

Characterization of pollen polygalacturonase encoded by several cDNA clones in maize

Marie-Françoise Niogret, Manuel Dubald, Paul Mandaron and Régis Mache*

Laboratoire de Biologie Moléculaire végétale, CNRS and Université J. Fourier, BP 53, 38041 Grenoble Cedex, France (*author for correspondence)

Received 20 February 1991; accepted in revised form 17 July 1991

Key words: cDNA family, maize, pollen-expressed genes, polygalacturonase, signal peptide

Abstract

A full-length cDNA clone, named PG1, abundantly expressed in late stages of pollen development, has been isolated from a cDNA library using a differential screening method with cDNA probes representative of microspores at early or late developmental stages. The encoded 410 amino acid polypeptide has significant homology with various polygalacturonases (PG) described elsewhere. Two polypeptides, of 49 and 53 kDa respectively, have been identified in the active PG fraction, isolated from mature pollen by immuno-cross-reaction with tomato PG antibodies. According to their N-terminal sequence, they can be identified as being mature peptides encoded by the PG1 cDNA clone. We propose that these two proteins derive from a unique precursor through several post-translational events, including the excision of a 22 amino-terminal signal peptide and glycosylation. PG-encoding genes form a small genomic family. Sequence analysis of three PG cDNA clones shows that they are closely related. The divergence of nucleotides between these three cDNA clones is 1%. They encode the same product.

Introduction

Polygalacturonase (PG) is one of the enzymes associated with cell wall degradation. It occurs in several biological processes such as the phytopathogenic virulence of *Pseudomonas solanacearum* [10, 20] and tomato fruit ripening [2, 6, 9, 12]. The enzyme has also been detected in pollen of grasses, where it has been identified by its activity [19]. Pollen PG is presumed to have a role in the growth of pollen tubes in the pistil.

Several studies have shown that PG is excreted from cells by mechanisms of cellular transport which remain unclear. The export of PG in *P. solanacearum* involves the cleavage of a typical 21 residue amino-terminal signal sequence and requires sequences from the mature PG protein for its export across the outer membrane [10]. In tomato fruit, two isozymes are present in the cell wall [8]. They derive from a single translational product which undergoes a number of processing events [7, 17]. These include the removal of a

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X57575, X57627, X57628, X57743.

signal sequence, and several post-translational modifications including glycosylation. These isozymes differ in size due to their differing extents of glycosylation.

Unlike tomato PGs, which randomly cut inside polygalacturonate chains (endo-PG) [18], PG in pollen of grasses is an exoenzyme that cleaves monomers in a stepwise fashion from the end of the substrate chains [19]. Physiological implications of this difference are still unclear.

A partial pollen cDNA has recently been isolated in *Oenothera* encoding a polypeptide homologous to tomato PG [4]. Information on the final product(s) is needed in order to further analyse the maturation process of pollen PG. We present here results concerning the characterization of a pollen-specific cDNA clone encoding a PG precursor in maize. Two mature glycosylated products have been identified, probably arising from post-translational modification of a single precursor.

Materials and methods

Plant culture and microspore isolation

Maize plants (MO17 inbred line) were grown in a culture room and pure microspores at different stages of development were isolated as described by Mandaron *et al.* [15]. Stages V1 and V2 correspond to early and late vacuolated microspores, respectively. Mitoses I and II take place and microspores start to accumulate starch. Stage S1 corresponds to the beginning of starch synthesis and the decrease in size of the vacuole in the tricellular pollen grain. Stage S2 corresponds to microspores in which starch is accumulated. At the mature stage (M) the pollen grain is full of starch and can be released from the anther.

RNA isolation and cDNA library construction

Fresh or frozen pollen or other plant tissues were ground in liquid nitrogen in a Spex 6700 Freezer Mill (Bioblock, France). The frozen powder was

solubilized in extraction buffer and total RNAs were prepared according to Westhoff *et al.* [24]. Poly(A)⁺ RNAs were isolated using oligo-dT cellulose (type 7, Pharmacia).

Two cDNA libraries were constructed from 2 µg of poly(A)⁺ RNA using the cDNA Synthesis Plus kit (Amersham) and the λgt10 Cloning System (Amersham). The first library (S1 cDNA library) was made using RNA extracted from microspores at stage S1. The second library (M cDNA library) was made using RNA extracted from mature pollen grains.

Screening for late-microsporogenesis-specific clones

³²P-labelled cDNA probes were made from poly(A)⁺ RNAs of either S1 or V1 microspores using a random priming kit (Boehringer, Mannheim) and were used to perform a differential screening of the S1 cDNA library. Hybridization conditions were those used by Cox *et al.* [5]. The PG4 cDNA clone was selected because of its strong hybridization with the S1 cDNA probe and lack of hybridization with the V1 cDNA probe. The 0.7 kb PG4 cDNA was subcloned into the plasmid vector pUC 18. The PG4 *Sma* I-*Sca* I restriction fragment (probe 1 in Fig. 1) was used as a probe for screening the M cDNA library. Prehybridization was carried out for 1 h at 65 °C in 6 × SSPE, 0.02% PVP, 0.02% Ficoll, 0.0002% NaN₃, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA (1 × SSPE = 0.15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA). Hybridization was performed for 16 h at 65 °C in 3 × SSPE, 0.02% polivinylpyrrolidone (PVP), 0.02% Ficoll, 0.0002% NaN₃, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA. Finally, filters were washed in 0.2 × SSPE, 0.1% SDS at 65 °C and exposed for autoradiography.

Northern blot analysis

Total RNA samples for microspores or other tissues were fractionated in a 1.2% agarose gel containing 5 mM methyl mercuric hydroxide [1] and

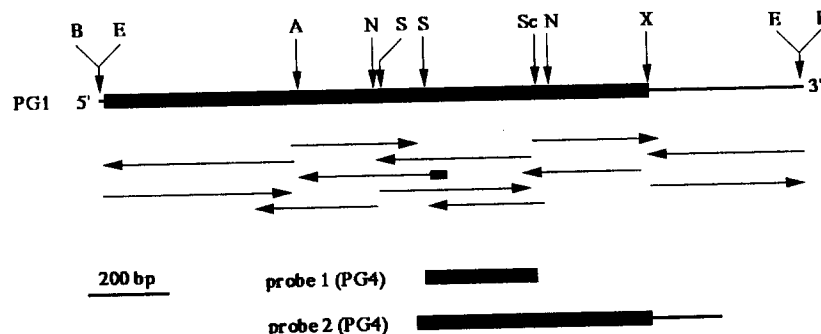


Fig. 1. Restriction endonuclease site map and sequencing strategy for the PG1 cDNA clone. A, *Apa* I; B, *Bam* HI; E, *Eco* RI; N, *Nar* I; S, *Sma* I; Sc, *Sca* I; X, *Xba* I. The PG1 open reading frame is indicated by a black filled-in box. The PG1 5' and 3' non-translated regions are indicated by thin lines. Direction and extent of sequencing is given by thin arrows. The black rectangle indicates the position of an oligonucleotide used as a primer for sequencing. PG4 cDNA sequences used as probes for M library screening (probe 1: *Sca* I-*Sma* I restriction fragment of PG4 cDNA) and northern experiments (probe 2: complete PG4 cDNA) are indicated above the PG1 cDNA restriction map.

transferred to GeneScreen Plus membrane (Du Pont-New England Nuclear) according to the manufacturer's instructions. Prehybridization was carried out at 42 °C for 30 min in 50% formamide, 1 M NaCl, 1% SDS, 100 µg/ml denatured salmon sperm DNA. Hybridization was carried out under the same conditions for 20 h using the PG4 cDNA insert as a probe (Fig. 1, probe 2). Final washes were for 1 h at room temperature in 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate).

DNA sequencing

Inserts from recombinant λgt10 phages were subcloned into M13 mp18 and mp19 phages using adequate restriction sites (Fig. 1), processed according to Amersham's instructions and sequenced on both strands using the Sequenase DNA Sequencing Kit (United States Biochemical Corporation) and the M13 universal primer or the oligonucleotide represented in Fig. 1. Sequence analysis was carried out using the facilities of CITI2 (Paris).

Purification of maize pollen polygalacturonase

Extraction, purification and enzymatic assay of maize polygalacturonase were carried out according to Pressey and Reger [19] except that reduc-

ing groups were measured by the arsenomolybdate method of Milner and Avigad [16].

Protein electrophoresis

Protein concentration was estimated by the Bradford assay [3]. One-dimensional SDS-PAGE was performed according to Laemmli [13]. Gels were either stained with Coomassie brilliant blue R250, used for western blotting or detection of glycoproteins as indicated by Kapitany and Zebrowsky [11].

Western blot analysis

Proteins from microspores at different stages of development were prepared by solubilization in 150 mM NaCl and 10 mM CaCl₂ of material ground in liquid nitrogen. After centrifugation, the proteins in the supernatant were precipitated with acetone. The pellet was resuspended in loading buffer and fractionated by SDS-PAGE according to Laemmli [13]. After separation, proteins were transferred electrophoretically to Hybond C nitrocellulose sheets (Amersham) and processed for immuno reaction using a standard procedure. Polyclonal antibodies raised against tomato polygalacturonase and preabsorbed with proteins extracted from immature-green tomato fruit were used [6] (a generous gift of Dr Ben-

nett). Immuno-reacting proteins were revealed using anti-rabbit sheep IgG conjugated to alkaline phosphatase (Promega).

N-terminal sequence analysis

10 µg of partially purified polygalacturonase was analysed by SDS-PAGE and transferred electrophoretically to an Immobilon PVDF membrane (Millipore). The membrane was stained with 0.2% Coomassie brilliant blue R250 in 45% methanol and 10% acetic acid, and then extensively washed with deionized water. The bands that corresponded to the two main polypeptides cross-reacting with antibodies raised against tomato polygalacturonase were cut out. Sequence analysis was performed by automated solid-phase Edman degradation [14].

Results

Identification of cDNA clones abundant in late stages of microsporogenesis

In order to select mRNAs primarily expressed in later stages of microsporogenesis a λgt10 cDNA was constructed using poly(A)⁺ RNAs purified from microspores at a stage corresponding to the beginning of starch accumulation. This stage of microspore development, named S1 [15], follows the two successive mitoses and occurs about 8 days after meiosis and about 4 days before the formation of mature pollen. The cDNA clones were submitted to a differential screening using two radiolabelled cDNA probes prepared from poly(A)⁺ RNAs isolated from either juvenile vacuolated microspores (stage V) or microspores at the beginning of starch accumulation (stage S1). A number of clones were found to hybridize selectively with the second probe. One of these, the PG4 cDNA clone, gave a strong hybridization signal and was consequently retained for further analysis. This clone was found to be incomplete. As the PG gene is expressed at its maximum in mature pollen (Fig. 2), screening of a M cDNA

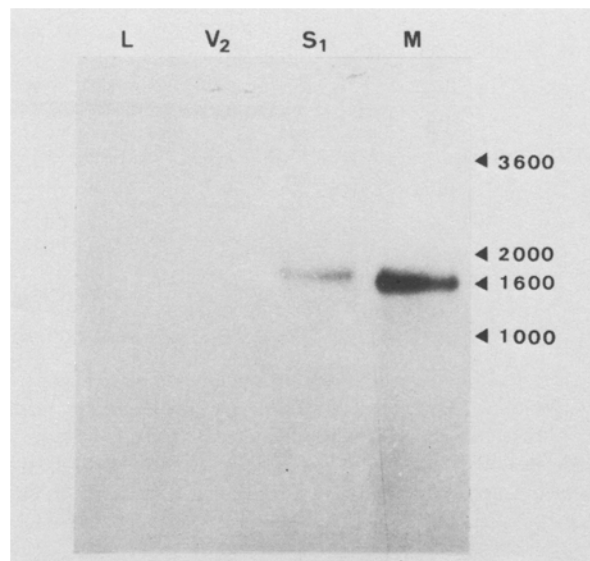


Fig. 2. Northern experiment. 10 µg of total RNA from leaves (L), vacuolated microspores (V2), microspores at the beginning of starch accumulation (S1) or from mature pollen (M) were fractionated by electrophoresis in denaturing conditions, blotted and hybridized with the ³²P-labelled PG4 probe (Fig. 1), which is a partial sequence of the PG1 cDNA. The autoradiogram was exposed for 6 h at -70 °C with an amplifying screen. No hybridization signal was detected either in V2 microspores or in leaf RNA, not even after longer exposure. The same negative result was observed with RNA prepared from roots, female organs or from coleoptiles.

library was performed using the PG4 *Sma* I-*Sca* I restriction fragment as a probe (probe 1 in Fig. 1) in order to select full-length PG cDNA clones. About 1% of the clones hybridized with this probe. Three cDNA clones, PG1, PG2 and PG3, were thus selected and further analysed. They are all strongly homologous to PG4 cDNA, as shown below.

The PG cDNA clones correspond to mRNAs abundantly synthesized in pollen after microspore mitosis

The expression pattern of the PG mRNAs was analysed during maize microsporogenesis using total RNAs isolated from microspores at several stages of development and using PG4 cDNA as a probe (Fig. 1, probe 2). Significant levels of the 1600 bp PG mRNAs were found in microspores at the beginning of starch accumulation (stage S1,

Fig. 2). One band only was detected by the northern blotting technique. The mature pollen contained the largest amount of mRNAs, showing that these mRNAs accumulate during a short period of time, in the late stages of pollen formation. No hybridization signal between PG4 cDNA and RNAs extracted from microspores at early stages of development (i.e. before the two successive mitoses) could be observed, not even after prolonged exposure of autoradiograms. The PG4 cDNA probe was hybridized to RNAs of different maize organs to determine the developmental expression of the PG genes. No hybridization signal was detected either in leaves of different ages (Fig. 2), in roots, in female organs, or in coleoptiles (not shown).

The polypeptide encoded by the PG1 cDNA is homologous to tomato and Oenothera polygalacturonases

Sequence analysis of the PG1 cDNA clone shows the presence of an open reading frame potentially coding for a 410 amino acid protein (Fig. 3). The first ATG codon must be the translation start codon since no other in-frame ATG codon was found in the upstream sequence of several of the genes corresponding to the PG cDNAs (in preparation). The stop codon is found at an identical position in the three other cDNAs sequenced (Fig. 3). Consequently, the ORF starting at position 8 and ending at position 1240 in the PG1 cDNA represents the full polypeptide encoded by this cDNA. The polypeptide has a calculated mass of 43457 Da and a calculated pI of 7.2. It has a high (27%) serine, threonine and glycine content. The amino acid sequence shares 34% identical amino acids with the tomato PG which plays a major role in the ripening of the fruit [22], and 44% identical amino acids with the partial sequence of the *Oenothera* pollen PG [4].

The comparison of the three amino acid sequences clearly shows the presence of highly conserved domains (Fig. 4). Some of these domains are also conserved in the PG of the prokaryote *P. solanacearum* (conserved amino acids are indicated by an asterisk in Fig. 4). The region com-

prising the residues 252 to 264 is particularly conserved and might be involved in the catalytic activity of the enzyme. The maize polypeptide is particularly enriched in methionine residues (12 versus 4) relative to the pollen PG of *Oenothera*.

Identification of pollen polygalacturonase encoded by the PG1 cDNA clone

A fraction containing PG activity has been purified from mature pollen as indicated in Materials and methods. Results of SDS-PAGE separation of the polypeptides contained in the purified fraction are shown in Fig. 5, lane 2. Three major polypeptides (of 56, 53 and 49 kDa) are observed. Immunoblot analysis using the tomato PG antibodies reveals a strong immuno reaction with the 53 and 49 kDa polypeptides (Fig. 5, lane 3). These two polypeptides have an identical N-terminal sequence which corresponds exactly to part of the amino acid sequence deduced from the PG1 cDNA, overlined in Fig. 3. The fact that the N-terminal sequences are identical strongly suggests that both polypeptides derive from a unique precursor. This is consistent with the northern experiment described above, in which all mRNAs detected were of a single size (Fig. 2). The two immuno-reacting polypeptides were also detected in microspores before pollen maturity, at stage S2, during which starch is accumulated. No immunological reaction was detected at earlier stages (Fig. 6). Thus, the accumulation pattern of polypeptides cross-reacting with PG antibodies follows the accumulation pattern of PG mRNAs (Fig. 2).

The N-terminal sequence of the two polypeptides strongly suggests that the PG-1 cDNA encoded protein is post-translationally cleaved releasing a 22 amino acid residue peptide and a mature product of 41 kDa. This hypothesis is strongly supported by the fact that the 22 N-terminal residues deduced from the PG1 ORF have strong hydrophobic properties (not shown), which are characteristic of signal peptides for endoplasmic reticulum targeting [23].

A positive Schiff reaction was observed for both the 53 kDa and the 49 kDa polypeptides (Fig. 7),

↓

```

      M A C T N N A M R A L F L L V L F C I V H G E K E E S K
1  ogacaagATGGCGTGCACAAACATGGGATGAGAGCCTTGTTCCTCCTGGTCTCTTCGTCATCGTGCAATGGTGAGGAGAGATCAAAG 91
2      .A.....
3      .....

      G I D A K A S G P G G S F D I T K L G A S G N G K T D S T K
1  GGCAATCGATGCGAAGCGTCCGGCCCTGGTGGGTCTTGGACATCACCAAGTTGGGCGCCTCCGGCAATGGCAAGACAGACAGCACGAG 181
2  .....
3  .....

      A V Q E A W A S A C G G T G K Q T I L I P K G D F L V G Q L
1  GCTGTGCAGGAGGCATGGGCATCGGCGTCCGGCGGCACTGGGAAGCAGACAATCTCATACCCAAGGGTGACTTCTTGTTCGGACAAC 271
2  .....C.....
3  .....

      N F T G P C K G D V T I Q V D G N L L A T T D L S Q Y K D H
1  AACTTCACAGGCCCTTGCAGGGCGAGCGTGACCATCCAGGTGGATGGCAATCTGCTGGCGACCACGGACCTAAGCCAGTACAAGGACCAT 361
2  .....
3  .....

      G N W I E I L R V D N L V I T G K G N L D G Q G P A V W S K
1  GGTAAATTGGATCGAGATTCTACGTGTGGATAACCTGGTCATCACCGGCAAGGGAAACCTTGACGGGCGAGGGCCCGAGCCGTGTGGAGCAG 451
2  .....C.....T.....
3  .....

      N S C T R K K Y D C K I L P N S L V M D F V N N G E V S G V T
1  AACTCCTGCACCAAGAGTACGACTGCAAGATCCTTCCCAACTCGCTGGTGGATGGACTTCGTGAACAACGGGGAGGTGTCCGGGGTCAAG 541
2  .....
3  .....

      L L N S K F F H M N M Y R C K D M L I K D V T V T A P G D S
1  CTGCTCAACTCCAGTCTCTCCACATGAACATGTACCGGTGCAAGGACATGCTGATCAAGGACGTGACCGTGAACGGCGCCCGGGGACAGC 631
2  .....
3  .....

      P N T D G I H M G D S S G I T I T N T V I G V G D D C I S I
1  CCCAACACGGATGGCATCCACATGGGCGACTCATCCGGGATCACGATCACCAACACCGTCAATGGCGTGGGTGACGACTGCTCATCTCCATC 721
2  .....C.....
3  .....

      G P G T S K V N I T G V T C G P G H G I S I G S L G R Y K D
1  GGCCCCGGGCTCCAGGTGAACATCACCGGCGTGAACCTCGGCCCCTGCCCAAGGACATCAGCATCGGCAGCTTAGGGCGGTACAAGGAC 811
2  .....A.....
3  .....

      E K D V T D I N V K D C T L K K T M F G V R I K A Y E D A A
1  GAGAAGGACGTACGGACATCAACGTCAAGGATTGCACTCTTAAGAAGACGATGTTGGCGTCCGCATCAAGGCGTACGAGGACGCGGCC 901
2  .....
3  .....

      S V L T V S K I H Y E N I K M E D S A N P I F I D M K Y C P
1  TCCGTGCTCACCGTCTCCAGATCCACTACGAGATATCAAGATGGAGGACTCAGCCAACCCCATCTTCATCGACATGAAGTACTGCCCC 991
2  .....
3  .....

      N K L C T A N G A S K V T V K D V T F K N I T G T S S T P E
1  AACAAATTGTGTACTGCCAACGGCCCTCCAGGTCAACCGTCAAGGATGTCACCTTCAAGAACATCACGGCACCTCTCCACCCCGGAG 1081
2  .....
3  .....

      A V S L L C T A K V P C T G V T M D D V N V E Y S G T N N K
1  GCGTTAGCTGCTCTGCACTGCCAAGGTCCCATGCAACGGCGTCAACATGGATGACGTCAACGTGAGTATAGCGGCACCAACAACAG 1171
2  .T.....
3  .....

```

	T M A I C T N A K G S T K G C L K E L A C F	
1	ACCATGGCTATATGCAACGACGCAAGGGCAGCACCAGGGTTGCCCTCAAGGAGCTTGCACTGCTTtagaccctccatgactgacccat	1261
2	g.....	
3		
1	ctctctagttataatttttctctctctctgctcattgcccattagatgctatccattggttaacgcacaacagtaaaacgacagacatccga	1351
2	C.....	
3		
1	cagctatattatgttcgaagggtgaacaocctgaatttgagggtat [▼] aaaattttctctccaaataccaaccaaatttaggggttaacctct	1441
2	t.....C...t.....	
3	C.....	
1	tgtctctctctctc ttttctttttgattaaaagtaagtgaatttaggcgaggggttaattattttttgtcaaaacttatgtgaag	1527
2	tctc.....	
3		
1	tcatgaaatgttgcatcatgttgagcctaaattattcttttgtttgatgcacatgtttgaattgg	1592
2	C.....	
3	C.....	

Fig. 3. Nucleotide sequence (lane 1) and deduced amino acids of PG1 cDNA clone. The N-terminal amino acid sequence of the 53 kDa and 49 kDa polypeptides (Fig. 5) are shown by continuous and dashed lines respectively. The putative signal peptide excision site is indicated with a vertical arrow. The four putative glycosylation amino acid motives are overlined. Comparison of the nucleotide sequence of 3 PG cDNA clones: PG1 (lane 1); PG2 (lane 2); PG3 (lane 3). Only different nucleotides of PG2, and PG3 towards PG1 are represented. The lower-case sequence represents the non-translated regions. Two putative polyadenylation sites are underlined. PG4 cDNA is completely identical to the PG1 cDNA; it begins at the first arrowhead and ends at the second one, where it is continued by a polyA(13) tail.

showing that they are probably glycosylated. Four putative glycosylation sites are overlined in Fig. 3. Glycosylation may explain the discrepancy between the molecular weight of PG as calculated from the cDNA and the apparent molecular weights found after SDS-PAGE. Similar results were obtained for the PG from tomato fruit [7].

Characterization of a PG cDNA clone family

PG4 cDNA (710 bp long, overlapping PG1 from base 697) contains only part of the open reading frame. It is identical to PG1 at the level of sequence, but is shorter in its 3' non-coding region. The PG4 sequence is terminated by a poly(A) tail just after the first polyadenylation site shown in Fig. 3. Sequence analysis of the three cDNA clones PG1, PG2 and PG3 showed that they belong to a closely related family of genes (Fig. 3). Comparison of the PG1 and PG2 clones showed seven silent mutations at positions 13, 250, 385, 439, 676, 703 and 790 in the PG1 nucleotide sequence, and one mutation at position 1053 resulting in an amino acid substitution of a valine (359)

for the equivalent alanine. Six bases are changed in the 3' non-coding region, and one insertion of 4 bases is observed. The PG3 cDNA clone (1555 bp overlapping PG1 from base 19) contain a partial coding sequence and diverge from the PG1 sequence in the 3' non-coding region only (Fig. 3, lane 3). The PG1, PG2 and PG3 sequences contain two identical putative polyadenylation signals underlined in Fig. 3.

We conclude that the PG1, PG2 and PG3 cDNAs correspond to a closely related family of genes.

Discussion

The results presented have allowed us to characterize polygalacturonase in maize pollen, which is the product of at least one cDNA clone containing the full coding sequence. Several points concerning this finding will be discussed.

Several of the findings described here are in agreement with those recently obtained for incomplete PG clones characterized in *Oenothera organensis* [4].



Fig. 4. Alignment of predicted amino acid sequences deduced from maize pollen PG1 cDNA clone (lane 1), *Oenothera organensis* pollen partially overlapping P26 and P22 cDNA clones [4] (lane 2) and tomato fruit PG-2A cDNA [21] (lane 3). Identical amino acids between these sequences are boxed. The comparison has been extended to *Pseudomonas solanacearum* PG [10] (lane 4); amino acids identical to the maize PG1 sequence are indicated with an asterisk.

– The polypeptide encoded by the PG clones is homologous to tomato fruit polygalacturonase.

– The mRNAs and the protein are abundant in pollen. The products are detected after pollen mitosis and the maximum accumulation is found in mature pollen.

– In both plants, evidence has been found indicating the presence of a small gene family encoding polygalacturonase. Sequencing of the three isolated cDNA clones PG1, PG2 and PG3 has shown that the divergence is very limited (about 1%) within this family. This result suggests that the selection pressure for the conservation of the PG genes is very high within one species or that duplications within the PG gene family have occurred recently.

PGs involved in tomato fruit ripening are known to be endoenzymes [18]. But all PGs so far isolated from pollen have been characterized as exoenzymes [19]. Evidence of a PG gene family expressed in *Oenothera* and maize pollen contrasts with the presence of only one tomato PG gene which is expressed during fruit ripening [2]. From this observation, it emerges that exoPGs would be the products of a gene family, whereas endoPGs would be encoded by a single gene. The large divergence in homology between genes coding for endo- and exoPGs could explain the fact that one band only is observed in the tomato genomic Southern experiment with an endoPG nucleic probe [6].

The sequence of the PG1 cDNA clone reported

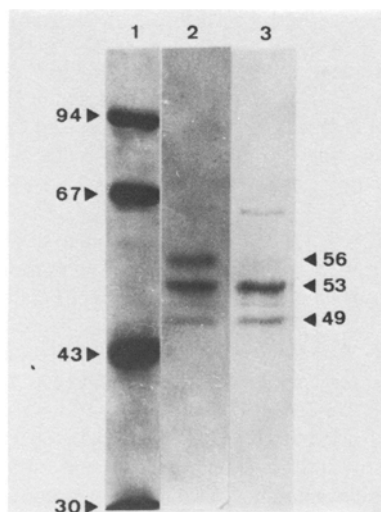


Fig. 5. Identification of polygalacturonase purified from pollen. 5 μ g of protein of a fraction purified as described in Materials and methods was separated by SDS-(12%)PAGE, stained with Coomassie brilliant blue (lane 2), or used for immunodetection with anti-tomato fruit PG antibodies (lane 3). Molecular masses of markers (lane 1) and of major bands of the purified fraction are given in kDa.

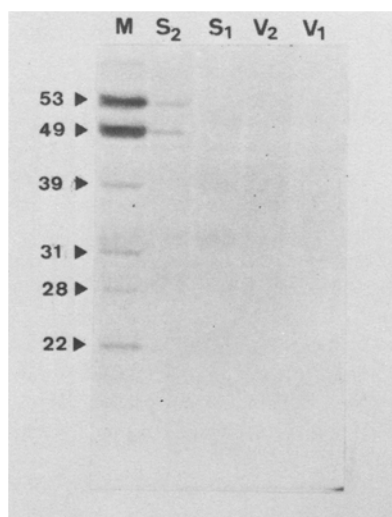


Fig. 6. Immunodetection of polygalacturonase during microspore development. Total proteins (30 μ g) from microspores were fractionated by SDS-(12%)PAGE and blotted on nitrocellulose for immunodetection using immunopurified anti-tomato fruit polygalacturonase antibodies. Developmental stages are: mature pollen (M), late starch accumulation (S2), early starch accumulation (S1), late vacuolated microspores (V2) and early vacuolated microspores (V1). Triangles indicate polypeptides cross-reacting with the antibodies. Molecular masses are given in kDa.

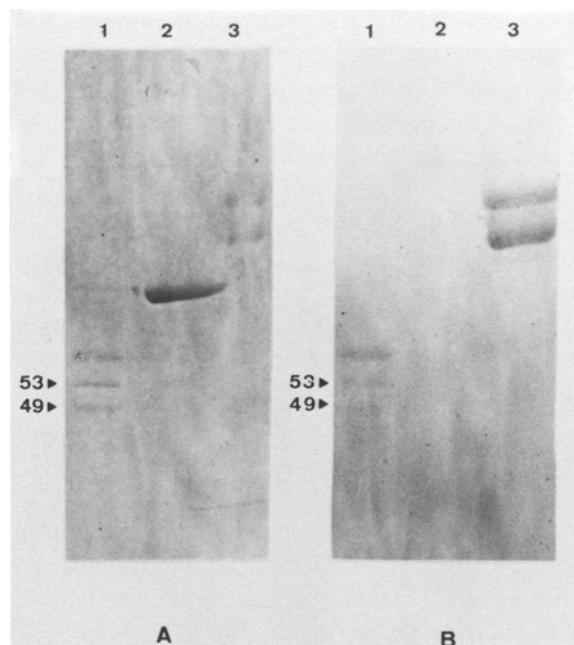


Fig. 7. Detection of glycoproteins after SDS-(12%)PAGE by Schiff staining. A. Coomassie brilliant blue staining of purified pollen PG (3 μ g, lane 1), bovine serum albumin (3 μ g, lane 2), C1 complement inhibitor (2 μ g, lane 3). B. Schiff staining of purified pollen PG (25 μ g, lane 1), bovine serum albumin (15 μ g, lane 2, negative control), C1 complement inhibitor (15 μ g, lane 3, positive control). Polypeptides 49 and 53 kDa cross-reacting with anti-tomato fruit PG antibodies are indicated (see Fig. 5).

here allows the characterization of the full-length amino acid sequence of PG. Two corresponding polypeptides are found in a catalytically active fraction prepared from mature pollen. These two polypeptides are glycosylated and we suggest that the size difference between them is due to differences in extent of glycosylation. The N-terminal sequence of each of these two polypeptides strongly suggests that they are the result of post-translational modification of native products. Three closely related PG cDNA clones have been characterized and only one amino acid difference has been observed between the translational products of these cDNA clones. The PG produced during pollen maturation is therefore composed of almost identical translational products of a family of cDNA clones. The N-terminal 22 residue sequence which ends at a glycine residue,

just before the maturation site, has properties of a signal peptide. We suggest that this signal peptide would target the growing polypeptide into the lumen of the endoplasmic reticulum, and the mature products would then be glycosylated.

The characterization of the pollen PG cDNA sequence should allow detailed analysis at the molecular level of the maturation process of the enzyme. Further work is needed to determine the subcellular location of pollen PG and its implication in the pollen germination process.

Acknowledgements

We thank Dr G. Freyssinet and Dr M. Lebrun for their support, Dr Bennet for the tomato PG antibodies, Dr Denoroy at the Centre National de la Recherche Scientifique (CNRS) at Vernaison (France) for N-terminal sequence analysis. M.D. is granted by Rhône-Poulenc Agrochimie, Lyon.

References

1. Bailey JM, Davidson N: Methyl mercury as a reversible agent for agarose gel electrophoresis. *Anal Biochem* 7: 75–85 (1976).
2. Bird CR, Smith JS, Ray JA, Moureau P, Bevan MW, Bird AS, Hugues S, Morris PC, Grierson D, Schuch W: The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. *Plant Mol Biol* 11: 651–662 (1988).
3. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254 (1976).
4. Brown SM, Crouch ML: Characterization of a gene family abundantly expressed in *Oenothera organensis* pollen that shows sequence similarity to polygalacturonase. *Plant Cell* 2: 263–274 (1990).
5. Cox F, Gestautas J, Rapoport B: Molecular cloning of cDNA corresponding to mRNA species whose steady state levels in the thyroid are enhanced by thyrotropin. *J Biol Chem* 263: 7060–7067 (1988).
6. DellaPenna D, Alexander DC, Bennett AB: Molecular cloning of tomato fruit polygalacturonase. Analysis of polygalacturonase mRNA levels during ripening. *Proc Natl Acad Sci USA* 83: 6420–6424 (1986).
7. DellaPenna D, Bennett AB: *In vitro* synthesis and processing of tomato fruit polygalacturonase. *Plant Physiol* 86: 1057–1063 (1988).
8. Grierson D, Tucker GA, Robertson NG: The molecular biology of ripening. In: Friend J, Rhodes MJC (eds) *Recent Advances in the Biochemistry of Fruit and Vegetables*, pp. 147–158. Academic Press, London (1981).
9. Grierson D, Tucker GA, Keen J, Ray J, Bird CR, Schuch W: Sequencing and identification of a cDNA clone for tomato polygalacturonase. *Nucl Acids Res* 14: 8595–8603 (1986).
10. Huang J, Schell MA: DNA sequence analysis of *pglA* and mechanisms of export of its polygalacturonase product from *Pseudomonas solanacearum*. *J Bact* 172: 3879–3887 (1990).
11. Kapitany RA, Zebrowsky EJ: A high resolution PAS stain for polyacrylamide gel electrophoresis. *Anal Biochem* 56: 361–369 (1973).
12. Knapp J, Moureau P, Schuch W, Grierson D: Organization and expression of polygalacturonase and other ripening related genes in Ailsa Craig 'Neverripe' and 'ripening inhibitor' tomato mutants. *Plant Mol Biol* 12: 105–116 (1989).
13. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685 (1970).
14. Laursen RA: Solid phase Edman degradation: An automatic peptide sequencing. *Eur J Biochem* 20: 89–102 (1971).
15. Mandaron P, Niogret MF, Mache R, Moneger F: *In vitro* protein synthesis in isolated microspores of *Zea mays* at several stages of development. *Theor Appl Genet* 80: 134–138 (1990).
16. Milner Y, Avigad G: A copper reagent for the determination of hexuronic acids and certain ketohexoses. *Carbohydrate Res* 4: 359–361 (1967).
17. Osteryoung Toenjes K, Hall B, Winkler V, Bennett AB: Analysis of tomato polygalacturonase expression in transgenic tobacco. *Plant Cell* 2: 1239–1248 (1990).
18. Pressey R, Avants JK: Two forms of polygalacturonase in tomato. *Biochim Biophys Acta* 309: 363–369 (1973).
19. Pressey R, Reger B: Polygalacturonase in pollen from corn and other grasses. *Plant Sci* 59: 57–60 (1989).
20. Schell MA, Roberts DP, Denny TP: Analysis of the *Pseudomonas solanacearum* polygalacturonase encoded by *pglA* and its involvement in phytopathogenicity. *J Bact* 170: 4501–4508 (1988).
21. Sheehy RE, Pearson J, Brady CJ, Hiatt WR: Molecular characterization of tomato fruit polygalacturonase. *Mol Gen Genet* 208: 30–36 (1987).
22. Tucker GA, Grierson D: Synthesis of polygalacturonase during tomato fruit ripening. *Planta* 155: 64–67 (1982).
23. Verner K, Schatz G: Protein translocation across membranes. *Science* 241: 1307–1313 (1988).
24. Westhoff P, Nelson N, Bünemann H, Hermann RG: Localization of genes for coupling factor subunits on the spinach plastid chromosome. *Curr Genet* 4: 109–120 (1981).