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Characterization of *ppEG1*, a member of a multigene family which encodes endo-β-1,4-glucanase in peach

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Abstract

Three cDNA clones (pCel10, pCel20 and pCel30), each encoding different endo-β-1,4-glucanases in peach, were obtained by RT-PCR and their expression investigated by northern analysis during leaf and fruit abscission and during fruit development. This analysis allowed the detection of only the pCel10-related mRNA. A 2.2 kb transcript accumulated in ethylene activated abscission zones of leaves and fruits, and *ppEG1* (*Prunus persica* endoglucanase 1) the gene coding for pCel10, was isolated and characterized. A cDNA (termed pCel1), containing the entire open reading frame of *ppEG1*, was obtained and its sequence used to define the structure of the gene and the exon/intron boundaries. *ppEG1* consists of 7 exons and encodes a 497 amino acid polypeptide including a putative signal peptide at the N-terminus. The similarity of this peach endo-β-1,4-glucanase (EGase, EC 3.2.1.4) is high (76.3%) with the ripening avocado and low (47.3%) with the bean abscission EGase. A 1639 bp region at the 5′ of the transcription start site shows regulatory functions in transgenic tobacco plants, as judged by its ability to drive GUS expression in cell separation-related events.

Introduction

In higher plants, endo-β-1,4-glucanases (EGases, EC 3.2.1.4) form a family of enzymes which share the ability to hydrolyse the β-1,4-glucan linkages of plant cell wall polymers [16]. However, while this ability has been demonstrated in the case of the hemicellulosic matrix [18], up to now no clear proof exists that they are able to degrade crystalline cellulose, as the bacterial and fungal glucanases do [27]. The primary structure of these enzymes shows a certain degree of divergency, so that on this basis they can be grouped into a number of subfamilies [27, 13].

Higher plant endo-β-1,4-glucanases (EGases) are involved in the weakening of the primary cell walls, a process which occurs whenever plant organs undergo cell separation phenomena. While the abscission of organs and the softening of fleshy fruits are the most characteristic and well studied examples (see [1]), cell separation events also occur during other phases of plant growth, for instance cell and tissue expansion [41], adventitious root formation [29, 23] and the development of the reproductive organs [9, 30].

In a number of cases it has been shown that the observed EGase activity may be due to the presence of various isoforms with different isoelectric points. So, in bean [8], in peach [4] and in pepper [13] two isoforms have been found, while a greater number of isoforms have been observed in avocado [22]. Molecular analyses have given additional evidence to the idea that multiple forms of EGases can be expressed throughout a plant's life cycle. It has recently been shown that at least three different mRNAs are expressed in pepper [13] and many more in tomato plants [27, 30, 7].

Different EGases respond differently to different hormonal stimuli, and this is yet further evidence of variability within this group of enzymes. The expression of EGases involved in the shedding of organs is

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X96853 (pCel1), X96854 (pCel20), X96855 (pCel30), X96856 (*ppEG1*).

typically up-regulated by ethylene [39, 4, 40], while the expression of EGases related to vegetative growth seems to be insensitive to this hormone but in some cases, may be increased by auxin treatment [41]. A mixed situation has been found in the case of the softening of fruits where it is possible to find both sensitivity [31, 37, 27] and insensitivity [2] to ethylene.

As seen before, EGases are involved in many physiological processes whose unifying motif appears to be cell separation. After the discovery that multigene families encode different EGases in a plant species, it has become evident that both developmental stage and tissue type play a role in the expression of EGase genes. For instance, in the same plant it has been possible to distinguish between abscission and fruit ripening EGases [13].

Research carried out in our laboratory has shown that in peach the shedding of leaves and fruits is preceded by an ethylene-induced increase in EGase activity. Anyway, the role played by the enzyme is different in the two different organs. In leaf solely the high level of EGase activity seems responsible for the phenomenon, whereas in fruit its lower level is accompanied by a marked increase in polygalacturonase activity [4]. In both processes the total enzyme activity is provided by two isoforms with different isoelectric points (6.5 and 9.5, respectively) whose levels are increased by ethylene treatment [4].

Different EGase isoforms in pepper plants have recently been related to the expression of multiple mRNAs [13]. So, also in peach the observed different isoforms may suggest that EGase is present in the form of a gene family with a number of divergent members. We have therefore endeavoured to study the molecular situation of EGase genes in this plant species and cloned three cDNA fragments which code for three different proteins. One of these cDNAs is preferentially expressed during the abscission of leaves and fruits and at a very late stage of fruit ripening, and *ppEG1*, the gene which encodes its cognate mRNA, has been isolated and characterized. A 1639 bp region upstream of the transcription start site exhibits regulatory functions in transgenic tobacco plants where it is able to drive GUS expression in cell separation-related events.

Materials and methods

Plant material

Plants of *Prunus persica* (L.) Batsch cv. Redhaven were grown in a field near Padua. Fruits at various stages of development (S1, S2, S3, S4 and overripe, see [42]) were collected and used without further treatment. Explants of both leaf and fruit abscission zones were prepared as already described [4]. The hormone treatment was provided by placing the explants, fixed on an agar support, in a sealed chamber and flushing them with ethylene (100 μl/l) in air at a flow rate of 6 l/h. The treatments lasted 48 h for the leaf abscission zones and 72 h for those of the fruit; thereafter abscission zones were excised from the explants. Both treated and untreated samples were frozen in liquid nitrogen and stored at -80 °C. Flushing with ethylene causes an acceleration and, especially, a synchronisation in the onset of cell separation events in a population of variously aged organs.

Peach cells were obtained by placing cotyledons on the following medium: Gamborg's B-5 salts 3.19 g/l, sucrose 20 g/l, naphthaleneacetic acid 2 mg/l,6-benzylaminopurine 2 mg/l, agar 8 g/l. Cells were then grown either in solid (as above) or liquid (as above but without agar) medium. They were subcultured every 7 days.

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were grown at the Botanic Garden of Padua under standard greenhouse conditions.

RNA extraction, northern analysis and ribonuclease protection assay (RPA)

Total RNA was extracted from both abscission zones and fruits as described in Schneiderbauer *et al.* [34]. For northern and RPA analyses RNA loading was checked by means of ethidium bromide staining of agarose gels. Total RNA was separated in 6% formaldehyde-1.2% agarose gels and blotted onto Hybond N membranes (Amersham International plc, Little Chalfont, England) using 20× SSC as blotting buffer. DNA probes were ^{32}P-labelled using a random-primed DNA labelling kit (Promega Co, Madison, WI). The membranes were prehybridized (2 h) and hybridized (16–20 h at 60 °C) in 5% SDS, 5× Denhardt's solution, 5× SSC, sonicated herring sperm DNA (100 μg/ml). After hybridization membranes were washed at 60 °C with solutions containing 1% SDS and decreasing concentration of SSC down to a

final wash with 0.5× SSC, and exposed to X-ray films (X-Omat S, Kodak) at −80 °C.

For the Ribonuclease Protection Assay, the radiolabeled RNA probes were transcribed in the antisense orientation. The cDNA clones were first linearized with *Xho*I (pCel10 in pCRII, Invitrogen, San Diego, CA) and *Apa*I (pCel 20 and pCel30 in pGEM-T, Promega), and then labelled using [α-^{32}P]UTP (800 Ci/mmol, Amersham) and SP6 RNA polymerase (Ambion, Austin, TX). Aliquots of the same RNA preparations used in the northern were also used for the ribonuclease protection assay (HybSpeed RPA, Ambion). Total RNA was probed with pCel10 (10 μg) and pCel20 and pCel30 (40 μg). Gels of the protected fragments were wrapped in plastic and exposed to X-ray films at −80 °C.

RT-PCR, cloning of the amplified cDNA fragments and isolation of genomic clones

Different oligonucleotides were chosen on the basis of known plant endo-β-1,4-glucanase sequences [36, 6, 38]. Those which gave successful results were: (C2): 5′-CCAGAAGACATGGATACACC-3′; (C4): 5′-GAGCAGTARAATGGGCA-3′; (C6): 5′-GASGAGCCTCTGTGATG-3′. Two oligos, (LT21) 5′-TTAATTAGGTCTCTGCTGCTA-3′ and (LT57) 5′-AGAAGTTGGAGCTAATTTGG-3′, were used to obtain pCel1, which is the cDNA clone containing the entire open reading frame of the *ppEG1* gene.

2 μg of total RNA were used as the starting material for the RT-PCR experiments. First-strand synthesis was carried out with the cDNA Cycle Kit (Invitrogen) using a specific internal primer (oligo C4 to obtain pCel20 and pCel30, oligo C6 to obtain pCel10 and oligo LT57 to obtain pCel1). 5 μl of the first-strand reaction were used for the subsequent PCR amplification. PCR reactions with 200 pM of each primer (C2-C4 for pCel20 and pCel30, C2-C6 for pCel10 and LT21-LT57 for pCel1) and 2 mM $MgCl_2$ were performed in 50 μl volumes using the DNA Thermal Cycler apparatus (Perkin Elmer, Foster City, CA). Denaturation, annealing and extension temperatures were 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, respectively. This cycle was repeated 30 times. The PCR products were separated by gel electrophoresis and the bands of interest cloned either in the pCR vector using the TA Cloning kit (Invitrogen) or in the pGEM-T vector (Promega).

The genomic clone λEG11 was isolated from a library constructed by the cloning of *Mbo*I partially digested peach DNA in the Bam HI site of the λEMBL3 SP6/T7 vector. The pCel10 partial cDNA was used as probe to screen the library following standard procedures [33]. DNA from the purified lambda clones was extracted with a Qiagen kit (Qiagen, Hilden, Germany), digested and, after electrophoresis and blotting, probed again with pCel10. The hybridizing bands were subcloned in the pGEM (Promega) plasmid vector.

Genomic DNA extraction and Southern analysis

DNA was extracted from peach and tobacco leaves as described in Dellaporta *et al.* [10]. Aliquots of 10 μg were digested with 100 u of the indicated endonucleases for 3 h. The restricted DNA was resolved on an 0.8% agarose gel and transferred onto a Hybond N^+ (Amersham) membrane using 0.4 M NaOH as blotting agent. Hybridization conditions were the same as for northern analysis. The membranes were washed at high stringency (15 min with 0.5× SSC, 0.1% SDS, 65 °C for the final wash) and afterwards they were exposed to X-ray films at −80 °C.

DNA sequencing and analysis

DNA sequencing was performed using the T7 DNA sequencing kit (Pharmacia Biotech, Uppsala, Sweden). For all the clones, sequences were determined on both strands using, when necessary, chemically synthesised oligonucleotides to cover the whole length of the clone. Sequence manipulations, analyses and alignments were performed using the Lasergene software package (DNASTAR, Madison, WI).

Transcription start determination

The transcription start site was determined using the AMV Reverse Transcriptase Primer Extension System (Promega). The primer used (LT59: 5′-GAGGAGTGAAGGCAGAGCAGCATAAACTGAG-CAAAC-3′) is a 36-mer and corresponds to bases 174–139 of the antisense strand. Each reaction was carried out with 200 fmol/μl of ^{32}P-end-labelled primer and 20 μg of total RNA from both ethylene activated leaf and fruit abscission zones. A control without RNA was also included. Annealing of the primer to its corresponding mRNA was performed at 65 °C. The reaction products were concentrated by ethanol precipitation, resuspended in a suitable volume and loaded onto a sequencing gel close to a sequencing reaction, performed with the same oligo LT59, to be used as a

standard. After electrophoresis the gel was dried and exposed to X-ray films.

Preparation of a glucuronidase gene construct and analysis of both transient and stable expression

The oligonucleotides LT27 (5′-CCAGTGTCCAAAC AACCATGTTTTAC-3′), annealing from −1794 to −1768, and LT90 (5′-TGGTGGGTTTGAAATGTGTT GAGG-3′), annealing from −13 to +10, were used in a PCR amplification to obtain the 5′ region of the *ppEG1* gene. After PCR, 5 units of Klenow DNA Polymerase were added to the mix at 37 °C for 15 min to fill in the termini. The reaction mix was then ethanol precipitated, resuspended and digested with *Hin*dIII. The digestion products were separated in an agarose gel and the band of interest, a 1649 bp fragment with a *Hin*dIII site at the 5′, was cloned in the pBI101.2 vector (Clontech Laboratories, Palo Alto, CA) prepared by a double digestion with *Hin*dIII and *Sma*I to produce plasmid p27-GUS. The correct fusion of the 5′ region of *ppEG1* with the GUS reporter gene was checked by sequencing. The double digestion of p27-GUS with *Hin*dIII and *Eco*RI released the cassette shown in Figure 4. This cassette was cloned in pUC18 and the resulting plasmid was named pUC27-GUS.

Four days after subculturing, peach cells were prepared for bombardment by placing them on a filter paper (embedded with the same medium) in the centre of an agar plate. Peach leaf abscission zones were prepared as described in 'Plant material' but for the fact that, in this case, no more than 1.5 mm of petiole was left. Nine abscission zones were placed in the centre of an agar plate. A Biolistic PDS-1000/He particle gun (BioRad, Hercules, CA) was used to bombard cells and explants. The pCaMVI$_1$CN plasmid containing the 35S promoter, a fragment of intron 1 from the alcohol-dehydrogenase 1 gene isolated from maize and the CAT reporter gene (gift of Dr Søren Knudsen, Carlsberg Laboratory, Denmark) [24] was used as an internal control for the particle gun experiments. Equal amounts (5 μg) of pCaMVI$_1$CN and pUC27-GUS were co-precipitated onto 1 μm gold microcarriers. Bombardment parameters included a helium pressure of 7.6 MPa, 0.6 mm distance between the rupture disc and macrocarrier and 5 cm distance between the stopping screen and the plate. After bombardments the plates were either kept in air or flushed with ethylene.

Plant transformation

The p27-GUS plasmid was inserted in *Agrobacterium tumefaciens* (strain LBA4404) cells [15]. These cells were then used for *A. tumefaciens*-mediated transformation of tobacco according to a modified leaf disc protocol [14].

Enzyme extractions and assays

To extract endo-β-1,4-activity, plant tissues were homogenized in 1.5 ml tubes in 4 volumes (w/v) of extraction buffer (20 mM sodium phosphate, 1 M NaCl pH 6.0, 0.3% (v/v) 2-mercaptoethanol, 5% (w/v) polyvinyl polypyrrolidone). After shaking for 2 h at 4 °C, the homogenate was centrifuged for 20 min at 14 000 × g, the supernatant was collected and the proteins were precipitated by the addition of ammonium sulphate (80% saturation). The precipitated proteins were stored at 4 °C.

Quantitative assays for enzyme activity were performed viscometrically according to the method of Durbin and Lewis [11]. A sample volume of 0.2 ml, containing a known amount of protein, was added to 1.8 ml of substrate consisting of 0.6% (w/v) carboxymethylcellulose in 20 mM phosphate buffer (pH 6.0). The flow rate of this solution was measured before and after a 2 h incubation at 37 °C. Changes in viscosity of the solution were converted to relative units of enzyme activity according to the method of Almin *et al.* [3]. The values of specific EGase activity (units per mg protein per hour) were normalized as a percentage of the maximum value (100%).

The 'Reporter Lysis Buffer' (Promega), which allows the simultaneous extraction of both glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT) activities, was used to extract protein from bombarded tissues. CAT activity, used to normalise the results from the shooting experiments, was measured with the phase extraction assay [35] using *n*-butyryl CoA as donor and [^{14}C]chloramphenicol (50 Ci/mmol; NEN). Fluorometric assays for GUS activity were performed as described by Jefferson *et al.* [19]. An histochemical assay for GUS [24] was performed on wild-type and transformed tobacco plants by incubating the plant organs in 50 mM sodium phosphate pH 7 containing 0.1% Triton X-100, 0.5 mm $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM EDTA, 20% methanol and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. To prevent browning of the tissues,

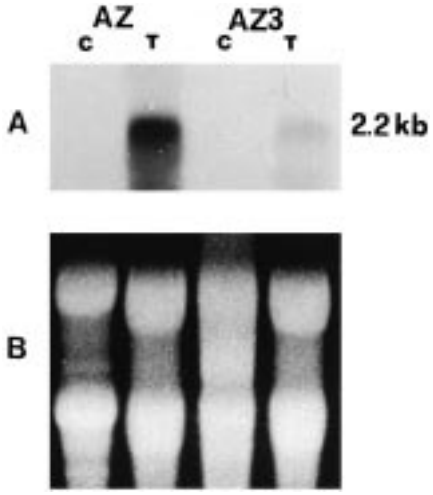

Figure 1. A. Northern analysis of pCel10-related mRNA expression in abscission zones of leaves (AZ) and fruits (AZ3). In both types of ethylene-treated abscission zones a 2.2 kb transcript is evidenced by the probe. 45 μg of total RNA was separated on formaldehyde-agarose gels, blotted and hybridized with pCel10 cDNA. C, abscission zones before activation; T, ethylene-activated abscission zones. B. Ethidium bromide staining of the gel shown in panel A before blotting.

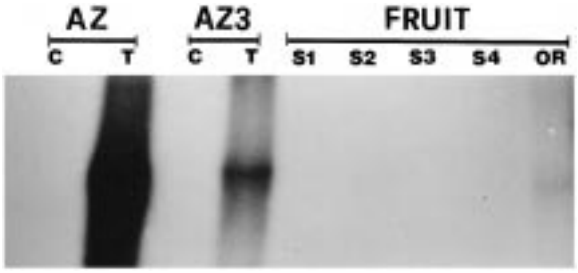

Figure 2. pCel10-related mRNA expression in various tissues analysed by RNase protection assay. RNA was isolated from leaf (AZ) and fruit (AZ3) abscission zones and from fruits at various stages of development and 10 μg of each sample were used. Besides confirming the expected high abundance of the pCel10-related mRNA in ethylene activated abscission zones, this analysis demonstrates low levels of expression also in overripe fruits. C, abscission zones before activation; T, ethylene-activated abscission zones. Fruits were harvested at 35 (S1), 60 (S2), 90 (S3), 110 (S4) days after bloom and at the overripe stage (OR).

100 mM ascorbate was added when the flowers were incubated for the histochemical assay.

The protein concentration was measured by the method of Lowry modified by Peterson [32] using bovine serum albumin (BSA, Fluka) as a standard.

Results

Preparation of three EGase-encoding cDNAs and analysis of their expression

RNA obtained from both leaf (AZ) and fruit (AZ3) abscission zones was used as the starting material to isolate EGase-encoding cDNAs by means of the RT-PCR technique. Primers C2 and C4 gave an amplification fragment of the same size with both types of RNA. Cloning and sequencing of the fragments demonstrated that they encode different EGases. One of them, named pCel20 (239 bp), was obtained from leaf abscission zone RNA, while another, named pCel30 (224 bp), was obtained from fruit AZ3 RNA. When employed with RNA from leaf abscission zones, primers C2 and C6 yielded a 788 bp fragment (pCel10) encoding a third EGase.

A divergence was evident when the deduced amino acid sequences of the three cDNAs were compared with those of the two most extensively studied EGases, that are the bean abscission [38] and the avocado ripening [36] EGases which are both ethylene-responsive but have quite different isoelectric points (9.5 and 4.7, respectively). The percent similarity among the considered sequences shows a striking 91% between pCel10 and avocado, a 73% between pCel30 and bean, while pCel20 shows a less significant similarity to both bean (60.8%) and avocado (64.6%). Interestingly, a high degree of divergency is observed among the three peach EGases whose percent similarity reaches a maximum of only 62% between pCel10 and pCel20.

Expression of the three different cDNAs was studied by northern analysis considering the most conspicuous events where cell separation occurs in peach, i.e. abscission of leaves and fruits and development of fruits. This type of analysis allows the detection of the mRNA transcripts which are relatively abundant, that is transcripts whose presence bears an evident significance for the studied physiological process. The only cDNA whose expression could be detected by this analysis was pCel10, although high amounts of RNA were used for the experiments. Its related mRNA (2.2 kb) is expressed in ethylene-activated abscission zones of both leaves and fruits, although much higher levels are found in the foliar zones (Figure 1). RNase protection assay experiments confirmed the low abundance of both pCel20 and pCel30-related mRNAs which were barely detectable in all the cell separation events of interest to us (not shown) and demonstrated that, besides the activated leaf and fruit abscission

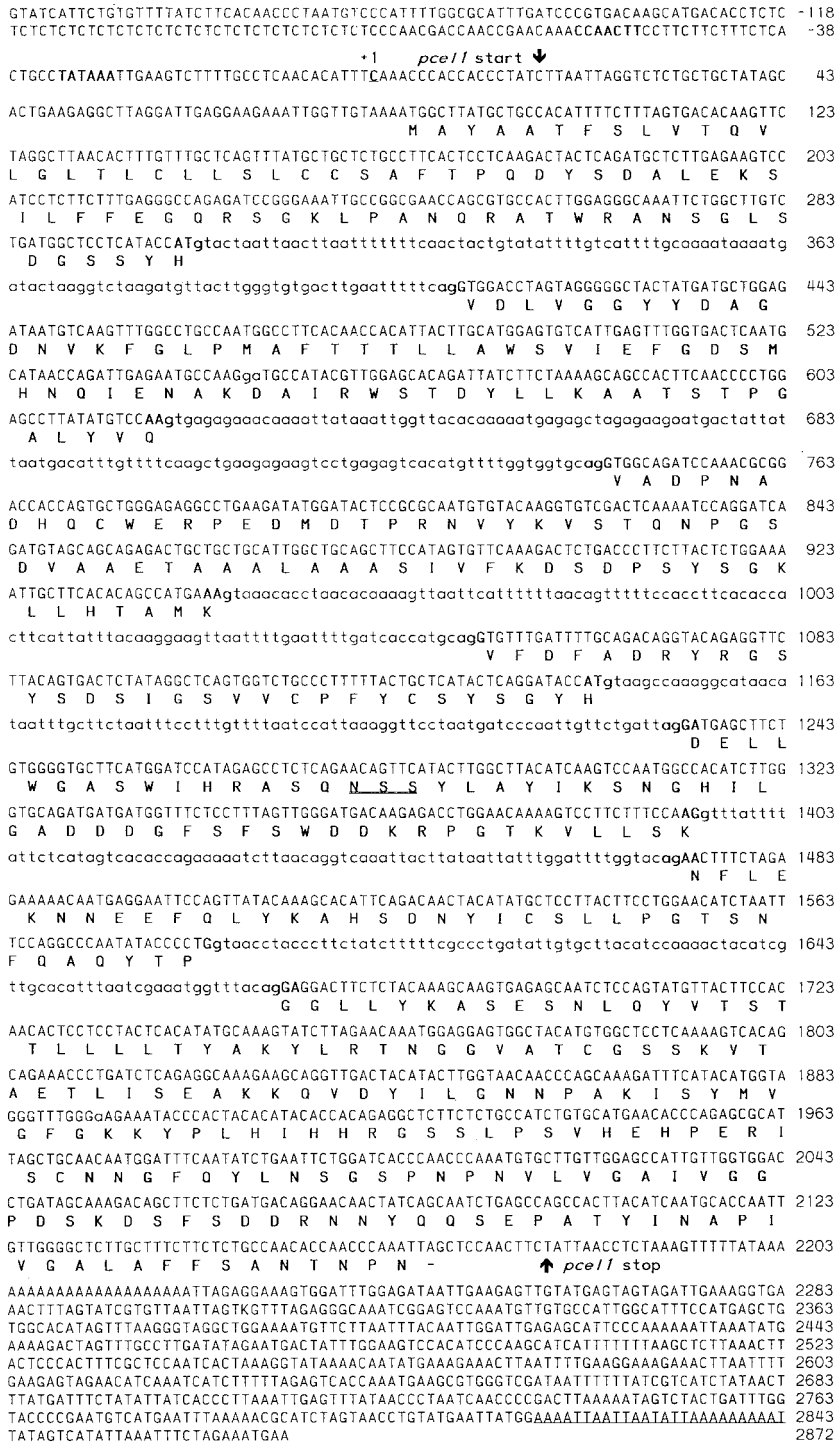

Figure 3. Nucleotide sequence of the *ppEG1* transcribed region plus part of the flanking DNA. Numbering is from the transcription start site, at the position +1. Arrows indicate the start and the end of the cDNA (pCel1) which covers the entire open reading frame of the *ppEG1* gene. Potential TATA and CCAAT sequences are shown in bold letters at the 5′ of the gene. A single underline at the 3′ of the gene indicates a possible polyadenylation site. Introns are evidenced by small characters, and exon/intron boundaries are marked by bold letters. The predicted amino acid sequence of the endo-β-1,4-glucanase encoded by *ppEG1* is shown below the nucleotide sequence; a potential site for glycosylation in the sequence of the polypeptide is indicated by a double underline.

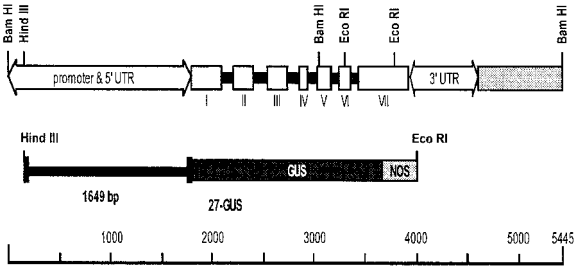

Figure 4. Restriction map and structure of the *ppEG1* gene. The sequence encompassing the EGase protein consists of 7 exons (roman numerals) and 6 introns. The *Bam*HI, *Eco*RI and *Hin*dIII restriction sites are indicated above the structure of the *ppEG1* and 27-GUS genes. Promoter and untranslated sequences (UTR) at the 5′ and 3′ ends are indicated by the double arrows, while 3′-flanking sequences are represented by the grey rectangle. The chimeric gene construct (27-GUS) used in the expression experiments is shown in the middle of the figure; it is formed by a 1649 bp region at the 5′ of the *ppEG1* gene fused to a GUS reporter gene and a NOS termination sequence. The ruler at the bottom of the figure shows the size, in base pairs, of the sequenced fragment of peach genomic DNA which contains *ppEG1*.

zones, softening peach fruits also show increased levels of the pCel10-related mRNA (Figure 2).

Characterization of the endo-β-1,4-glucanase gene ppEG1 and its cognate open reading frame (ORF)

The cDNA clone pCel10 was used to screen a peach genomic library in λEMBL3 SP6/T7. The phage clone λEG11 was purified and two fragments released by *Bam*HI digestion were subcloned in plasmids. The 5445 bp region, which contains the two *Bam*HI fragments (3055 and 2390 kb, respectively), was sequenced on both strands. Analysis of the sequencing data revealed that this region contains the gene, named *ppEG1*, which encodes the peach endo-β-1,4-glucanase corresponding to pCel10.

Based on the sequence of *ppEG1*, two oligos, located at the 5′ (position 18–39) and the 3′ (position 2179–2162 of the lower strand) of the region which contains the open reading frame (Figure 3), were prepared and used in RT-PCR experiments with RNA from ethylene-activated leaf abscission zones in order to obtain the cDNA of the *ppEG1* ORF. The result of these experiments was a 1579 bp fragment, named pCel1, which was cloned and sequenced on both strands. No differences at the nucleotide level were found between the exon regions of the gene and the PCR-obtained cDNA. According to the sequence analysis and by comparison with the pCel1 cDNA the *ppEG1* gene contains 7 exons.

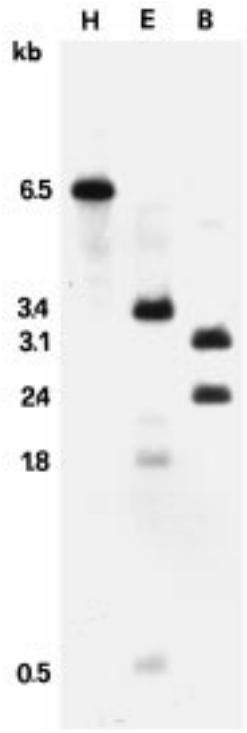

Figure 5. Southern analysis of the *ppEG1* gene. 10 μg of genomic DNA cut with *Hind*III (H), *Eco*RI (E), and *Bam*HI (H) endonucleases was hybridised at high stringency to its cognate cDNA pCel1. The hybridisation pattern of the pCel1 probe confirms the fragment size predicted by the restriction map of *ppEG1* and indicates that this gene is present as a single copy in the peach genome. The numbers on the left indicate the fragment sizes in kilobases.

The open reading frame of *ppEG1* encodes a polypeptide of 497 amino acids (Figure 3) whose predicted molecular mass is 54.3 kDa. This predicted value is consistent with the values of other known higher-plant EGases. The Kyte and Doolittle [26] hydrophobicity plot indicates that, similarly to the avocado, bean and pea enzymes [38, 41], the peach EGase also has a predicted signal peptide (28 amino acid long) at the N-terminus, characteristic of secreted proteins.

A comparison of the deduced isoelectric points (pI) of the avocado and the bean EGases with that of the peach protein shows that also at this level the peach EGase has a higher similarity to avocado than to bean. In fact, the protein encoded by *ppEG1* has a deduced pI of 5.9 which is very close to pI 5.3 deduced for the avocado EGase, while the deduced pI for the bean protein is 7.8 [38]. Finally, also the presence of one consensus sequence for potential glycosylation (Asn-X-Ser/Thr) makes *ppEG1* similar to the avocado sequence which

contains two such sites, whereas the bean EGase does not contain this consensus sequence.

A primer extension analysis was performed in order to define the putative promoter region of the *ppEG1* gene. The transcription start site of the *ppEG1* transcript was mapped 84 bases upstream of the translational initiation codon. The sequence surrounding this putative transcription start is TTTCAAA with cytosine being the first nucleotide (Figure 3). Sequences similar to the CAAT and TATA boxes are present in the *ppEG1* promoter region at position -59 to -53 and -32 to -27, respectively, upstream of the putative transcriptional initiation point.

To further characterise the endo-β-1,4-glucanase gene, genomic DNA was cut with *Hin*dIII, *Eco*RI and *Bam*HI endonucleases and studied by southern analysis using the pCel1 cDNA as a probe. A restriction analysis of the sequenced genomic DNA fragment showed that one *Bam*HI and two *Eco*RI sites are present in the region corresponding to the pCel1 probe, while a *Hin*dIII site is present in the promoter region (Figure 4). Therefore, should *ppEG1* be present as a single copy gene, the southern analysis would yield one fragment larger than 5.3 kb in the *Hin*dIII-cut DNA, two *Bam*HI fragments sizing 3.1 and 2.4 kb, respectively, and three fragments in the *Eco*RI-cut DNA. This predicted hybridisation pattern was confirmed by the southern experiment which shows all the expected bands, and demonstrates that *ppEG1* is present as a single-copy gene in the peach genome (Figure 5).

Analysis of ppEG1 promoter activity using a GUS reporter gene

Attempts to study the regulatory activity of the 1639 bp region at the 5′ of the transcription start site of *ppEG1* were first made with the particle delivery technique using peach cells from liquid and solid cultures as a homologous transient expression system. However, the shooting of the 27-GUS constructs resulted in random and too low levels of GUS expression to make this system suitable for analysis of promoter activity.

Another attempt with the particle delivery system was done using leaf abscission zones as a target. However, contrary to the results obtained by Koehler *et al.* [25] with the abscission zones of bean leaves, it was not possible to measure either CAT or GUS activity even when driven by the 35S-CaMV promoter, probably because of the high polyphenolic content of the peach tissues.

Tobacco was therefore used as a heterologous expression system because of the well established techniques to transform it with *Agrobacterium tumefaciens* and to regenerate the transformed cells to plants for analysis of the promoter activity *in vivo*. Moreover, tobacco has flowers whose pedicels contain an abscission zone [20] promptly activated by ethylene.

The DNA fragment used for the promoter analyses consisted of 1649 bp at the 5′ end of the cloned *ppEG1* gene, that is 1639 bp 5′ to the transcription start plus the first 10 bp of the mRNA untranslated region (Figure 4). This fragment was used to prepare a chimerical gene by fusing it to a GUS reporter gene followed by a NOS terminator sequence (Figure 4). The construct was stably inserted in tobacco via *A. tumefaciens* transformation. Kanamycin-resistant regenerates were screened by Southern blot analyses to confirm insertion of the 27-GUS chimeric gene into their genome and three independent transformants gave the typical blue GUS staining. Histochemical staining for GUS activity indicates that, as expected, the *ppEG1* promoter induces expression of GUS in the abscission zones of flowers (Figure 6B). A very intense staining was also observed at the level of the floral stigma (Figure 6D) and at the sites of lateral root formation (Figure 6F). The lack of staining in wild type plants (Figure 6A, C, E) confirms the specificity of the blue colour observed in the tobacco transformants.

A possible relation among the three different tissues exhibiting GUS activity was found by analysing their level of EGase activity which, in all cases, was much higher than that of control tissues (Figure 7). In the cases of the stigma and the lateral roots the controls consisted of homologous tissues where the blue staining did not appear, such as ovary parietal tissues and root tissues without lateral roots. As for the flower abscission zones, which are known to be activated by ethylene, the comparison was made between non activated and ethylene activated zones. In fact, this comparison might also provide information about the presence of ethylene responsive elements in the analysed promoter region. A disappointing discrepancy in the pattern of GUS expression was revealed by the ethylene activation of the flower abscission zones. In fact, while the hormone increased the amount of tobacco EGase activity, it did not increase the expression of the 27-GUS chimeric gene. On the contrary, as evidenced by histochemical staining, the GUS activity appeared to be decreased in the ethylene activated zones (not shown). A barely detectable blue staining was sometimes also observed at the proximal end of

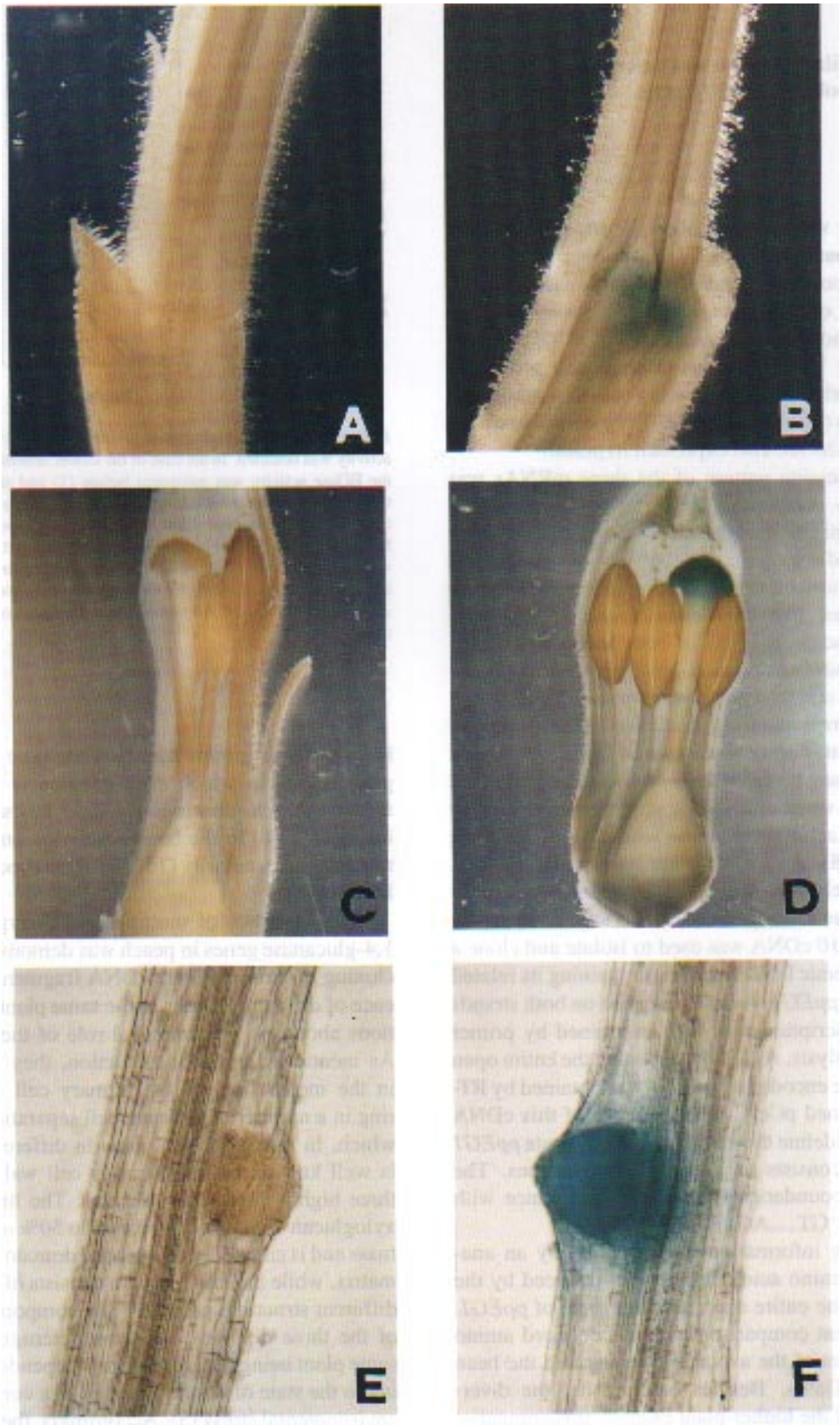

Figure 6. Histochemical localisation of *ppEG1* promoter activity in transgenic tobacco containing the chimeric 27-GUS gene. Expression of the GUS reporter gene was at the level of abscission zones of flowers (B), stigmas (D) and newly formed lateral roots (F). A, C, E show the respective controls which consisted of wild type tissues subjected to the same experimental procedure for detection of GUS activity.

the leaf petioles, similarly to what was described by Eyal *et al.* [12] for the tobacco *PRB-1b* gene.

Discussion

In this study we have described the structure and the expression pattern of *ppEG1*, a member of a multigene family coding for endo-β-1,4-glucanase in peach. The cloning of this gene was made possible by the use of a homologous cDNA probe (pCel10) obtained by RT-PCR. This experimental approach proved to be particularly useful, allowing us to obtain two more cDNAs (pCel20 and pCel30, respectively) whose related mRNAs are also expressed in peach.

The expression pattern of the three mRNAs was examined by northern analysis during leaf and fruit abscission and also during fruit development. This analysis revealed expression of the only pCel10-related mRNA. In particular, increased amounts of a 2.2 kb pCel10-related transcript were found in ethylene-activated abscission zones of both leaves and fruits. These data indicate that its cognate gene *ppEG1* is up-regulated by ethylene and codes for the EGase which is highly expressed during abscission of leaves and, to a lesser extent, during abscission of fruits. An RNase protection assay revealed that *ppEG1* is also expressed at a very late stage of fruit ripening, thus co-operating with polygalacturonase in the softening of the fruit mesocarp [28]. As for the other two EGase cDNAs, their extremely low abundance makes them of little interest for the cell separation events studied by us.

The pCel10 cDNA was used to isolate and clone a 5445 bp genomic DNA fragment containing its related gene, named *ppEG1*. It was sequenced on both strands and the transcription start site determined by primer extension analysis. A cDNA containing the entire open reading frame encoded by *ppEG1* was obtained by RT-PCR and named pCel1. The sequence of this cDNA was useful to define the structure of its cognate *ppEG1* gene which consists of 7 exons and 6 introns. The exon-intron boundaries are well in accordance with the proposed 'GT.....AG' rule [17].

Interesting information was obtained by an analysis of the amino acid composition, deduced by the sequence of the entire open reading frame of *ppEG1*, and subsequent comparison with the deduced amino acid sequences of the avocado ripening and the bean abscission EGases. Besides emphasising the divergent nature of the higher-plant EGases, this comparison raised doubts about the usefulness of constructing homogeneous groups based on the type of physiological events in which each enzyme is involved. In fact, the peach 'abscission' EGase bears a poor similarity (47.3%) to the bean abscission enzyme, and a much higher similarity (76.3%) to the avocado ripening EGase [38].

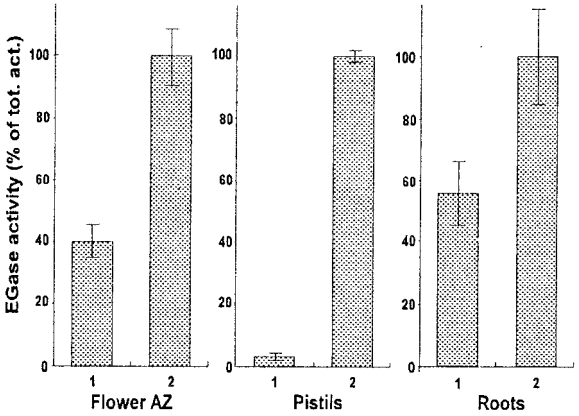

Figure 7. Endo-β-1,4-glucanase activity in tissues where also GUS activity was detected. In the case of the flower abscission zones (AZ), the EGase activity was measured before (1) and after (2) ethylene treatment. EGase activity in pistils refers to ovary parietal tissues (1) regarded as the control and to stigmas (2). In roots the measured EGase activity refers to primary root tissues (1) and to radical zones where lateral roots were being formed (2). The amount of EGase activity was normalised to the maximum activity measured for each set of analysed tissue. All values are the average of 5 independent measurements.

The existence of multiple and divergent endo-β-1,4-glucanase genes in peach was demonstrated by the cloning of three different cDNA fragments. The presence of different EGases in the same plant raises questions about the physiological role of these enzymes. As mentioned in the Introduction, they are involved in the metabolism of the primary cell walls occurring in a number of different cell separation processes which, in turn, can take place in different organs. It is well known that the primary cell walls consist of three highly interacting domains. The first cellulose-xyloglucan domain can make up to 50% of the parietal mass and is embedded in a second domain of the pectin matrix, while the third domain consists of a number of different structural proteins. The components of each of the three domains can show heterogeneity in the same plant being able to change independently according to the state of development or as a consequence of environmental stress [5]. Accordingly, the presence of different EGase forms might supply the biochemical

variability required to bring about different cell separation events in different organs.

In some cases it has been shown that the expression of the different EGase genes can be state of development- and/or tissue-specific [9, 39, 27, 13, 7]. This requires the presence of regulatory sequences which are able to modulate the expression of the various genes. *ppEG1* is a peach gene which is highly expressed in the abscission zones of leaves and fruits, therefore its promoter is expected to specifically drive expression of the gene at the level of abscission zones.

This hypothesis has been confirmed by the functional analysis of the 5′ promoter region of *ppEG1* carried out with transgenic tobacco plants. In fact, also in this heterologous system, the promoter was able to induce expression of GUS activity at the level of the flower abscission zones.

Interestingly, some barely detectable GUS activity was also found at the proximal end of the leaf petiole, thus confirming the observation of Eyal *et al.* [12] with the tobacco *PRB-1b* promoter. In most plants this is the region where abscission zones of leaves are differentiated, therefore, since tobacco leaves do not abscise, such a result appears quite surprising. One might hypothesise that a long time ago the abscission of leaves was also possible in tobacco plants but that this ability was later lost, as testified by the low, but still detectable, expression of GUS activity induced by at least two abscission-related promoters (i.e. *ppEG1* and *PRB-1b* [12]).

The 27-GUS chimeric gene induced expression of GUS activity also at the level of the stigma and in the regions where the newly formed lateral roots must find their way out through the cortical parenchyma. It has been reported that in bean an EGase immunologically related to the 9.5 form is also expressed at high levels in stigmatic tissues [9]; moreover, expression of an EGase mRNA has been demonstrated in tomato pistils, although in this case no distinction was made among the various parts of the pistil [30]. Increased amounts of EGase activity have also been associated with the phenomenon of adventitious root formation in soybean [23]. However, while in bean stigmas a western analysis demonstrated *de novo* synthesis of enzyme [9], in soybean the abscission EGase cDNA gave no hybridization signal in correspondence with the observed increase in EGase activity [23].

The finding that also in tobacco high levels of EGase activity are expressed in abscission zones, stigmatic tissues and sites of lateral root formation, suggests that the *ppEG1* promoter must contain some regulatory sequence able to respond to general ‘cell separation’ stimuli. Since no GUS activity was observed in young expanding tissues, it is probable that those stimuli are senescence-related ones.

As for the intriguing decrease in GUS activity observed in the flower abscission zones after ethylene treatment, this might mean that the possible ethylene regulatory elements (ERE) of *ppEG1* are located outside of the analysed fragment, although we cannot rule out the possibility of co-suppression phenomena as reported by Jorgesen [21] for other GUS chimeric genes in transgenic tobacco plants. Another possibility might be that upon ethylene treatment the tobacco EGase promoter(s) would successfully compete for their own *trans*-acting factors with the heterologous peach promoter, thus decreasing its ability to induce expression of GUS activity.

In conclusion, tobacco has proved to be a suitable system to carry out the future dissection of the *ppEG1* gene promoter. The results of ethylene treatments suggest that a longer sequence needs to be analysed to better understand the regulatory properties of this promoter. The specificity of the expression pattern, as evidenced by the histochemical staining for GUS activity, makes it feasible to use this promoter to regulate cell separation events in plants other than peach. To this purpose, it will be of particular interest to analyse its possible activity in plants that, unlike tobacco, produce fleshy fruits as, for instance, tomato.

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