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RESEARCH PAPER

Pectin esterase gene family in strawberry fruit: study of *FaPE1*, a ripening-specific isoform*

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Abstract

Pectin esterases (PE, EC 3.1.1.11) catalyse the demethylation of pectin. As a result of its activity, structural interactions among cell wall components during cell wall turnover and loosening are affected. In plants, PEs are typically encoded by a gene family. This family has been studied in strawberry (*Fragaria*×*ananassa* Duch.) in order to investigate the role of distinct PE genes during fruit ripening and senescence. By a combination of a PCR-based library screening and RT-PCR four different strawberry PE cDNAs, termed *FaPE1* to *FaPE4*, have been isolated. Differential expression of each *FaPE* gene in various organs and during fruit development was revealed by northern blot. *FaPE1* is specifically expressed in fruit, showing an increasing expression during the ripening process up to a maximum in the turning stage. Concerning hormone regulation, auxin treatment increased *FaPE1* mRNA levels in green fruit, whereas exogenous ethylene decreased *FaPE1* mRNA levels in ripe and senescing fruits. It is proposed that this repression of *FaPE1* expression could be involved in textural changes occurring during fruit senescence.

Key words: Auxin, cell wall, ethylene, fruit ripening, pectin esterase, strawberry.

Introduction

Fruit ripening in strawberry (*Fragaria* spp.) is a genetically programmed process of development that, in the late stage, overlaps with senescence (Perkins-Veazie, 1995). Characteristics and composition of the ripe fruit are the result of biochemical and physiological changes. These changes include a pronounced decrease in fruit firmness that is associated with increased susceptibility to physical damage and disease. Fruit softening is mainly the result of the action of hydrolytic enzymes that modify the carbohydrate components of the cell wall. As in all non-climacteric fruits, ethylene is considered to have little or no effect on the development of the strawberry receptacle (pseudo-fruit; Perkins-Veazie, 1995). However, a role for this hormone during strawberry fruit senescence could not be excluded as it has been shown that treatment of ripe fruits with ethylene enhanced fruit softening (El-Kazzaz *et al*., 1983). As there is little indication that ethylene is directly involved, auxin has been proposed to be the primary hormone controlling strawberry fruit ripening. Auxin is synthesized in the achenes (true fruits embedded in the receptacle) and positively effects the initial growth of the receptacle (Given *et al*., 1988). A gradual decline in the supply of auxin from achenes in the latter stages of growth has been proposed to be the basis of ripening (Perkins-Veazie, 1995). It has also been reported that auxin regulates the transcription of many ripening-related strawberry genes (Manning, 1994; Medina-Escobar *et al*., 1997*a*; Aharoni *et al*., 2002).

Pectin is the most abundant class of macromolecule within the cell wall matrix. It is also abundant in the middle lamellae between primary cell walls, where it functions in regulating intercellular adhesion, being the major adhesive material between cells. Fruit cell walls are usually highly enriched in pectins, often more than 50% of the wall.

* The sequences reported in this paper have been deposited in the GenBank database under the accession numbers AY324809 (*FaPE1*), AY357182 (*FaPE2*), AY357183 (*FaPE3*), and AY357184 (*FaPE4*).

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Abbreviations: PE, pectin esterase; HGA, homogalacturonic acid.

During fruit softening, pectins typically undergo solubilization and depolymerization that are thought to contribute to wall loosening and disintegration (Fischer and Bennett, 1991). However, continued synthesis of cell wall polyuronides has also been suggested (Perkins-Veazie, 1995). Several pectin-modifying genes have been cloned in strawberry fruit such as pectate lyase (Medina-Escobar *et al*., 1997*b*), endopolygalacturonase (Redondo-Nevado *et al*., 2001) or β-galactosidases (Trainotti *et al*., 2001). Pectin esterase (PE) activity has also been reported in ripening strawberry fruits (Neal, 1965; Barnes and Patchett, 1976). In fruit and vegetative tissues PE occurs in multiple isoforms, and individual isozymes can be distinguished by their physical and biochemical properties (Gaffe *et al*., 1994; Bordenave, 1996). Molecular studies have further demonstrated that plant PEs exist in multigene families whose members have different patterns of expression. Therefore, it is speculated that heterogeneity of PE isozymes reflects divergence in functional specialization.

The cloning of four *PE* genes in strawberry, *FaPE1, FaPE2, FaPE3*, and *FaPE4*, is reported here. Expression of the corresponding cDNAs was examined in vegetative tissues and throughout fruit development. It was shown that the expression of *FaPE1* was induced by auxin at the onset of fruit ripening and down-regulated by ethylene during fruit senescence. It is proposed that the ethylene-mediated decrease of *FaPE1* expression could be an important factor determining strawberry fruit post-harvest decay.

Materials and methods

Plant materials

Strawberry plants (*Fragaria×ananassa* Duch.) were grown under field conditions in Huelva, in the south-west of Spain. The cultivar used was a commercial variety registered by the University of California (Davis) as Chandler. Plants of *Fragaria vesca* were provided by the *Fragaria* germplasm collection from the Churriana Center (Málaga, Spain). *Fragaria×ananassa* fruits were selected at seven developmental stages that had previously been established (Agius *et al*., 2003): F1, young flower receptacle; F2, mature flower receptacle; F3, green fruit (green receptacle and green achenes); F4, white receptacle and green achenes; F5, white receptacle with some rosy spots and brown achenes; F6, turning receptacle; and F7, red receptacle. Other tissues were stolons, roots, and expanding leaves. All analyses were performed using tissue that was harvested, immediately frozen in liquid nitrogen, and stored at –80 °C.

To analyse *FaPE1* expression in senescent fruits, red ripe fruits were tagged on the vine, sprayed with an antifungal solution consisting of 0.05% carbendazim, and maintained on the vine until they showed evident symptoms of senescence. Alternatively, fruits were harvested at the ripe stage and left to overripen for 2 d at 21 °C on the laboratory bench.

Hormone treatments

In order to eliminate the endogenous auxin source, achenes were removed from F3 berries that were still attached to their parent plant. Deachenation was conducted by removing the achenes from longitudinal halves using sharp tweezers, maintaining the other half-fruit cointaining achenes as the control. For hormone treatment, NAA (1-napthaleneacetic acid) was applied to longitudinal halves of similarly staged, but otherwise intact, fruits (possessing achenes) at a concentration of 1 mM in a lanolin paste containing 1% (v/v) DMSO. Control fruits were treated solely with the lanolin–DMSO paste without NAA. Three and five days after deachenation and 3 d after the auxin/lanolin treatment, the two halves were separated from each other and frozen separately. A minimum of five fruits were used in each experiment.

For the ethylene treatment five F7 detached fruits were placed in 1.0 l airtight containers and subjected to a constant flow of air containing 50 μl l^{-1} of ethylene. Another set of F7 detached fruits were treated with 600 μl l^{-1} of the ethylene-perception inhibitor 1-methyl cyclopropene (1-MCP). Control (untreated detached fruits) were left in air. All treatments were performed for 24 h at room temperature.

cDNA cloning of PE from strawberry fruit

An alignment of deduced amino acid sequences corresponding to the C-terminal region of four plant pectin esterases [*L. esculentum* LePME2 (U70675); *A. thaliana* AtPME2 (U25649); *V. radiata* VrPECMEST (U94443), and *P. persica* PpPEESTR (X95991)] was used to identify two conserved domains that allowed the degenerate PCR primers to be designed. The 5′ primer FP2 [AAGCA(C/T)CAAGCAGTGGC(A/T)CT(A/C/T)] corresponded to the sequence KHQAVAL and the 3′ primer RP2 [CCACTCTG(C/G)CCA(C/T)CCTTC(A/T)GG] to amino acids PEGWXEW.

PE-like cDNAs were cloned from a red-ripe strawberry fruit (*F.×ananassa* cv. Chandler) cDNA library prepared in the *Lambda ZAP Express* vector (Stratagene). Different combinations of the degenerate primers FP2 and RP2, and T3 and T7 universal primers were employed. Resulting PCR fragments were gel-purified and cloned in the pGEM-T Easy vector (Promega). The primer combination FP2 and RP2 led to *FaPE1* amplification and the reaction performed with RP2 and T7 yielded the *FaPE2* cDNA fragment.

In parallel, reverse transcriptase-PCR was performed to amplify PE transcripts expressed in immature strawberry fruit. First-strand cDNA was synthesized from 1 μg of total RNA extracted from green fruit using Expand reverse transcriptase (Roche) following the manufacturer's instructions. The primer employed for the reverse transcription was the oligonucleotide RT1 [5′-GCGAATTCTC-GAGTCGACAC$(T)_{18}$-3′], which includes a poly-T sequence preceded by a 20 nucleotide sequence recognized by the primer RT2 employed in the PCR amplification. An aliquot (2 μl) of the first strand reaction was used for the subsequent PCR amplification using the primer combination FP2 (see above) and RT2 (5′-GCG-AATTCTCGAGTCGACAC-3′). The resulting PCR products were separated by gel electrophoresis, gel-purified and cloned into pGEM-T Easy vector (Promega).

Cloning of full-length FaPE1 *cDNA*

The full-length cDNA *FaPE1* was obtained by screening the red-ripe fruit cDNA library with the PCR fragment corresponding to the partial cDNA of *FaPE1*. The probe was radiolabelled by random priming using [α-^{32}P] dCTP and the Klenow fragment of DNA polymerase and the screening performed using standard procedures (Sambrook *et al*., 1989). Seven out of 48 positive phagemids were excised *in vivo* by means of a helper phage and the longest one was chosen for further studies.

Isolation of FaPE1 *genomic clones and promoter cloning*

Genomic clones were isolated from a strawberry (*F.×ananassa* cv. Chandler) genomic library constructed in the *Lambda FIX II* vector

(Stratagene). The screening was carried out following standard procedures (Sambrook *et al*., 1989) and using the partial length *FaPE1* cDNA as probe. Several positive genomic DNA clones were obtained and ten of them were selected for further analysis.

Phage DNA was isolated from the selected genomic clones as described in Sambrook *et al*. (1989) and used as the template to amplify the promoter region of the *FaPE1* gene. DNA from each clone was subjected to PCR using the T3 or T7 universal primers in combination with a specific primer complementary to a 21-mer sequence upstream the initial ATG codon (5′-GAAGAAAGA-AGTGATGGAGGA-3′).

Nucleid acid isolation and analysis

Genomic DNA was isolated from *F.×ananassa* young leaves as described in Poresbski *et al*. (1997) and from young leaves of *F. vesca* using the Nucleon PhytoPure system (Amersham Pharmacia Biotech) following the manufacturer's recommendations. For Southern blot, 10 µg aliquots of genomic DNA from *F.×ananassa* and *F. vesca* were digested with *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I, fractionated on a 0.8% agarose gel, and transferred to Hybond-N membrane (Amersham Pharmacia Biotech) using 10× SSC as blotting buffer. The blot was hybridized with cDNA probes obtained by *Eco*RI digestion of the pGEM-T Easy plasmids containing the four partial *FaPE* cDNAs and labelled with ^{32}P dCTP as indicated before. Membranes were prehybridized in Church buffer [0.5 M $NaPO_4$, pH 7.5, 7% (w/v) SDS, 1 mM EDTA, 1% (w/v) BSA] for 2 h at 65 °C. Labelled probes were added to the prehybridization buffer (1×10^6 cpm ml^{-1} buffer), and hybridization performed overnight at 65 °C. Membranes were washed four times: (1) in 2× SSC containing 0.1% SDS, at room temperature; (2) as wash 1 at 65 °C; (3) in 0.2× SSC containing 0.1% SDS at 65°C; (4) as wash 3 using fresh wash-buffer. Each wash-time was 15 min. Autoradiographs were prepared by exposing X-ray film (X-Omat AR, Kodak) to the membranes at –80 °C.

Total RNA isolation was performed as previously described (Manning, 1991) and separated in 6% formaldehyde–1.2% agarose gels and blotted onto Hybond-N membrane (Amersham Pharmacia Biotech) using 20× SSC as blotting agent. RNA loading was checked by means of ethidium bromide staining of agarose gels. The membranes were hybridized with cDNA probes that were obtained as indicated for Southern blot analyses and the same prehybridization, hybridization, washing, and exposition conditions were used.

Results

Isolation of strawberry PE cDNA clones

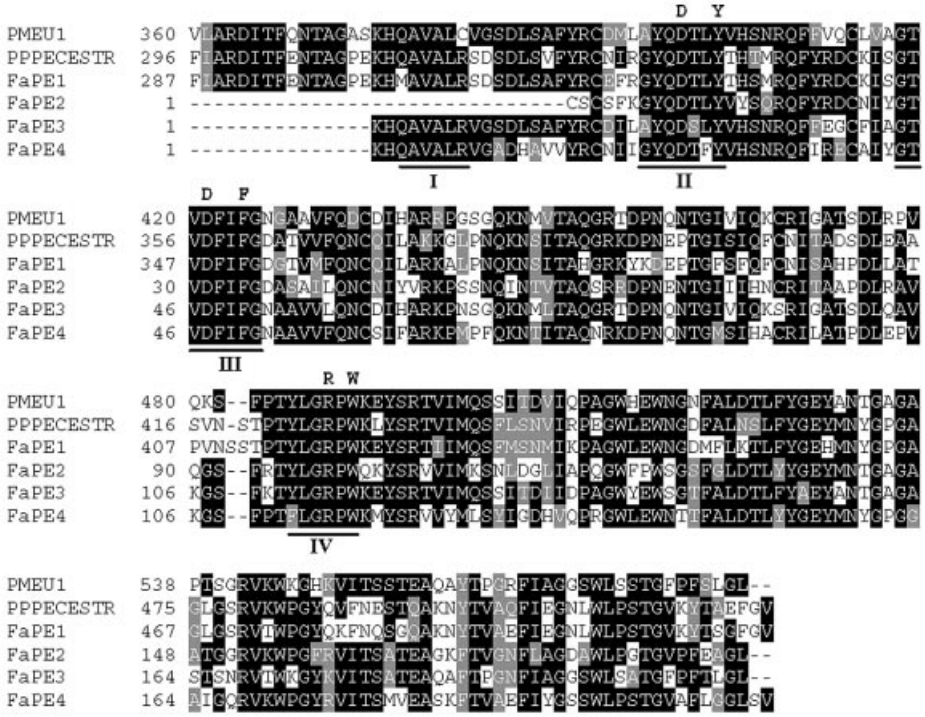
In order to study the role of PEs during strawberry fruit development, PE cDNAs were cloned from green and ripe fruits. Degenerate primers were deduced from highly conserved PE domains and employed in RT-PCR from green fruit and in several PCR reactions using a red ripe cDNA library as template. Sequencing of the amplified fragments yielded four different cDNAs which, on the basis of comparisons with other sequences from databanks, code for different PEs named *FaPE1, FaPE2, FaPE3*, and *FaPE4* (Fig. 1A). *FaPE1* and *FaPE2* were isolated from the PCR-based screen of a ripe strawberry cDNA library whereas *FaPE3* and *FaPE4* were obtained by RT-PCR from green stage fruit. The four cloned sequences shared a high degree of sequence similarity with PE of other plant species and possess the putative PE catalytic domain defined by Albani *et al*. (1991). This esterase-like domain includes the catalytic triad Asp–Asp–Arg as well as several aromatic residues that are thought to be important in the interaction with pectins (Johansson *et al*., 2002; Fig. 1A). Identity and similarity percentages among the encoded amino acid sequences are shown in Fig. 1B, where it is shown that FaPE1 is the most divergent among all FaPE proteins (less than a 60% of identity) and that this isoform is highly similar to a fruit-specific PE from *P. persica* (PPPECESTR).

Southern analysis was conducted to determine the copy number of *FaPE* genes in the genome of the octoploid strawberry variety *F.×ananassa* cv. Chandler. The complex pattern of bands obtained did not provide an accurate estimation of the copy number (Fig. 2) as found for other *F.×ananassa* cv. Chandler genes (Trainotti *et al*., 1999). However, Southern analyses of *F. vesca,* a diploid wild relative species, indicated that each *FaPE* is encoded by a single gene per diploid genome (Fig. 2). This result may be suggesting that each *FaPE* gene from *F.×ananassa* belongs to a divergent multigene family whose members are distinguishable by restriction fragment polymorphism. Interestingly, hybridization with *FaPE3* and *FaPE4* yielded the same band pattern with all the restriction enzymes employed, with the exception of *Eco*RV, since *FaPE3* contains an *Eco*RV recognition site.

Expression pattern of the FaPE *genes*

The spatial and temporal expression of the four strawberry *PE*-related genes has been analysed by northern blot carried out under very stringent conditions (Fig. 3). *FaPE1* mRNA was only detected in fruit tissue, with higher levels during the final stages of ripening. The expression of *FaPE1* follows a dramatic increase from F3 to F4, coinciding with the beginning of the ripening process. During ripening *FaPE1* mRNA expression shows two peaks at F4 and F6. This double peak of *FaPE1* expression was routinely reproduced in a replicated northern blot experiment which included a different set of fruits. After F6, *FaPE1* mRNA levels decreased although it was still detectable in red ripe fruits (F7). Like *FaPE1*, *FaPE2* cDNA was also isolated from a cDNA library of ripe fruits. However, *FaPE2* mRNA was not detected during fruit development by northern blot, its expression only being detected in leaf tissue (Fig. 3). *FaPE3* and *FaPE4* transcripts were detected in all the tissues analysed (Fig. 3). During fruit development *FaPE3* and *FaPE4* showed a similar up-down-up expression profile which has also been observed in other genes expressed during strawberry fruit development (Manning, 1998). However, *FaPE3* seems to be more highly expressed than *FaPE4* (Fig. 3). In vegetative tissues *FaPE3* signal was notably more intense in leaves whereas *FaPE4* expression predominated in stolons.

A)



B)

	FaPE2	FaPE3	FaPE4	LYCES PMEU1	PPPECESTR
FaPE1	**50%** 74%	**50%** 75%	**56%** 77%	**52%** 74%	**81%** 93%
FaPE2		**61%** 83%	**61%** 83%	**60%** 82%	**55%** 78%
FaPE3			**62%** 78%	**82%** 93%	**55%** 76%
FaPE4				**62%** 77%	**61%** 81%
LYCES PMEU1					**56%** 77%

Fig. 1. (A) Alignment of the C-terminal region of strawberry pectin esterases (FaPE1–FaPE4) predicted proteins and two other PEs from *P. persica* (PPPECESTR, X95991) and *L. esculentum* (PMEU1, AY046596). The four conserved regions of the pectin esterase domain are underlined. The consensus residues Asp–Arg–Asp that form the catalytic triad and three aromatic residues responsible for substrate interaction are highlighted. (B) Identity (bold face) and similarity percentages between overlapping regions of the above aligned PE proteins.

Isolation of FaPE1 *full length cDNA and sequence analysis*

A full length *FaPE1* cDNA (GenBank accession number AY324809) was isolated by screening of a cDNA library with the partial *FaPE1* cDNA as probe. *FaPE1* cDNA is 1848 bp long with a major open reading frame extending from nucleotide 55–1599. At position –48 relative to the predicted initial ATG codon, a stop codon was found, indicating that the isolated fragment contained an entire open reading frame. The 1545 bp ORF of *FaPE1* encodes a protein that shares high homology with plant PE and it is most similar (83% identical) to a PE expressed in peach fruit (PPPECESTR X95991). The amino acid sequence deduced of *FaPE1* contains eight putative Asn–X–Ser/Thr glycosylation sites usually found in proteins to be secreted. The Kyte and Doolittle (1982) hydrophobicity plot (not shown) revealed the presence of a short hydrophobic domain around 25 amino acids long at the N-terminus, corresponding to the putative signal peptide and a predicted signal peptide cleavage site at Cys^{27} (Nielsen *et al*., 1997). Its removal would result in a 53.4 kDa polypeptide. Previous reports on other plant PEs have proposed an additional post-translational proteolytic cleavage (Hall *et al*., 1994; Bordenave *et al*., 1996). In the case of FaPE1 it would be expected in the vicinity of Val^{196},

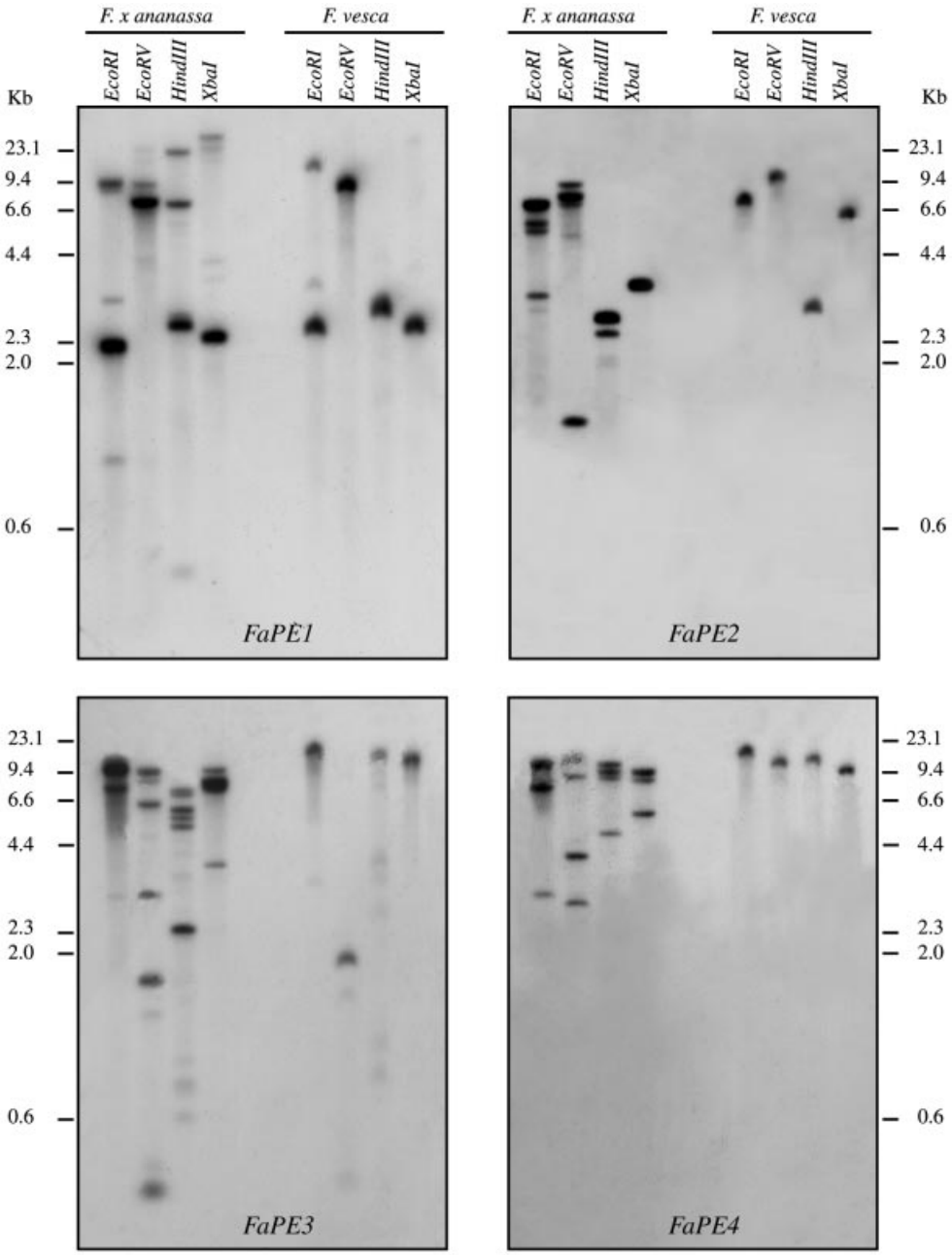


Fig. 2. Genomic Southern blot analysis of DNA from the strawberry species *F.×ananassa* and *F. vesca.* Genomic DNA (10 μg per lane) was digested with the indicated restriction enzymes and hybridized with ^{32}P-labelled fragments of *FaPE1–FaPE4* probes.

releasing a mature protein of 35.8 kDa with a theoretical isoelectric point of 8.52. This putative mature protein which includes the esterase-like domain corresponds to the carboxy-terminal region of the precursor and its molecular mass is similar to that of the 33.5 kDa PE recently purified from ripe strawberry fruit (Nguyen *et al*., 2002*a*).

Effect of auxin on FaPE1 *expression*

The role of auxin during fruit development was analysed in F3-stage fruits. At this stage, the endogenous peak of auxin occurs in the berry (Archbold and Dennis, 1984) and it also precedes an increase in the expression of *FaPE1*. Because it is well established that achenes are a rich source of auxins, in order to test whether *FaPE1* mRNA expression is affected by the removal of endogenous auxin, northern blot was conducted in deachened green fruits. As shown in Fig. 4A, *FaPE1* mRNA expression was undetectable in fruits 3 d after the elimination of the achenes. By contrast, *FaPE1* mRNA levels were higher in tissue with achenes 3 and 5 d after the treatment, suggesting that auxin is

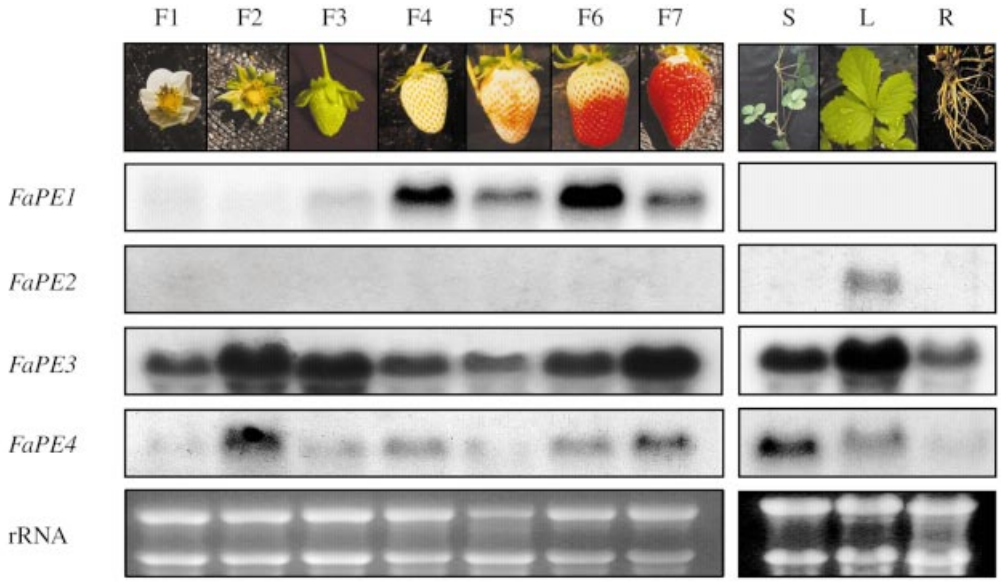


Fig. 3. Northern hybridization analysis of pectin esterase gene expression during strawberry fruit development (F1–F7), and in stolons (S), leaves (L), and roots (R). 15 μg of total RNA were used in these analyses.

required for *FaPE1* expression during fruit ripening. To examine the auxin effect further, green fruit halves were covered with a lanolin paste with or without NAA. Northern blot analysis showed that NAA induces an increase in *FaPE1* expression 3 d after the auxin application compared with the untreated control (Fig. 4B). Fruits treated with lanolin did not show changes in *FaPE1* mRNA abundance (Fig. 4B). As a control for the auxin treatment, the same blots were stripped and subsequently hybridized with a probe for *FaPE3* and no changes in gene expression were observed (data not shown).

Ethylene regulation of FaPE1 *expression*

To gain insight into the regulation of *FaPE1* gene expression the promoter region of *FaPE1* was isolated from a *F.*×*ananassa* genomic library constructed in λ FIX II. Library screening was performed using the partial *FaPE1* cDNA as probe. An amplified fragment of 1.6 kb was cloned and sequenced; its identity was confirmed by identical overlapping with the 5′ UTR sequence of *FaPE1* cDNA. The 1.6 kb fragment included a putative TATA box located at nucleotide –145 relative to the ATG initiation codon. Sequence analysis of the *FaPE1* promoter region using PlantCare software (Rombauts *et al*., 1999) led to the identification of putative *cis*-acting elements that could be involved in *FaPE1* transcriptional regulation. Three ethylene responsive elements (EREs) were located at positions –386, –812, and –1065 (Fig. 5A). Noticeably, no auxin-responsive elements were found in the 1.6 kb region analysed. Each ERE consists of an 8 bp motif with the sequence A(A/T)TTCAAA and they have previously been identified in the promoter region of ethylene-stimulated genes (Montgomery *et al*., 1993; Itzhaki *et al*., 1994).

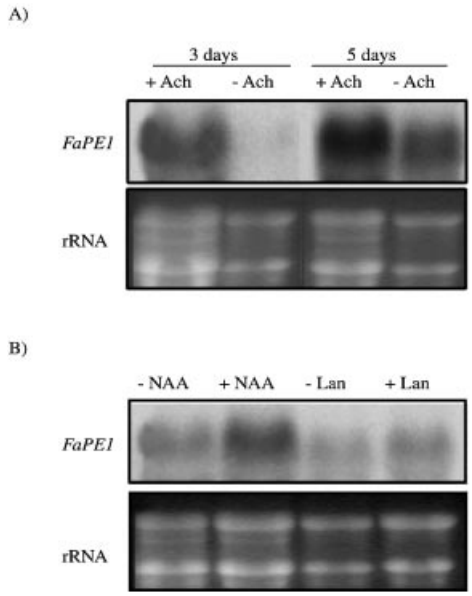


Fig. 4. Effect of auxins on *FaPE1* expression. Northern blot analysis of total RNA (15 μg) extracted from green fruits under different auxin conditions. (A) *FaPE1* expression in the presence (+Ach) or absence (–Ach) of achenes 3 d and 5 d after deachenation. (B) *FaPE1* expression in untreated (–NAA) and 3 d after the application of 1 mM NAA in a lanolin paste (+NAA). As a control for the auxin treatment, another set of fruits were treated with the lanolin paste only (+Lan) or left untreated (–Lan).

To test the biological significance of the putative EREs in the promoter of *FaPE1*, the effect of the hormone ethylene in *FaPE1* expression was analysed. Treatment was performed in F7 strawberry fruits, coinciding with an endogenous peak of ethylene production (Iannetta *et al*., 2000). When treated for 24 h with 50 μl l^{-1} ethylene, fruits

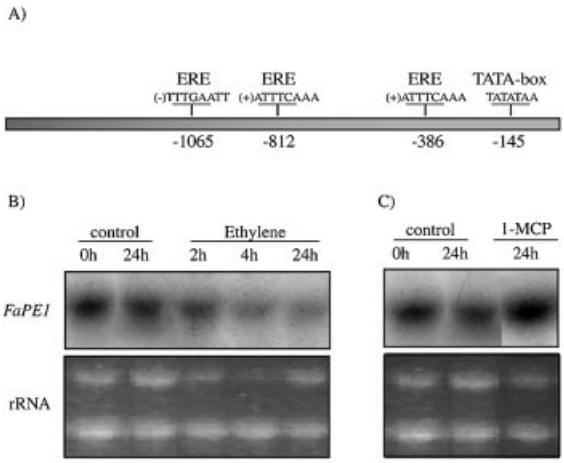


Fig. 5. (A) Schematic representation of the 1.6 Kb *FaPE1* promoter region. The putative TATA-box and the predicted ethylene responsive elements (EREs) have been highlighted. Numbering is relative to the first nucleotide of the translation initiation codon. (B) Northern blot analysis of *FaPE1* expression. Total RNA (15 µg) was extracted from ripe strawberry fruits that were treated with 50 µl l^{-1} ethylene for 24 h. Control fruits were kept in air. (C) *FaPE1* expression in ripe strawberry fruits that were treated for 24 h with 600 µl l^{-1} of 1-MCP. Control fruits were the same as in (B).

showed a decrease in *FaPE1* mRNA accumulation compared with the control air-treated fruits (Fig. 5B). F7 fruits were also treated with the inhibitor of ethylene action 1-methyl cyclopropene (1-MCP). After 24 h of treatment, *FaPE1* mRNA levels were higher in 1-MCP treated fruits (Fig. 5C), thus strongly supporting the fact of a negative regulation of *FaPE1* expression exerted by the hormone ethylene.

FaPE1 *expression in senescing strawberry fruits*

FaPE1 expression was also analysed in overripe fruits. Firstly, several fruits were harvested at the ripe stage (F7) and left to overripen on the shelf for 24 or 48 h at 21 °C. A second set of fruits were maintained on the vine and harvested after 48 h and several days later when fruits showed clear symptoms of senescence i.e., a semi-melted texture. During senescence, fruits that were maintained on the plant showed a progressive reduction in *FaPE1* transcripts, being undetectable at the overripe stage (Fig. 6). Similarly, in detached fruits maintained at room temperature *FaPE1* mRNA levels also decreased. However, the kinetics of the process was faster and *FaPE1* transcripts were hardly detected after 24 h (Fig. 6).

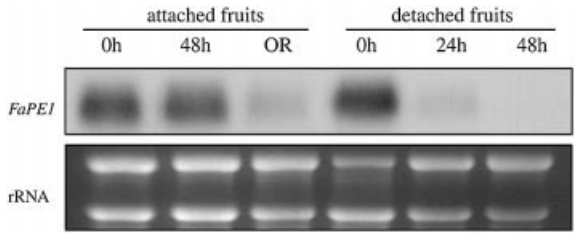


Fig. 6. Northern blot analysis of *FaPE1* expression during senescence of strawberry fruit whilst either detached or attached to their parent plant. Attached fruit were allowed to senesce until overripe (OR), whilst detached fruit were allowed to ripen for 48 h.

Discussion

Changes in the pectin matrix are regarded as an important factor that affects cell wall structure during ripening and senescence. In this context, PE activity is a key control point for both the assembly and disassembly of pectin networks. Homogalacturonic acid (HGA) is an abundant and widespread component of the polysaccharide complex, commonly termed 'pectin', that appears to be synthesized in the Golgi apparatus in a highly methyl-esterified state (Doong *et al*., 1995). Subsequent to its incorporation into the extracellular matrix, HGA often become enzymatically de-esterified by the action of the enzyme PE. De-esterification of HGA increases the negative charge density in the cell wall microenvironment. Therefore HGA can be cross-linked by divalent cations, such as calcium, resulting in supramolecular assemblies and gels. The formation of these calcium-mediated pectin gels significantly affects the mechanical properties of the cell wall and adds rigidity to the wall (Jarvis, 1984; Willats *et al*., 2001). However, the degree and pattern of methyl esterification is also important in regulating the cleavage of HGA by pectinolytic enzymes, such as polygalacturonase and pectate lyase, and may influence the activities of other wall-associated enzymes by altering the pH of the local environment (Pressey and Avants, 1982; Almeida and Huber, 1999).

In this paper, the existence of four strawberry *PE*-related genes is reported, three of them expressed during fruit development at a level that could be detected by northern blot hybridization. The occurrence of multiple isoforms of PE in strawberry fruit was expected as several PE isoforms have previously been described in different fruits such as tomato, peach, and banana (Hall *et al*., 1994; Glover and Brady, 1994; Nguyen *et al*., 2002*b*), and also in a strawberry fruit EST collection (Aharoni and O'Connell, 2002). It is possible that the different genes may encode proteins with slightly different functions in the cell wall or temporally associated with a particular cell type, tissue or organ through plant development. Each *FaPE* gene showed a unique expression profile and it was possible to classify them into functionally distinct groups as determined for other PE gene families (Micheli *et al*., 1998; Gaffe *et al*., 1994). The first *FaPE* group comprises genes showing an organ-specific expression, at least at a level detectable by northern blot; this includes *FaPE1* in ripening fruit and *FaPE2* in leaves. Ripening stages where *FaPE1* expression were detected coincided with the PE activity profile described by Barnes and Patchett

(1976), starting from small green berries up to the overripe berries. Maximum activity was found around ripening time and, like *FaPE1* mRNA, the activity decreased at the overripe (senescent) stage, suggesting that FaPE1 activity highly contributes to the overall PE activity during fruit development. *FaPE3* and *FaPE4* belong to the second group, which includes those *FaPE* genes that are ubiquitously expressed throughout the plant. Thus, *FaPE3* is most identical (82%) to PMEU1 (AY046596), another ubiquitous PE from tomato (Gaffe *et al*., 1997). The expression pattern of this last group of PE genes supports the existence of 'house-keeping' PEs involved in maintaining cell wall integrity throughout plant development (Micheli *et al*., 1998). In strawberry, up to four PE-like ESTs have been reported whose expression was followed by DNA microarrays (Aharoni and O'Connell, 2002). They were receptacle-specific and transcript level varied along fruit ripening stages. Sequence information is available only on two of them (CB934832, CB934833) and did not show similarity to any of the strawberry cDNAs here reported.

Southern blot analysis of genomic DNA from *F.*× *ananassa,* an octoploid species, and that from the diploid species *F. vesca* was conducted. In the genome of the diploid species each *FaPE* represents a single-copy gene. It can be observed that all the bands in *F. vesca* seem to be a subset of the bands detected in *F.*×*ananassa*. This fact agrees well with the genetic origin of *F.*×*ananassa*. It is known that a diploid set of the eight genomes that comprise the species *F.*×*ananassa* is homologous to the modern diploid species which includes *F. vesca* (Senanayake and Bringhurst, 1967). Thus, the elevated allele polymorphism observed in *F.*×*ananassa* may reflect divergence among the *F.*×*ananassa* progenitors. The parallel band patterns using the *FaPE3* and *FaPE4* probes suggest that these genes may be in a tandem array. In tomato, PE genes have also been reported to be present in the genome as a tandem repeat (Hall *et al*., 1994).

The control of ripening in non-climacteric species, such as strawberry, is poorly understood. In strawberry it has been shown that auxin delays ripening by altering the expression of many ripening-associated genes (Given *et al*., 1988). Most of the strawberry ripening-related genes are negatively regulated by auxin, although a few auxin up-regulated genes have also been described (Manning 1998; Aharoni *et al*., 2002). This last report (Aharoni *et al*., 2002) included a pectin esterase-like EST (H163, CB934833) whose short sequence did not show similarity to *FaPE1*. It is known that free and conjugated indole-3-acetic acid (IAA) reaches a peak at the green fruit stage of fruit development and subsequently declines (Archbold and Dennis, 1984). Removal of the endogenous auxin source in halved green fruit resulted in a reduced *FaPE1* transcript accumulation, whilst exogenous auxin application caused an enhancement of *FaPE1* expression.

Strawberry is considered a non-climacteric fruit, however, a small increase in ethylene production has been reported at late ripening stages (Perkins-Veazie *et al*., 1996; Iannetta *et al*., 2000). An *in vivo* role for ethylene is supported by the finding that exogenous application of ethylene accelerates strawberry fruit senescence and causes a 50% decrease in fruit firmness (El-Kazzaz *et al*., 1983). Recently, Jiang *et al*. (2001) also demonstrated that 1-MCP treatment of ripe strawberry fruits tended to maintain fruit firmness and lowered increases in anthocyanin and phenolic contents. The observed changes in fruit firmness could be influenced by changes in FaPE1 activity. The promoter region of the *FaPE1* gene has been shown to contain three putative EREs. This element has previously been found in ethylene-responsive promoters such the carnation *GST* and the tomato *E4* gene promoters (Montgomery *et al*., 1993; Itzhaki *et al*., 1994). The responsiveness of the *FaPE1* promoter region to ethylene was proven, as *FaPE1* mRNA levels decrease after ethylene treatment in ripe fruits. Inhibition of ethylene perception with 1-MCP resulted in an increase of *FaPE1* transcripts, also supporting a role for ethylene in regulating *FaPE1* expression. Although auxin is known to regulate the expression of many ripening-related strawberry genes, as far as the authors are aware this is the first time that an ethylene-responsive gene is characterized in a non-climacteric fruit such as strawberry. Previously, some other ethylene-responsive genes have also been reported in non-climacteric fruits (Alonso and Granell, 1995; Cazzonelli *et al*., 1998). However, the effect of this hormone has not been described for any strawberry gene. In particular, both an expansin (*FaExp2*) and a cellulase (*FaCel1*) appeared to be ethylene-insensitive (Civello *et al*., 1999).

It is possible that the repression of *FaPE1* mRNA expression at the late stages of fruit ripening and, in particular during senescence, could be an important factor determining the post-harvest life of the strawberry fruit. In strawberry (Barnes and Patchett, 1976), as in many other fruits such as peach (Glover and Brady, 1994), banana (Kanellis *et al*., 1989), and melon (Fils-Lycaon and Buret, 1991), a decrease in total PE activity has been observed at the end of the ripening process, overlapping with senescence. In tomato fruit, the role of pectin esterase in fruit softening has been studied by constitutive expression of a fruit-specific PE antisense gene (Tieman *et al*., 1992). Normal ripening occurred in the transgenic plants with reduced PE activity, but there was a significant decrease in tissue integrity during fruit senescence. Cell walls from these fruits with a lower degree of methyl esterification showed an altered cation-binding capacity as a consequence of the lower frequency of anionic charges on fruit pectins (Tieman and Handa, 1994). The role of the tomato fruit-specific PE isoform that affects tissue integrity during senescence (Brummell and Harpster, 2001) could be

common to FaPE1. During strawberry fruit senescence, the inhibition of *FaPE1* following the endogenous ethylene burst would increase the degree of methyl esterification of pectic polysaccharides and thus reduce pectin stabilization mediated by calcium cross-links. The inability to form calcium cross-bridges would lead to cell separation and have a negative effect on fruit integrity. In agreement with this proposal, the calcium content of many fruits has been related to the quality of fruit in storage. In the case of strawberries it has been proven that dips in calcium delay fruit post-harvest decay and maintain its firmness (García *et al*., 1996).

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