins have been isolated from microorganisms [8,9] and citrus peel [10] but no information is available concerning the possible function of this activity in vivo. Recently, a protein exhibiting pectinacetylesterase activity was extracted from cell wall fragments isolated from mung bean hypocotyls [11]. This acetylesterase was purified and some internal peptide sequences were obtained. Physicochemical properties of the protein were also determined. The present paper reports on the sequence of a cDNA coding for the mung bean PAE and its expression in the different parts of 3-day-old seedlings.

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2. Materials and methods

2.1. Biological material

Vigna radiata (L.) Wilzeck seeds were rehydrated for 3 h in distilled water at 37°C, washed and sown on moist vermiculite. Seedlings were germinated for 3 days, in darkness at 25°C, and harvested for DNA and RNA isolation.

2.2. Isolation of full-length PAE cDNA by the RACE method

As a first step towards RACE-mediated cloning of the PAE cDNA, total RNA was prepared according to the guanidinium thiocyanate method [12], and was used to produce cDNA (first-strand cDNA synthesis kit, Pharmacia). Two synthetic degenerated oligonucleotide primers, prPAE1 (5'-GARAAYGCNGTNGCNAARGG-3') and prPAE4 (5'-GGRTCNACNGCYTCNACRTC-3'), were designed with regard to the N-terminal (ENAVAKG) and internal (DVEAVDP) peptide sequences of the PAE protein previously characterized [11]. PCR performed with 2 µl of the cDNA as template permitted amplification of a fragment of 317 bp (PCR317) after 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. Once purified, the PCR317 fragment was integrated into the pCR-Script vector (Stratagene) and further sequenced.

Specific primers designed from the PCR317 sequences were used in combination with non-specific primers purchased commercially (5'RACE and 3'RACE system, Gibco BRL), to obtain the full-length PAE cDNA. The 3'RACE was carried out using an oligo(dT) and a specific primer (prPAE5: 5'-ATTGTTCACTTTGAGGGTGG-3') corresponding to the region IVHFEGG of the PAE. These two primers allowed amplification of a 1180-bp fragment (PCR1180) that has been cloned into the pMOS*Blue* vector (Amersham) and sequenced. For 5'RACE, an antisense primer designed from the region upstream of the poly(A) tail of PCR1180 was used in combination with the anchor primer of the 5'RACE kit to get a full-length PAE cDNA after PCR. This last step led to the amplification of a 1.4-kb fragment which was cloned into pMOS*Blue*. Two recombinant plasmids were sequenced and shown to be identical.

All PCR products described above were sequenced on both strands according to the dideoxy chain termination method of Sanger et al. [13], using Sequenase (United States Biochemical).

2.3. DNA and RNA analyses

Genomic DNA was isolated from 3-day-old hypocotyls, according to the procedure described [14], and digested overnight with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I or *Sal*I for Southern blot analysis. Restriction fragments were fractionated on a 0.75% agarose gel, and transferred onto nylon-charged membrane (Hybond-N$^+$, Amersham) under the conditions described [15].

Total RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform extraction method [12], from leaves, cotyledons, hypocotyl segments (A, B, C, D and E from the apex to the base), and roots of 3-day-old seedlings. For Northern blot analysis, it was fractionated on a formaldehyde agarose gel and transferred as described for Southern blot.

Hybridization procedures used for Southern as well as Northern analysis were carried out under similar conditions, using as a probe the ^{32}P-labelled PCR1180 fragment (Prime It Kit, Pharmacia). DNA and RNA blots were hybridized at 65°C under conditions described [16], and then washed at a final stringency of 0.1×SSC, 0.1% SDS at

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65°C for 20 min. All the miscellaneous molecular biology techniques were performed following the procedures described in [15].

2.4. Extraction and purification of pectinacetylesterase

Cell walls were isolated from the upper 2.5 cm of hypocotyl tissue according to a previously described procedure [17]. Pectinacetylesterase was then extracted from cell wall fragments with NaCl 1 M and purified as reported [11].

2.5. Protein sequencing

The tryptic digests of the purified PAE were obtained and sequenced as previously described [11].

2.6. Protein sequence analysis

The search for sequence homologies was performed using the FASTA program of the Sequence Analysis Package by Genetics Computer, Inc. (the GCG Package) in the protein databases Swiss-Prot (release 32), PIR (release 47) and GenPept (release 92).

3. Results

3.1. Molecular cloning and nucleotide sequencing

Different oligonucleotide primers were designed according to previously reported amino acid sequences of the pectinacetylesterase of *V. radiata* [11]. Using the primers prPAE1 and prPAE4, a 317-bp fragment was amplified by PCR from the cDNA. Upon sequencing, this was shown to include the N-terminal region as well as two regions coding for internal peptides characterized from the PAE. The RACE technique helped the isolation of a full-length PAE cDNA we named *VrPAE1*. The sequence of *VrPAE1* is 1453 bp in length and contains an open reading frame of 1197 bp encoding a polypeptide of 399 amino acids (Fig. 1). The 5′- and 3′-untranslated regions are 99 bp and 156 bp long, respectively. The PAE precursor protein contains a signal peptide of 24 amino acids, as deduced from the N-terminal sequence of the mature PAE [11]. The mature protein (375 amino acids) has a calculated molecular mass of 41 166 Da and an estimated pI of 8.4. These values are in good agreement with those previously reported [11]. Two potential *N*-glycosylation sites were found at positions 325 and 359 of the mature protein.

As shown in Fig. 2, the amino acid sequences obtained after tryptic hydrolysis are in agreement with the protein sequence deduced from the cDNA. Some of these sequences exhibit minor differences when compared to those already published [11]. This is ascribed to analytical difficulties when distinguishing histidine from alanine residues and to tryptophan identification in the experiments.

The search for homologies throughout the protein databases failed to reveal any significant identity of the pectinacetylesterase with any known protein. Thus, the pectinacetyles-

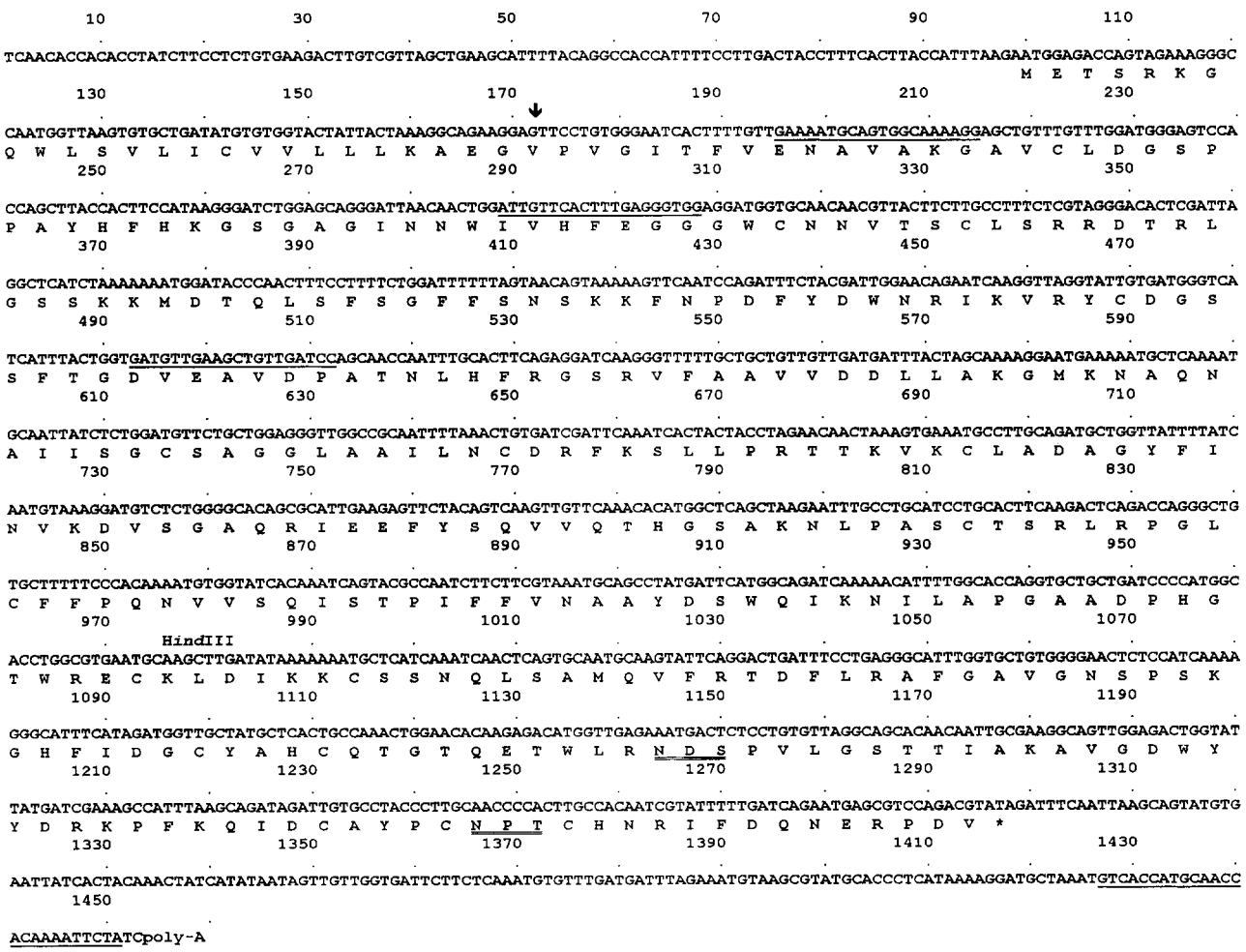

Fig. 1. Nucleotide and the deduced amino acid sequence of the *VrPAE1* cDNA of *V. radiata*. The arrow indicates the signal peptide cleavage site. The regions used for primer design are underlined. The two potential *N*-glycosylation sites are double underlined.

terase from *V. radiata* represents a novel protein, which is unrelated to other cell wall enzymes such as pectinmethylesterase.

3.2. Southern blot analysis

High-molecular-weight genomic DNA was isolated from 3-day-old mung bean hypocotyls and digested with various restriction enzymes. After fractionation, DNA was hybridized with the labelled PCR1180 fragment. This fragment does not contain any *Bam*HI, *Eco*RI, *Pst*I or *Sal*I site but contains a single *Hin*dIII site located at position 976 of the *VrPAE1* sequence. As shown in Fig. 3, hybridization revealed two homologous restriction fragments whatever the enzyme used in the experiment. For *Bam*HI, *Eco*RI, *Pst*I as well as *Sal*I digestion, the occurrence of two hybridizing bands might be due to the presence of intron(s) containing a restriction site for each of the four enzymes. However, preliminary results of PCR performed on genomic DNA with primers flanking the mature coding region of PAE did not demonstrate the presence of such introns (data not shown). These results might then denote the existence of two highly homologous *PAE* genes within the genome of *V. radiata*, but further characterization of the gene is needed for unambiguous interpretation of the hybridizing bands obtained with *Hin*dIII and *Eco*RI digestion.

3.3. PAE mRNA expression throughout organs of mung bean seedlings

In order to determine the pattern of expression of the *PAE* gene, Northern blot analysis was performed with total RNA isolated from the first two leaves, the cotyledons, the hypocotyl segments A–E (from the apex to the base), and the roots. Data illustrated in Fig. 4 reveal a strong tissue specificity of the *PAE* gene expression for some parts of the hypocotyl, while expression in leaves and roots was significantly lower and almost absent in the cotyledons.

From the apex to the base of the hypocotyl, the *PAE* mRNA levels increase from the crosier (segment A) up to the middle part (segment C) of the hypocotyl, and decrease towards the base (segment D), until reaching a level as low as the ones found for leaves and roots (segment E). The expression pattern of the *PAE* gene appears to be slightly asymmetrical on both sides of the segment C where the maximum expression occurred. Densitometric analysis reveals a 2-fold higher amount of *PAE* mRNA in the segment A at the apex, than in the symmetrically opposite segment E at the base of the hypocotyl.

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                                                    50
VPVGITFVENAVAKGAVCLDGSPPAYHFHKGSGAGINNWIVHFEGGGWCN
VPVGITFVENAVAKGAV?LDGSPPAYHFHKG
(N-ter)                                            100
NVASCLSRRDTRLGSSKKMDTQLSFSGFFSNSKKFNPDFYDWNRIKVRYC
            GSSKKMDTQLSF          FNPDFYDWNR    Y?
                                               150
DGSSFTGDVEAVDPATNLHFRGSRVFAAVVDDLLAKGMKNAQNAIISGCS
DGSSFTGDVEAVDPATNLHFR
                                               200
AGGLAAILNCDRFKSLLPRTTKVKCLADAGYFINVKDVSGAQRIEEFYSQ
              SLLPR                      IEEFYSQ
                                               250
VVQTHGSAKNLPASCTSRLRPGLCFFPQNVVSQISTPIFFVNAAYDSWQI
VVQT
                                               300
KNILAPGAADPHGTWRECKLDIKKCSSNQLSAMQVFRTDFLRAFGAVGNS
 NILAPGAADPHGT                      TDFLR
                                               350
PSKGHFIDGCYAHCQTGTQETWLRNDSPVLGSTTIAKAVGDWYYDRKPFK
                                    AVGDWYYDR
QIDCAYPCNPTCHNRIFDQNERPDV
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Fig. 2. Comparison and alignment of the cDNA deduced amino acid sequence of *V. radiata* PAE with the N-terminal sequence and the internal peptides of the protein [11]. (?) represents residues which could not be unambiguously identified.

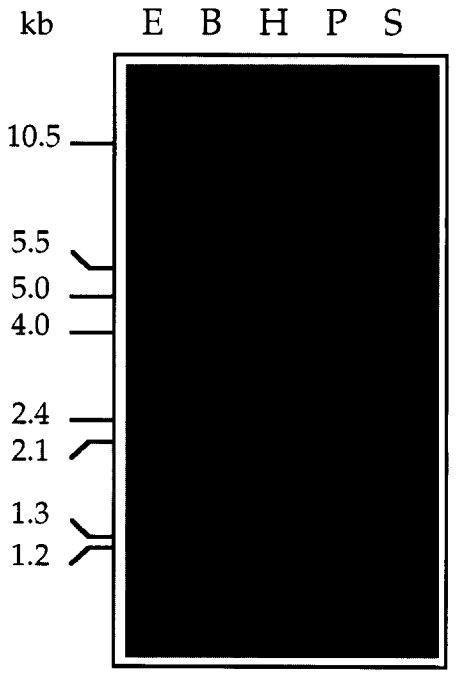

Fig. 3. Southern blot analysis of *V. radiata* genomic DNA. Plant DNA (100 μg) was digested with either *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Pst*I (P) or *Sal*I (S), fractionated and hybridized with the ^{32}P-labeled PCR1180 fragment. The 1-kb ladder (Gibco BRL) was used to determine the molecular size of the hybridizing bands.

4. Discussion

An enzyme exhibiting acetylesterase activity was previously characterized from cell walls isolated from 3-day-old mung bean hypocotyl [11]. This enzyme was able to release acetate from triacetin and *p*-nitrophenylacetate as well as from beet and flax pectins. The rate of deesterification was shown to increase upon prior demethylation of pectins. The N-terminus and some partial peptide sequences from the purified enzyme were also characterized. Here, we report on the first cloning and characterization of a cDNA (*VrPAE1*) encoding PAE in mung bean. Since only one electrophoretic band was observed following SDS-PAGE and IEF of mung bean PAE [11], and also because all sequenced peptides can be aligned with the cDNA-deduced protein sequence, we can conclude that the PAE previously characterized is encoded by the *VrPAE1* gene. Southern analysis might indicate the existence of two *PAE*-related genes within the genome, while only one PAE has been detected in mung bean seedlings up to now. Further molecular and biochemical approaches should allow us to determine whether PAE is encoded by a duplicate gene or by a multigene family.

The PAE enzyme is a novel protein sharing no significant homology with any known protein in the databases. It is

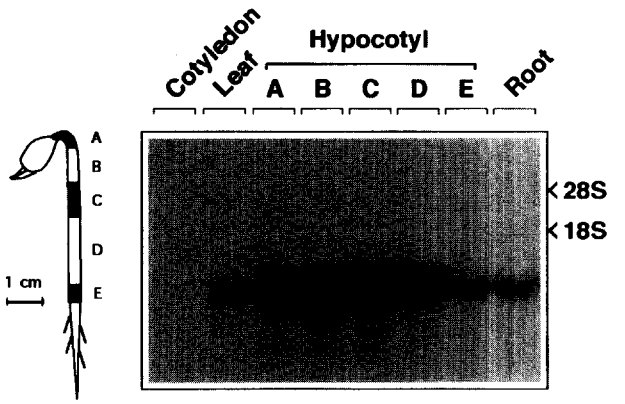

Fig. 4. Organ-specific expression of the mung bean *PAE* gene. 50 μg of total RNA samples was used for Northern analysis of *PAE* mRNA levels in organs of 3-day-old seedlings. Segments of the hypocotyl were cut off as indicated on the left. The ^{32}P-labeled PCR1180 fragment was used as a probe. Location of the ribosomal RNA 28S and 18S is shown on the right.

totally unrelated to the other cell wall pectinesterase enzymes. Recently, several PAEs have been isolated from orange peel [10] and *Aspergillus aculeatus* [9]. The mung bean PAE differs from the PAE isolated from citrus peel since the latter has a M_r of 29 kDa and a pI of 5.1. However, both enzymes are more active on galacturonyl residues which are not methoxylated. It is also different from the rhamnogalacturonan acetylesterase from *Aspergillus aculeatus* (~40 kDA), which is highly specific for the acetyl esters of ramified pectic regions but inactive towards triacetin, and whose activity is not affected by the pretreatment of pectins with pectinmethylesterase. Moreover, the cDNA-deduced amino acid sequence of the *A. aculeatus* acetylesterase [18] does not exhibit any significant identity with the mung bean PAE. The mung bean Pae seems also different from other microbial esterases capable of liberating acetate from various acetylated carbohydrate polymers, such as xylan, since they differ in their physicochemical properties or amino acid sequence [19–21].

Northern blot analysis gives new insight about the possible involvement of the PAE in the general mechanism of growth. As observed in 3-day-old hypocotyls, the highest expression level of the *PAE* gene is found in an intermediary segment between the upper part of the hypocotyl which still elongates, and the basal part which has lost its ability for growth. This region has been shown to be more responsive in term of elongation, with regard to exogenous applied auxin or to acidification, than other parts of the hypocotyl [22]. Moreover, PAE gene expression pattern fits rather well with the PAE activity which increase gradually from the apex to the base of the hypocotyl (results not shown). All these data suggest that PAE might contribute to the limitation of the cell growth capacity which develops along the hypocotyl. Further investigations on seedlings of various stages should allow us to determine whether these changes take place at a relative distance defined from the apex in the time course of hypocotyl development. The consequences of the PAE-mediated changes on the structural and the functional properties of the pectins are not yet clearly understood. Nevertheless, recent theoretical calculations indicate that the presence of acetyl groups does not alter the conformational behaviour of the homogalacturonan backbone but induces significant changes in the capacity for the chains to associate [23]. PAE activity might then contribute to the loss of cell wall extensibility by enhancing aggregation processes between galacturonan chains inside the apoplasm. Chain associations might explain the decrease of hydrophobicity observed after pectin deacetylation [6]. The way PAE acts in concert with other pectinolytic enzymes to modulate cell growth, offers a wide field of investigation.

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