Plant Molecular Biology **55:** 763–780, 2004.

A strawberry fruit-specific and ripening-related gene codes for a HyPRP protein involved in polyphenol anchoring

Rosario Blanco-Portales[1], Juan A. López-Raéz[1], Mari Luz Bellido[1], Enriqueta Moyano[1], Gabriel Dorado[1], José A González-Reyes[2], Jose L. Caballero[1] and Juan Muñoz-Blanco[1,*]
[1]*Dpto. de Bioquímica y Biología Molecular e Instituto Andaluz de Biotecnología Edificio Severo Ochoa. Universidad de Córdoba, Campus Universitario de Rabanales, 14071 Córdoba, Spain (*author for correspondence; e-mail bb1@uco.es);* [2]*Dpto. de Biología Celular, Fisiología e Inmunología, Edificio Severo Ochoa. Universidad de Córdoba, Campus Universitario de Rabanales, 14071 Córdoba, Spain*

Received 26 March 2004; accepted in revised form 8 August 2004

Key words: auxins, condensed tannins, HyPRPs protein, polyphenols, ripening genes, strawberry

Abstract

A strawberry (*Fragaria x ananassa* cv. Chandler) fruit cDNA (*Fahyprp*-cDNA) and its corresponding gene (*Fahyprp*) showing sequence homology to higher plant *hyprp* genes have been isolated. The cDNA contains an open reading frame encoding a 16 kDa protein with 156 amino acids. The peptide has an amino-terminal signal sequence, a repetitive proline-rich sequence, and a cysteine-rich carboxy-terminal region homologous to other HyPRP proteins. Northern blot and QRT-PCR analysis have shown that the strawberry transcript is specifically expressed in fruit, not being detected in other plant tissues. "*In situ*" hybridization and immunolocalization studies have indicated that the *Fahyprp* gene is strongly expressed in achene sclerenchyma cells, in the vascular and receptacle cells of immature green fruit and in the vascular cells of mature red fruits. The achenes removal from unripe green fruits induced the expression of this *Fahyprp* gene. This induction was reverted by treatment of deachened fruit with the auxin NAA, supporting the idea that *Fahyprp* gene expression is regulated by auxins. Furthermore, the HyPRP protein has been localized in parenchymatic cells of immature fruits associated to structures containing condensed tannins. The results are discussed supporting a putative role of this protein in the anchoring of polymeric polyphenols in the strawberry fruit during growth and ripening.

Abbreviations: CAD, cinnamyl alcohol deshydrogenase; Ct, threshold cycle: DMACA, *p*-dimethylamino-cinnamaldehyde; FaHyprp, strawberry hybrid proline-rich protein; GRPs, glycine rich proteins; HyPRPs, hybrid proline-rich proteins; KLM, keyhole limpet hemocyanine; NAA, 1-naphthaleneacetic acid; pfu, plaque formation units; pI, isoelectric point; PRPs, proline-rich proteins; QRT-PCR, quantitative real time PCR; RER, rough endoplasme reticulum; RT-PCR, retrotranscriptase polymerase chain reaction

Introduction

Different classes of proteins containing abundant proline residues have been described in plants. They include extensins, proline-rich proteins (PRPs) and glycine-rich proteins (GRPs). All these are structural cell wall proteins with signal peptide which permits their sortening into the rough endoplasmic reticulum (RER), other cellular organelles or secretion out of the cell. The signal peptide is followed by a proline-rich domain that presents different repetitive motives (Josè and

The nucleotide sequence data reported corresponds to the accession number AF 026382 (cDNA) and AY530533 (genomic DNA).

Puigdomènech, 1994). A second family of proteins containing a second hydrophobic carboxy-terminal domain in addition to the amino-terminal proline-rich domain has also been described (Josè and Puigdomènech, 1994). Such domain does not contain repetitive elements but presents a series of invariable cysteine residues in a conserved distribution pattern. These proteins have been named either hybrid proline-rich proteins (HyPRPs) or hybrid glycine-rich proteins (HyGRPs) (Josè and Puigdomènech, 1994).

The HyPRPs proteins may have different repetition patterns in their proline-rich domain. However, when the second hydrophobic domain is analyzed, the HyPRPs can be subdivided in two different groups (A and B), according to the cysteine residue positions in the peptidic sequence (Josè and Puigdomenéch, 1994). Proteins belonging to group A present a signal peptide and 4–6 cysteine residues in their hydrophobic domain with the conserved distribution pattern ...CXXC... C...C...C...C... On the other hand, proteins belonging to group B present an optional signal peptide and a hydrophobic carboxy-terminal domain containing eight cysteine residues distributed according to the pattern ...C...C...CC...CXC ...C...C... (Josè and Puigdomènech, 1994). This hydrophobic domain is well conserved among the group B proteins mainly around the positions of the cysteine residues (Josè and Puigdomènech, 1994).

It has been observed that the expression pattern of plant genes encoding HyPRPs is quite diverse. For example, in rice it has been shown that the expression of a gene which encodes a HyPRP occurs in the root cap and the elongation zone of the root, being restricted to vascular and cortical tissues (Xu *et al*., 1995). However, in leaves, the expression occurs mainly in vascular and mesophyll cells (Xu *et al*., 1995). On the other hand, in maize it has been observed that the expression of a gene that encodes for HyPRP is restricted to developing cortical cells. Accordingly, a role in transporting molecules from or towards the vascular tissues has been proposed for this protein (John *et al*., 1992).

In bean, a gene encoding a HyPRP is strongly expressed in the root apex, showing decreasing expression levels from the root apex towards the upper parts of the root (Choi *et al*., 1996). "*In situ*" hybridization experiments have determined that the maximum expression levels for this gene take place in the cortical ground meristem, specifically in those zones where the maximum cellular division is being carried out. However, expression of this gene was not detected in the root cap, procambium, vascular cylinder, epidermis or lateral roots. Also, low expression levels were observed in the apical meristem. Thus, it has also been suggested that the protein encoded by this gene could be involved in the cortical cell differentiation in bean (Choi *et al*., 1996). On the other hand, a gene encoding a HyPRP protein has been shown to be expressed in alfalfa plants acclimated to cold and in response to physical damage. Furthermore, the intensity of the expression correlated to the degree of the damage (Castonguay *et al*., 1994). However, the expression of this gene was not increased under hydric or thermal shock (Castonguay *et al*., 1994). Also, a root salt-inducible alfalfa gene encoding a putative HyPRP has been described (Deutch and Winicov, 1995). It was proposed that this protein was involved in retarding dehydratation of the plant roots during salt stress. Alternatively, a putative structural role as wall-to-membrane linkers was also suggested (Deutch and Winicov, 1995). Additionally, a tobacco 14 kDa tumor induced HyPRP protein has been reported (Fujita *et al*., 1994). In spite of all these findings, there are no reports of any purified protein containing the domains described in these HyPRPs. Furthermore, the possible biological functions of these putative proteins remain elusive.

In the present paper, we report the isolation and characterization of a strawberry gene encoding a protein which presents clear identity and similarity with the HyPRP class B proteins of higher plants. We demonstrate that the expression of the corresponding gene is fruit specific and varies along the fruit ripening process being probably negatively regulated by the auxins synthesized by the achenes. The gene expression analysis, as well as the "*in situ*" hybridization, immunolocalization and histochemical experiments suggest that the strawberry *Fahyprp* gene product is likely related to the anchoring of polyphenols (lignin and condensed tannins) to cell membranes. To our knowledge, this is the first exhaustive report on the correlation of molecular biology and protein experiments that makes

feasible to propose a physiological role for Fa-HyPRP in strawberry fruits.

Materials and methods

Plant material

The strawberry fruit (*Fragaria* x *ananassa* c.v. Chandler, an octaploid cultivar) was harvested at different developmental stages: small-sized green fruits (G1), middle-sized green fruits (G2), full-sized green fruits (G3), white fruits with green achenes (W1), white fruits with red achenes (W2), turning stage fruits (T), and full-ripe red fruits (R).

Media, bacterial strains and bacteriophages

Unless otherwise indicated, all the bacterial strains, phages, media and growing conditions used in this work were those recommended in the manufacturer's protocols as indicated below.

Auxin treatments

Achenes of two sets of the G2-stage strawberry fruits were carefully removed in the growing plant using the tip of a scalpel blade. One set of deachened fruits was treated with the synthetic auxin 1-naphthaleneacetic acid (NAA) as a lanoline paste with 1 mM NAA in DMSO 1% (v/v). The other set of deachened fruits (reference group) was treated with the same paste but without NAA. Fruit samples were harvested at 0, 24, 48, 72 and 96 h after treatment and immediately frozen in liquid nitrogen and stored at −80 °C until used. As control, normal G2 untouched (with achenes) fruits were used.

Oxidative stress treatments

For the oxidative stress experiments, white stage fruits (attached to the plants) were carefully injected using a hypodermic syringe with a sterile needle, with 1 ml of a sterile solution of 0.3 mM plumbagine, 1 mM menadione or 0.5 mM peroxide hydrogen. Control fruits were injected with 1 ml of sterile water. Afterwards, fruits were harvested at 2, 4, 8 and 24 h and immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

Strawberry Fahyprp gene cloning

The cloning and isolation of the full length *Fahyprp*-cDNA from strawberry was performed by differential screening of a cDNA subtractive library according to Medina-Escobar *et al.* (1997).

The isolation of the strawberry *Fahyprp* genomic clones were performed by plaque hybridization screening of about 1.5×10^5 pfu of a strawberry genomic library (*Fragaria x ananassa* c.v. Chandler) in the phage λ-FixII (from Stratagene, La Jolla), using the ^{32}P-labelled *Fahyprp*-cDNA previously isolated from strawberry, as a probe. Filters were prehybridized and further hybridized at 65 °C in hybridization solution: 5 × SSC, 5 × Denhard't, 200 μg/ml salmon sperm and 0.5% SDS. After hybridization, the filters were washed twice, 15 min each, at room temperature in a 0.2 × SSC, 0.5% SDS solution. Afterwards, the filters were washed for 15 min at 65 °C, in a 0.2 × SSC, 0.1% SDS solution. Four positive clones ranging between 12 and 17 kb in size were isolated and their inserts were analyzed by restriction mapping. Then, suitable clones for DNA sequencing were obtained by subcloning into the *pBluescript* vector (Stratagene) and their DNA inserts were completely sequenced on both strands.

RNA isolation and gene expression analysis

Total RNA from a pool of six to seven strawberry fruits at different ripening stages and from roots, leaves, flowers and runners was isolated according to Manning (1991).

To investigate the differential expression of the strawberry *Fahyprp* gene during strawberry ripening process, both Northern blot and Quantitative Real Time (QRT-PCR) analysis were carried out.

Northern blot analysis

The insert from the *Fahyprp*-cDNA clone was used as template for generating a radioactive probe. A cDNA corresponding to the 18S ribosomal RNA was used to control equal loading of RNA samples. The probes were labelled to a specific activity of approximately 10^8 cpm/μg using a commercial random priming kit (Amersham Biosciences).

Twenty micrograms of total RNA per sample were routinely used for the Northern analysis. The

Hybond N^+ filters (Amersham Biosciences) were prehybridized at 65 °C for 1 h, in 15 ml of hybridization solution (0.25 M NaH_2PO_4, 7% SDS and 0.1 mM Na_2EDTA). Denatured probes were added to the same hybridization solution and hybridization was carried out at 65 °C for 14–16 h. Filters were washed (twice) at 65 °C, for 15 min in 100 ml of 0.2 × SSC, 0.1% SDS, and then exposed to X-ray film, at −70 °C for 24–48 h.

RT-PCR analysis

Studies related to determine the expression of the *Fahyprp* gene in vegetative tissues were carried out by RT-PCR. Briefly, two specific primers corresponding to the untranslated 3′ end of the *Fahyprp*-cDNA sequences (5′-CCAGGTCCCA AGAGGGTTCCAATGTTCC-3′ and 5′-CCAC TGCATAATTCAGGCAAACCCACC-3′) were used. Total RNA was extracted from vegetative tissues leaves, and 1.0 μg of total RNA was DNase I treated (Amersham Biosciences) and the resulting RNA was retrotranscribed using Superscript II (Invitrogen) reverse retrotranscriptase and the antisense primer, using the following conditions: 42 °C 5 min, 50 °C 50 min, 70 °C 15 min. Afterwards, the resulting single stranded cDNA was PCR amplified with the following conditions: denaturation at 94 °C for 2 min; 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. A 72 °C for 5 min final step of extension was also added. The RT-PCR mixtures were the ones recommended by the manufacturer (Invitrogen).

QRT-PCR analysis

The Quantitative Real Time-PCR (QRT-PCR) studies of gene expression were carried out as previously described for others strawberry fruit ripening-related genes (Blanco-Portales *et al*., 2002; Benítez-Burraco *et al*., 2003).

In QRT-PCR analysis, quantification is based on Ct (threshold cycle) values. The Ct is a mesurement taken during the exponential phase of amplification when limiting reagents and small differences in starting amount have not yet influenced the PCR efficiency. The Ct is defined as the cycle at which fluorescence is first detectable above background and it is inversely proportional to the logarithm (log) of the initial copy number. In our system, each 10-fold difference in initial copy number produced a 3.2 cycle difference in Ct. Each reaction was done by triplicate and the corresponding Ct values were determined. The Ct values of each QRT-PCR reaction were normalized using the Ct value corresponding to a strawberry housekeeping gene. Moreover, the efficiency of each QRT-PCR was also calculated. All these values were utilized to determine the increases in gene expression according to the following expression:

$$\text{Fold change} = 2^{-\Delta(\Delta C_t)},$$

$$\Delta C_t = C_t(\text{'target'gene}) - C_t(\text{'housekeeping'gene}).$$

$$\Delta(\Delta C_t) = \Delta C_t(\text{condition 1}) - \Delta C_t(\text{condition 2}).$$

DNA extraction and Southern blot analysis

Strawberry genomic DNA was extracted as follows: young leaves were kept in distilled water in the dark at 4 °C for 2 days. Afterwards, 2 g of leaves were ground under liquid nitrogen into a fine powder and gently resuspended in 25 ml of a warm (65 °C) buffer solution (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 50 mM EDTA, 1% (w/v) β-mercaptoethanol, 4% (w/v) SDS and 6% (w/v) polyvinylpolypyrrolidone (PVPP). The β-mercaptoethanol, SDS and PVPP were added fresh just before used. The mixture was incubated in an oven at 65 °C during 1 h with gentle rotation. Then, 8 ml of 3 M potassium acetate (pH 4.8) were added. The resulting mix was incubated in ice and then centrifuged at 10 000 rpm in a microfuge for 10 min. The supernatant was gently filtered through a double layer of Miracloth (EMD Biosciences). Two volumes of cold-freeze 100% (w/v) ethanol were added and DNA was extracted with a microcapillar, washed 2–3 times in fresh cold-freeze 70% ethanol and dried at room temperature.

Genomic DNA (2 μg) was digested with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, and *Hin*dIII, fractionated on 0.7% (w:v) agarose gels and then alkaline transferred to Hybond-N^+ membranes (Amersham Biosciences). The DNA was fixed by UV light using the Stratalinker (Stratagene) and the blot was hybridized using the *Fahyprp*-cDNA as radioactive probe (single stranded-PCR (α-^{32}P)-dCTP-labelled *Fahyprp*-cDNA with a specific activity of 10^8 cpm/mg). Hybridization and washing conditions were as in Northern blot experiments.

"In situ" *hybridization*

Probes were made from cDNA cloned in p*Bluescript* SK(-) using T7 or T3 promoters to generate sense or antisense RNA. The methods used for digoxigenin labelling of RNA probes, tissue preparation and "*in situ*" hybridization were as described by Jackson (1991) with the following modifications: in the treatment of tissues prior to hybridization, samples were incubated firstly with 3 μg/ml of proteinase K for 30 min, and then 5 min with 0.2% (w/v) glycine to block the protease.

Hybridization of the samples were performed, at 50 °C for 12–14 h, in hybridization solution, 50% (v/v) formamide, 6 × SSC, 3% SDS, 100 μg/ml tRNA, and 100 μg/ml Poly-A. Afterwards, the samples were washed with 2 × SSC and 50% formamide at 50 °C for 30 min, and twice with the same wash solution for 1 h 30 min. Then, the samples were rinsed twice for 5 min with NTE solution (10 mM Tris–HCl, pH 7.5, 500 mM NaCl and 1 mM Na_2-EDTA), at 37 °C and incubated at 37 °C for 30 min, in a prewarmed NTE solution containing RNase A (20 μg/ml). Samples were then rinsed twice for 5 min with NTE solution, and then washed (twice) for 20 min with 1 × SSC and again (once), for 1 h, with 0.1 × SSC at room temperature. The hybridized probes were detected using an alkaline phosphatase antibody conjugate. After the final color development, slides were dehydrated through an ethanol series, dried, mounted with Entellan (EMD Biosciences) and viewed using brightfield microscope.

Antibody production

To obtain antibodies against FaHyPRP proteins, an antigenic peptide deduced from the putative protein sequence was deduced and synthesized (CPRPKPKPRPRPRPTPSPGTPS). The peptide was individually conjugated to a keyhole limpet hemocyanin (KLH) carrier protein by the m-maleimidobencil-N-hidroxisuccimida (MBS) ester (from Pierce Biotechnology).

Removal of cross-reactive anti-carrier KLH antibodies

The presence of anti-KLH antibodies in serum may cause non-specific background in immunohistochemical staining of tissues of some plant species (Porankiewicz *et al*., 2000). The problem was avoided by removing anti-KLH antibodies from the immune serum. For this purpose, total immune serum was applied to an affinity column coupled with KLH. The column was eluted according to Porankiewicz *et al*. (2000). Afterwards, the purified anti-FaHyPRP antibodies were directly used in the immunolocalization experiments.

Immunolocalization of the strawberry HyPRP protein

For morphological and immunohistochemical analysis, samples of fruit receptacles and achenes at different stages of the ripening were processed as described below.

Light microscopy

For the cytolocalization of the FaHyPRP polypeptide, tissue sections were prepared as follows: small portions of strawberry achenes and receptacle tissue were fixed in formaldehyde 4%, dehydrated through an ethanol–tertiary butanol series, and embedded in "Paraplast Plus" (from Sherwood Medical Co.). Sections of about 5 μm were cut with a microtome, mounted on slides covered with gelatin, deparaffinized in xylene and rehydrated through an ethanol series. For immunolocalization purposes, sections were blocked with 2% (w/v) non-fat dried milk in TBS and immunological detection was then performed using a primary anti-strawberry FaHyPRP polyclonal antiserum diluted 1/10, and a secondary anti-rabbit alkaline phosphatase-conjugated antibody (Sigma) diluted 1/250. The reaction of alkaline phosphatase was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3 indolyl-phosphate (BCIP) for 30 min. The sections were dehydrated through graded ethanol series, cleared in xylene and mounted in "Entellan New" (EMD Biosciences). An Olympus AH-2 photomicroscope was utilized for sample visualization and photography.

For structural and histochemical analysis some sections were stained with 1% Bismarck Brown Y and then dehydrated and mounted. Additionally, some sections were stained with *p*-dimethylaminocinnamaldehyde (DMACA). These techniques have been reported to stain tannins, and

condensed tannins respectively (Schneider, 1981; Gutmann and Feucht, 1991; Abrahamns *et al.*, 2002).

Lignified structures were visualized using the phloroglucinol/HCl test (Weisner reagent). Sections of strawberry fruits were incubated in a solution of 1% (v/v) phloroglucinol in 70% ethanol until they were totally cleared. Then, a few drops of concentrated HCl were added and covered with a coverslip. Lignified structures appeared pink–red about 5 min later. Micrographs were taken immediately, since the color faded in about 30 min (Clifford, 1974).

Electron microscopy

For electron microscopy, samples were fixed in a mixture of 0.5% (v/v) glutaraldehyde and 2% (v/v) p-formaldehyde in 100 mM cacodylate buffer (pH 6.8) for 2 h. Then, samples were washed in the same buffer and postfixed in aqueous 1% (v/v) osmium tetroxide for 1 h at room temperature. Several samples were processed without osmium postfixation. After washing in buffer, samples were dehydrated in an ethanol series and embedded in LR-White using acetone as vehicle. Blocks were obtained by polymerization at 65 °C for 24 h. Silver sections were obtained in a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems) and mounted using nickel grids. In some cases, sections were stained with 4% (v/v) aqueous uranyl acetate and Reynold's lead citrate while in other samples were viewed and photographed without staining. Observations were carried out in a Philips EM 300 electron microscope. Semithick sections (about 0.5–1 μm thickness) were also

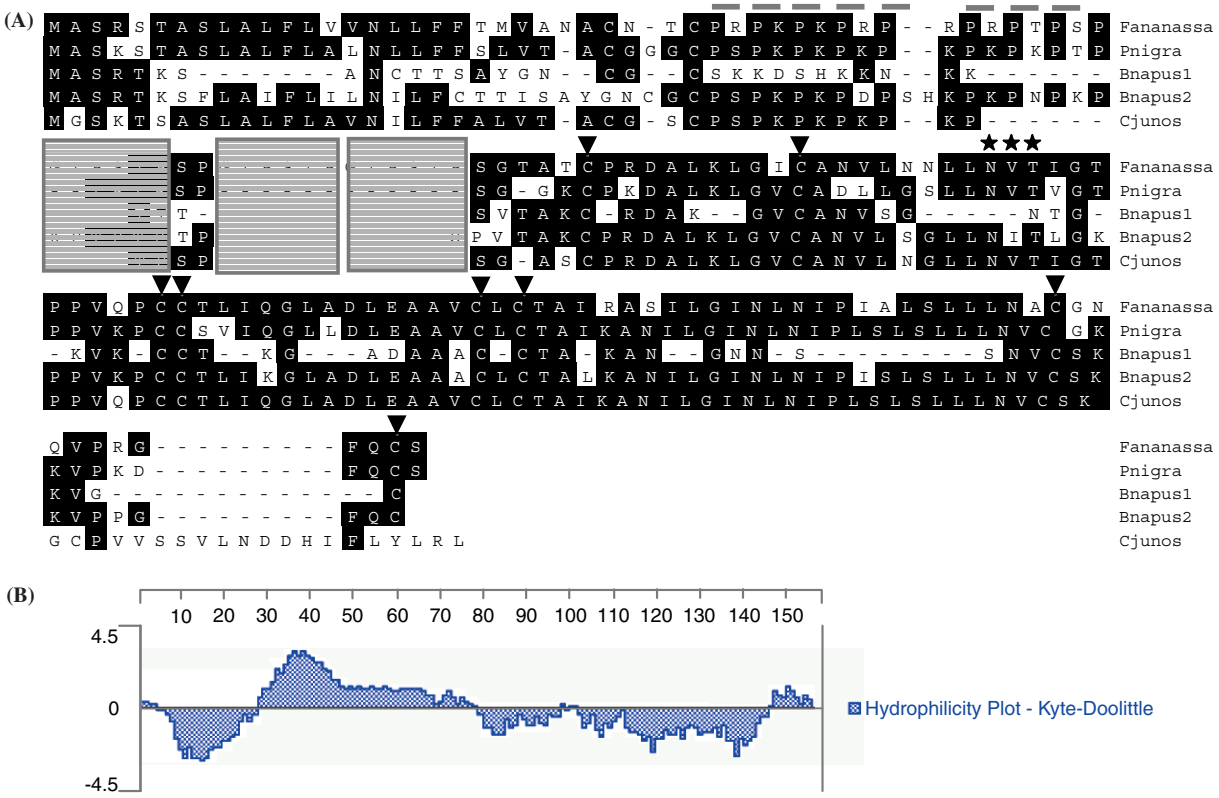

Figure 1. (A) PrettyBox comparisons of the putative strawberry FaHyPRP protein vs. other higher plants HyPRPs. Consensus amino acids are in black. Arrowheads show the positions of the highly conserved cysteine residues in all HyPRPs. Upper bars indicate the proline repeats on the hydrophilic domain of the HyPRP. Specific repetitive motifs (GTPSTP) are included in boxes. The stars indicate the predicted glycosilation site. The accesion numbers for the sequences used in the alignment are: AF026382 for *Fragaria x ananassa*; D83226 for *Populus nigra*; X71618 for *B. napus* (B.napus1); S68113 for *SAC51 gene* of *B. napus* (B.napus2) and AY00448 for *Citrus junos*. The alignment program used was *MegAlign* (LaserGene). (B) Hydrophilicity plot of the deduced amino acid FaHyPRP sequence.

obtained from this material and then stained with 1% toluidine blue in 1% borax solution or with 1% Bismarck Brown Y solution as previously explained. Then, sections were dehydrated and mounted in Entellan New.

Results

Sequence comparison and structural features of the Fahyprp *strawberry gene and protein*

By differential screening of a strawberry subtractive cDNA library, a *Fahyprp*-cDNA clone has been isolated. The comparison of the sequence with other sequences present in the nucleotide and protein data bases (GenBank, EMBL, PIR and SWISSPROT) revealed a significant homology of the strawberry sequence to other genes encoding HyPRP proteins of higher plants (Figure 1A). The identity values, at the amino acid level, varied between 64.8% and 39.2%. Furthermore, when the sequence comparisons were restricted to the carboxy-terminal hydrophobic domain, the overall identity raised and oscillated between 78.3% and 51.2% at the amino acid level. These results support that the isolated cDNA corresponds to a mRNA encoding a putative strawberry HyPRP protein. The *Fahyprp*-cDNA is 798 bp long what agrees with the mRNA size estimated by Northern blot. The cDNA carries an open reading frame encoding a small peptide of 156 amino acids. The *Fahyprp*-cDNA contains a 3′-untranslated region of 229 bp including the stop codon and 16 residues of the poly(A^+) tail. The stop codon (*TAG*) also matches the preferred one in plants (Gallie, 1993). Two putative polyadenylation sites AATAAG are found in the 3′-UTR sequence. These sequences are identical to the polyadenilation sequence found in the *hyprp* genes of alfalfa and maize (John *et al*., 1992; Castonguay *et al*., 1994). However, the polyadenylation signal is AATAAA in the *hyprp* genes of bean and maize (Jóse-Estanyol *et al*., 1992; Choi *et al*., 1996). Such sequence is not present in the 3′ UTR sequence of the strawberry *Fahyprp*-cDNA sequence.

The cDNA clone was used as a probe to isolate the corresponding genomic clone. Comparison of the full-length cDNA sequence with the corresponding genomic sequence of the *Fahyprp* gene showed the absence of introns in the strawberry gene (data not shown). Such genomic structure is very similar to those previously described in two of the *hyprp* genes cloned so far (Josè-Estanyol *et al*., 1992; Salts *et al*., 1992).

The deduced translation product includes 156 amino acids with a predicted molecular mass of 16 kDa. These values of the strawberry protein are close to those described for the HyPRPs proteins of alfalfa, bean, rice, soybean, corn, *Brassica napus* L. and *Catharantus roseus* (John *et al*., 1992; Castonguay *et al*., 1994; Hotze *et al*., 1994; Choi *et al.,* 1996). The pI value for the deduced strawberry protein is 8.75, practically identical to that deduced for the HyPRP protein from alfalfa (Castonguay *et al*., 1994). Also, as other HyPRP proteins, the strawberry FaHyPRP protein is rich in proline (14.1%), leucine (12.8%), threonine (10.2%), alanine (9.6%) and serine (7.7%) residues. In addition, the predicted strawberry protein shows three clearly differentiated domains as occur in all other HyPRPs (Figure 1B). First domain corresponds to the 29 residues amino-terminal signal peptide. The length of this signal peptide is similar in size to the signal peptides found in other HyPRP proteins (John *et al*., 1992; Josè-Estanyol *et al*., 1992; Coupe *et al*., 1993; Castonguay *et al*., 1994; Deutch and Winicov, 1995; Xu *et al*., 1995). A putative processing site of the signal peptide is observed in the N-terminal domain between the amino acid residues 29 (Thr) and 30 (Cys). The signal peptide is followed by a central hydrophilic proline-rich domain with the amino acid sequences P–X and GTPSTP repeated eight times (amino acid residues 30–46) and three times (amino acid residues 47–63), respectively. In the C-terminus of the protein, there is a long hydrophobic domain containing the eight highly conserved cysteine residues (Figure 1A). This is a characteristic of the HyPRP proteins, and potentially may form up to four disulfide bridges. Similar to other HyPRPs of higher plants, this hydrophobic carboxy-terminal domain presents putative transmembrane helix regions: residues 76–96 and 104–143 (Hotze *et al*., 1994).

A potential glycosylation site (NVT) is found among the residues 92–94 (Figure 1A). This unique glycosylation site can also be observed in other HyPRPs, although in variable positions. Thus, in the case of the HyPRP of *B. napus* L. a similar putative glycosylation site (NIT) is located, as in the case of the strawberry FaHyPRP, in the

carboxy-terminal hydrophobic domain (Coupe *et al*., 1993). However, in the case of the HyPRP of maize, the putative glycosylation site (NCS) is found before the hydrophilic proline rich domain (John *et al*., 1992).

Putative regulatory elements in the 5′-flanking region of the strawberry Fahyprp *gene*

We have analyzed the 5′-flanking region of the strawberry *Fahyprp* gene, searching for *cis* regulatory sequences (Figure 2). Inspection of 832 bp upstream the putative transcription start site (+1) revealed the presence of several sequences matching *cis* regulatory elements described in other plant genes. Thus, a clear TATA box (TATATT), close to the consensus TATAAT box, is located at nt. −74. Furthermore, some putative abscisic acid (ABRE) *cis* elements (Busk and Pages, 1998) were located at positions -18 (sequence GTACGTG) and −787 (sequence ATTACGTGT) (Baker *et al*., 1994; Hattori *et al*., 1995). In addition, a motif that matches the consensus I-box sequence (GAGATGA) found in promoters of genes regulated by light (Yamaguchi-Hinozaki *et al*., 1994), was located at position -698 (sequence GAGATGA), on the reverse complementary strand. Also, two methyl jasmonate responsive element-like (Lee *et al*., 1997) were observed at positions -408 and –589.

Southern blot analysis

Southern blot analysis of genomic strawberry DNA digested with *BamH*I, *Bgl*II, *Eco*RI and *Hind*III generated DNA fragments higher than 1.6 kb in size (Figure 3). However, the cDNA sequence corresponding to the strawberry *Fahyprp* gene does not present restriction sites for such enzymes. This pattern suggests that the strawberry fruit *Fahyprp* gene belongs to a small multigene family as it has been shown for other higher plant *hyprp* genes (John *et al*., 1992; Coupe *et al*., 1993; Deutch and Winicov, 1995; Xu *et al*., 1995; Choi *et al*., 1996). Only, in the case of TPRP-F1, that encodes an HyPRP form tomato, a single copy gene has been reported (Salts *et al*., 1992). It should be noted that *Fragaria* x *ananassa* c.v. Chandler is an octoploid cultivar. Thus, the hybridization pattern observed could be due to the ploidy level of this strawberry cultivar.

Gene expression studies

The spatial and temporal expression pattern of the *Fahyprp* gene has been studied by Northern-blot, RT-PCR and QRT-PCR. A mRNA transcript of about 0.8 kb in size was observed throughout fruit development and ripening stages (Figure 4A). The gene was specifically expressed in fruit, and exhibited a different expression pattern during

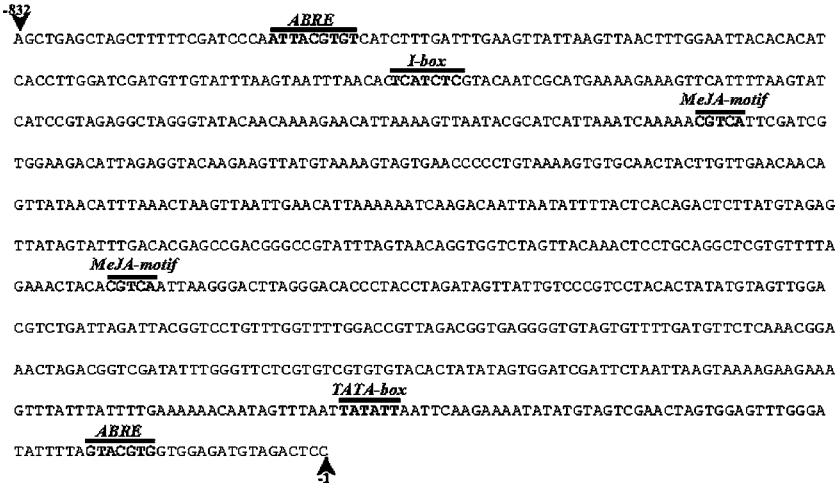

Figure 2. Nucleotide sequence of the promoter fragment corresponding to the *Fahyprp* gene. Upper bars indicate the putative *cis*-sequences found in the promoter fragment. ABRE is a putative abscisic acid *cis* element (Baker *et al*., 1994; Hattori *et al*., 1995), I-box is envolved in the regulation by light (Yamaguchi-Hinozaki *et al*., 1994), and putative MeJA-motif is linked to regulation by jasmonic acid pathway (Lee *et al*., 1997).

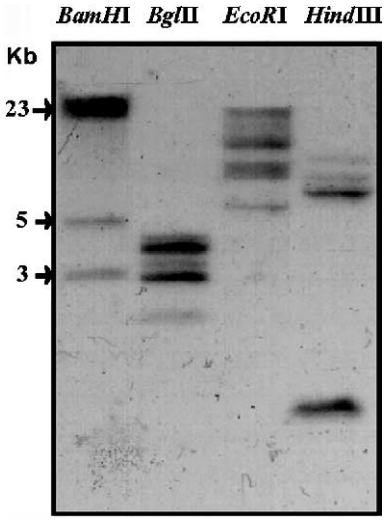

Figure 3. Southern blot of *Fragaria x ananassa* c.v. Chandler. Genomic DNA (2 μg) digested with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III and hybridized with a ^{32}P-labelled strawberry *Fahyprp*-cDNA probe. Hybridization and stringency conditions are described in Materials and methods (Southern blot analysis).

ripening process, with a clear decrease during fruit elongation followed by an increase during the ripening stages. The maximum expression level was found in the mature red fruit receptacle (R stage) (Figure 4A and B). No detectable expression level was found by Northern blot analysis or by RT-PCR experiments in roots, flowers, leaves or runners (Figure 4C). Moreover, a minor and constant expression level was observed in achenes along all the stages of fruit development and ripening (Figure 4B). Similar results were obtained both by Northern analysis and QRT-PCR technology and so corroborating that the later methodology represents a powerful and accurate approach to determine changes in gene expression as it has been previously demonstrated in strawberry fruits (Blanco-Portales *et al*., 2002, Benítez-Burraco *et al*., 2003).

The expression of some fruit-specific strawberry genes have been shown to be controlled by auxins (Moyano *et al*., 1998; Trainotti *et al*., 1999; Aharoni and O'Connell, 2002; Blanco-Portales *et al*., 2002; Benitez-Burraco *et al*., 2003). Thus, in order to determine whether the expression of the *Fahyprp* gene was also under this hormonal control, we performed gene expression analysis on de-achened green fruit (G2 stage). Thus, Northern

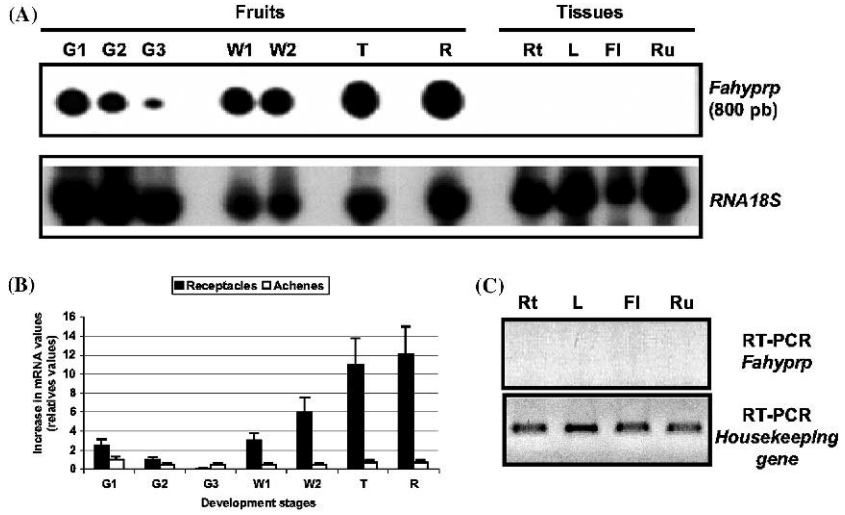

Figure 4. Developmental and spatial expression of the strawberry *Fahyprp* gene. Northern, RT-PCR and QRT-PCR analysis were performed as described in Material and methods. (A) Northern analysis of 20 μg of total RNA isolated from pooled samples of small-sized green fruits (G1), middle-sized green fruits (G2), full-sized green fruits (G3), white fruits with green achenes (W1), white fruits with red achenes (W2), turning stage fruits (T) and full-ripe red fruits (R) stages and from roots (RT), leaves (L), flowers (F) and runners (Ru). Size of hybridizing transcript is indicated in kb. The amount of RNA loaded on the gel was controlled by hybridization of the same blot with a ribosomal probe corresponding to an 18S gene. (B) Changes in gene expression, in both receptacle and achene tissues, at the different fruit developmental and ripening stages by QRT-PCR analysis. Real-time quantitation is based on C_t values as described in Materials and methods. The relative gene expression was determined using a strawberry fruit housekeeping gene as the endogenous control gene. Mean values ± SD of five independent experiments are shown. (C) RT-PCR studies of the *Fahyprp* gene in different vegetative studies. RT-PCR analysis was performed as described in Materials and methods.

blot and QRT-PCR analyses were performed with mRNA isolated from normal fruits (control fruits), deachened fruits and from deachened fruits treated with the auxin NAA. A clear increase in gene expression was detected in fruits after removing the achenes (Figure 5A and B). However, this increment in *Fahyprp* transcript level was partially reverted in G2 deachened fruits treated with NAA (Figure 5A and B), indicating that the expression of the *Fahyprp* gene is auxin regulated, as it has been reported previously for other strawberry fruit ripening-related genes (Moyano *et al*., 1998; Trainotti *et al*., 1999; Aharoni and O'Connell, 2002; Blanco-Portales *et al*., 2002; Benitez-Burraco *et al*., 2003).

Oxidative stress has been proposed as a triggering event for strawberry fruit ripening (Aharoni *et al*., 2002). In order to determine a possible relationship between *Fahyprp* gene expression and oxidative stress conditions, we carried out experiments with free radical generating compounds (hydrogen peroxide, menadione and plumbagine). As it is shown in Figure 6, the expression of the *Fahyprp* gene was clearly induced by oxidative stress as previously demonstrated for other strawberry ripening-related genes (Aharoni *et al*., 2002). These findings further support the possibility that the oxidative stress can play a relevant role in the strawberry fruit ripening process.

Localization of Fahyprp *mRNA and FaHyPRP protein in strawberry fruits*

The localization of *Fahyprp* mRNA by "*in situ*" hybridization fitted with the results obtained using antibodies, showing that the corresponding transcript was found mainly at the endocarp of achenes (Figure 7I). A control performed with the sense probe resulted in no detectable reaction at these tissues (Figure 7J).

The immunolocalization of the FaHyPRP polypeptide was carried out in those tissues and stages in which the maximum expression of this gene had been detected (G1 and R stages). In

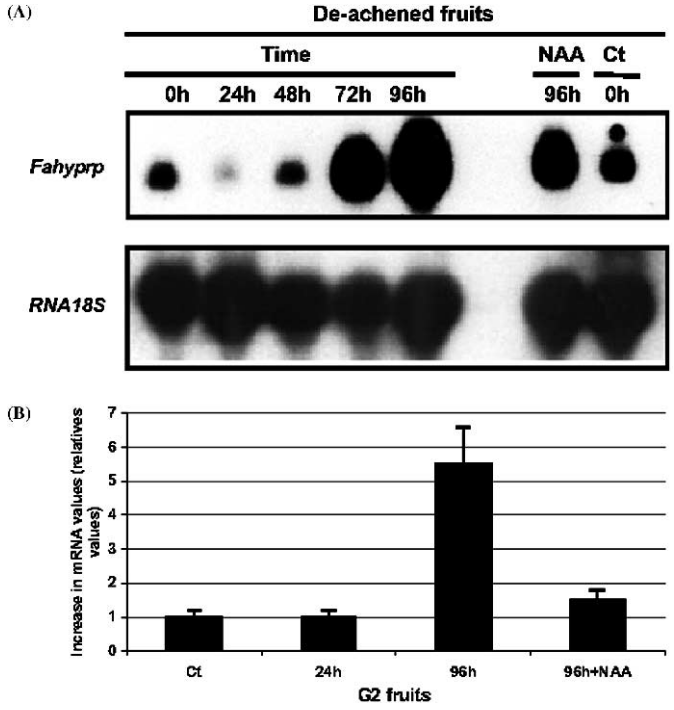

Figure 5. Effect of removing achenes and treatment with auxins on the *Fahyprp* gene expression. (A) Northern analysis of 20 μg of total RNA isolated from G2-stage strawberry fruit after removing the achenes at: 0, 24, 48, 72 and 96 h; (NAA) 96 h G2-stage strawberry de-achened fruit treated with NAA; (Ct) Control fruits, G2-stage strawberry fruit with achenes. Blot was hybridized as described in Material and Methods. The amount of RNA loaded on the gel was controlled by hybridization of the same blot with a ribosomal probe as in Figure 4. (B) Expression studies performed by QRT-PCR in de-achened, auxin treated and control fruits. As above described, quantification was carried out as described in Materials and methods.

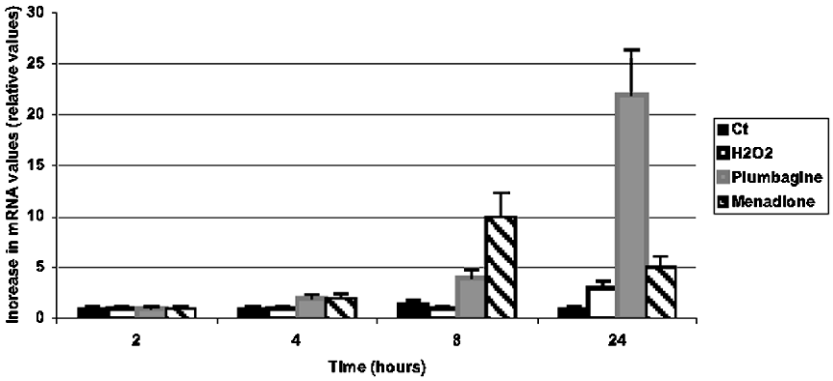

Figure 6. Changes in *Fahyprp* gene expression by oxidative stress in strawberry fruits after treatment with free radical generator compounds (hydrogen peroxide, plumbagine and menadione). Chemicals treatment and QRT-PCR analysis where done as indicated in Material and Methods. Mean values ± SD of five independent experiments are shown.

achenes, the labelling, was found associated to the testa and endocarp tissues (Figure 7C–F). In both cases the immunolocalization occurred inside the cells whereas cell walls were not stained with the anti-FaHyPRP antibody (Figure 7D–F). In the endocarp, the labeling was localized in the schlerenchymatic tissue (Figure 7F). Controls performed with preimmune sera at the different stages showed no appreciable reaction in the tissues (Figure 7G and H).

Interestingly, the immunolocalization of the FaHyPRP polypeptide at the testa was associated to some globular structures. A staining of semithick sections with the dye toluidine blue, highly stained these globular structures (Figure 7A). Such cell layer staining was also observed with Bismarck Brown Y, a stain that has been reported to be effective for tannins (Schneider, 1981). In this case, the cells showed dense brown-colored spots inside, while the cell walls remained basically unstained (Figure 7B).

Finally, a histochemical technique with phloroglucinol specific for lignin showed the lignification of the endocarp (Figure 7K and L) revealing a relationship between the lignification process and the presence of FaHyPRP polypeptide.

In receptacle at the G1 stage, the immunolocalization of the anti-FaHyPRP antibody was restricted to the cortical parenchymatic cells and some cells associated to vascular bundles. In most of the parenchymatic cortical cells the antibody was attached to the globular structures located inside of the vacuole associated to the tonoplast. Using a general staining procedure such as toluidine blue, we found dark spots coincident to those observed in immunolocalization staining and also to those found in achene testa cells (Figure 8A). Bismarck Brown Y and DMACA staining confirmed that the dense spots corresponded to tannins and condensed tannins (Figure 8B and C, respectively). In cortical cells, the immune reaction was found as dense deposits in several cell layers which resembled the tannins deposition (Figure 8D). In vascular bundles, the reaction was associated to tracheary xylematic elements and other cells associated to vascular bundles (Figure 8E). On the other hand, in receptacle at the R stage, the immunolabelling reaction was observed only in cells associated to vascular bundles, whereas no staining was detected at the parenchyma cells (results not shown). Preincubation of parenchyma and vascular tissues with preimmune sera yielded pictures with no detectable reaction (Figure 8G and H, respectively).

The localization of *Fahyprp* mRNA by "*in situ*" hybridization in receptacle, revealed a similar pattern to that obtained with the antibody. However, a weak reaction was found at the cytosolic peripheral region of the parenchymatic cells (Figure 8I) and associated to xylematic tracheary elements of the vascular bundless (Figure 8J and K). As expected, no reaction was detected when the sense probe was used as control (Figure 8L).

Finally, the dense deposits associated to vacuoles in achenes and receptacle were studied. Histochemical staining had pointed out the possibility

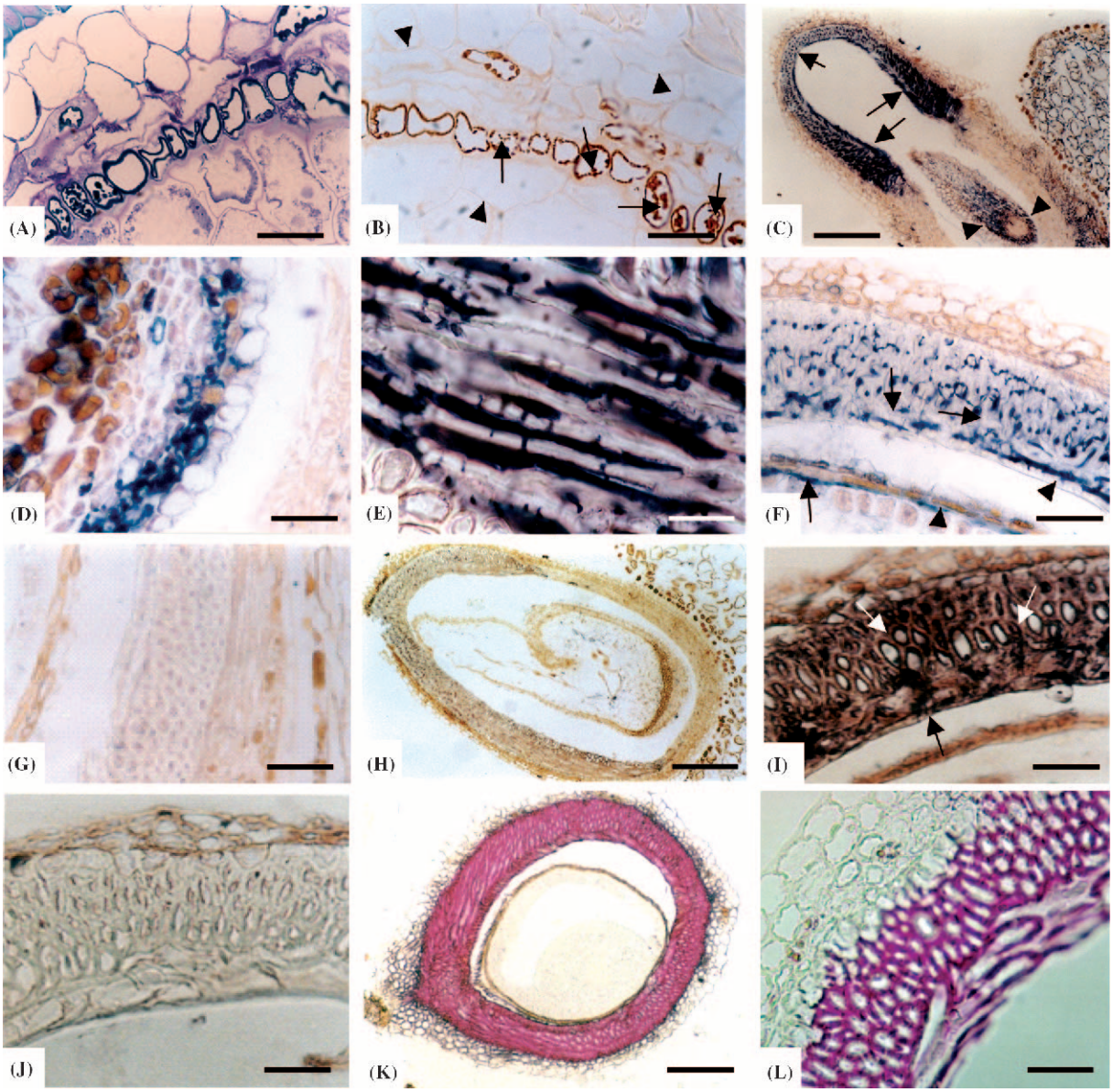

Figure 7. Histochemical analysis, "*in situ*" hybridization and immunohistochemical analysis of strawberry achenes from G1 to R stages of fruit ripening. (A) is a semithick section stained with toluidine blue showing an overview of the testa tissue. (B) is a semithick section stained with Bismarck Brown Y revealing dense brown spots identified as tannins (arrows). Cell walls were very lightly stained with this technique (arrowheads). (C-G) are sections from different stages of the achenes stained with the FaHyPRP antibody. (C) is an overview of a G1 achene showing the immunolocalyzation of FaHyPRP. The reaction was found throughout the endocarp (arrows) as well as in the testa tissues (arrowheads). (D) and (E) are details of the immunolocalization at the G1 achene tissues (D, testa; E, endocarp schlerenchymatic tissue). (F) is an achene at the R stage in which the immunoreaction was found at the testa (arrowheads) and schlerenchyma (arrows). (G) and (H) are controls from G1 and R stages, respectively, using a preimmune serum. (I) shows FaHyPRP expression at the achene endocarp by "in situ" hybridization (arrows). (J) is a control obtained with the sense probe. (K) shows an achene stained with phloroglucinol, which reveals lignin deposits (as pink-red stain) at the endocarp tissue. A more detailed view of this lignification is shown in (L). Magnifications are as follow: (A) and (B), bar = 4 μm; (C) and (H), bar = 66 μm; (D), (F), and (G), bar = 25 μm; (E), bar = 16 μm and (I), (J) and (L), bar = 20 μm.

that these structures were tannins. Therefore, we performed a fixation of both tissues in which postfixation with osmium tetroxide was omitted in some samples based on the fact that tannins have

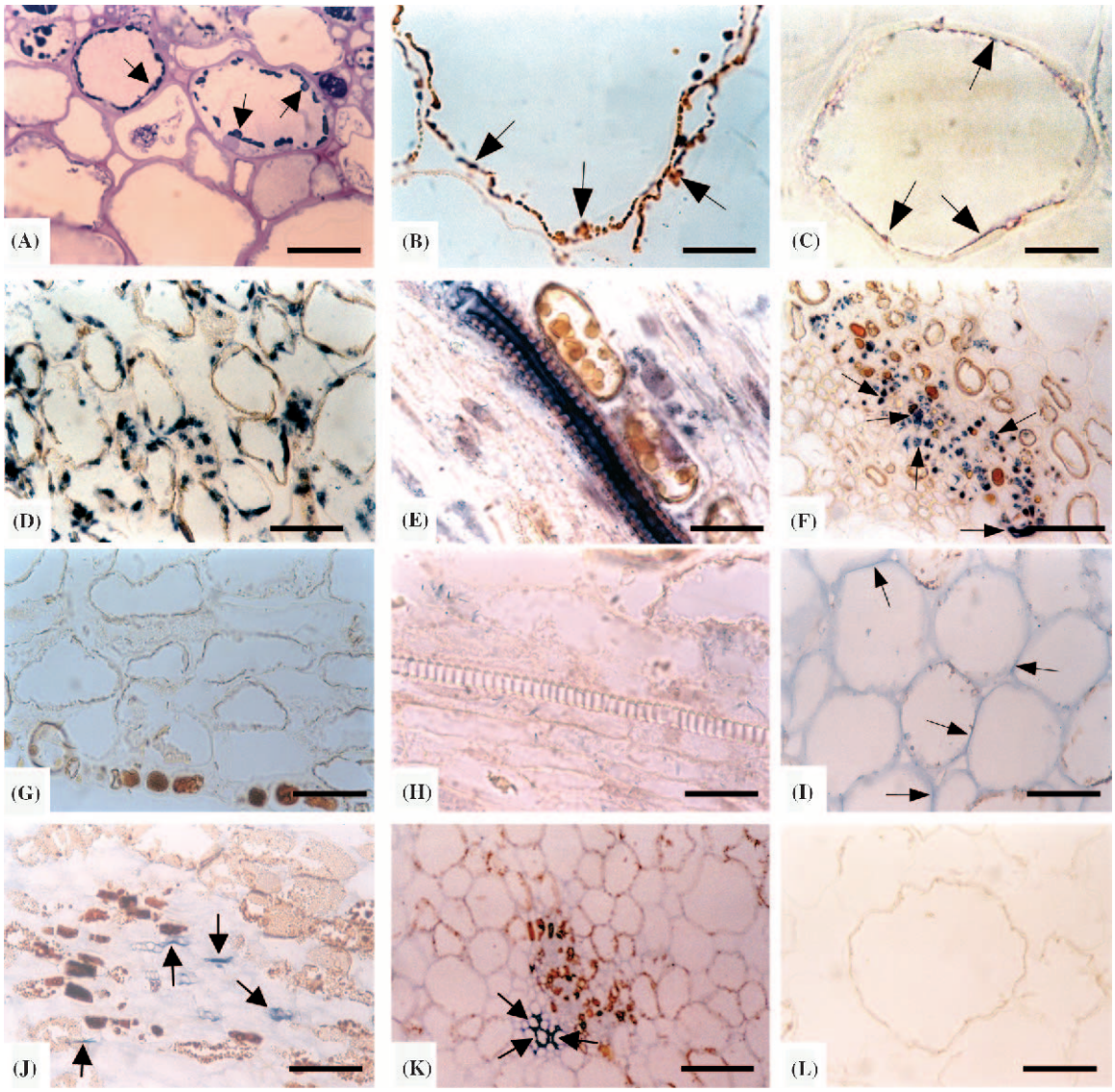

Figure 8. Histochemical analysis, "*in situ*" hybridization and immunohistochemical characterization of the strawberry *Fahyprp* gene and FaHyPRP protein in fruit receptacle. (A) is a semithick section of a portion of the cortical parenchyma stained with toluidine blue. Dark spots are seen in some cells (arrows). (B) is a semithick section of the same area stained with Bismarck Brown Y which reveals the same spots as in (A) (arrows). (C) is a paraffin section of a cortical cell stained with DMACA, specific for condensed tannins. A thin brown-stained line next to the cell wall can be observed (arrows). (D) and (E) are paraffin sections from G1 fruit stained with the anti-FaHyPRP antibody. (D) is subepidermical parenchymatic tissue and (E) shows the reaction associated to a tracheary element of the xylem. (F) corresponds to the R stage in which the reaction with anti-FaHyPRP was found only in cells associated to vascular bundles (arrows). (G) and (H) are controls using pre-immune sera performed in tissues from G1 and R stages. (I), (J) and (K) show the *Fahyprp* gene expression during ripening by "*in situ*" hybridization. (I) is parenchymatic tissue from G1 stage showing the reaction as blue ribbons at the peripheral cytosol (arrows). (J) is a longitudinal section of vascular tissue from the same stage. The reaction was found as blue spots associated to tracheary xylematic elements (arrows). (K) is a transversal section of vascular tissue from R stage. The reaction had the same localisation as in G1 stage; i.e. associated to tracheary elements (arrows). (L) is a control using the sense probe. Magnifications are as follow: (A) and (B), bar = 18 μm; (C), bar = 25 μm; (D), (E), (G), (H) and (L), bar = 12.5 μm; (F), bar = 76 μm; (I), (J) and (K), bar = 12 μm.

been reported to be highly osmiophilic structures (Gutmann and Feucht, 1991). The omission of osmium revealed the presence of discretely gray electron-dense spots located inside the parenchymatic cells (Figure 9A). However, these spots were practically electron-opaque when fixation was followed by postfixation with osmium (Figure 9B) indicating that such structures corresponded to tannins. Furthermore, very similar structures had been previously found in other plants material and have been identified as tannins (Lamhamedi *et al*., 2000; Grundhöfer *et al*., 2001). In strawberry receptacles, the tannins were found tightly associated to the inner surface of the tonoplast, being the surrounding cytosol free from these compounds (see Figure 9C). In some cases, tannins formed very large structures (Figure 9D) attached to the tonoplastic inner surface (Figure 9E).

Discussion

Although different physiological functions have been suggested for HyPRP proteins in higher plants, including their involvement in developmental processes and responses to different stress, so far no specific function for this class of proteins has been concluded.

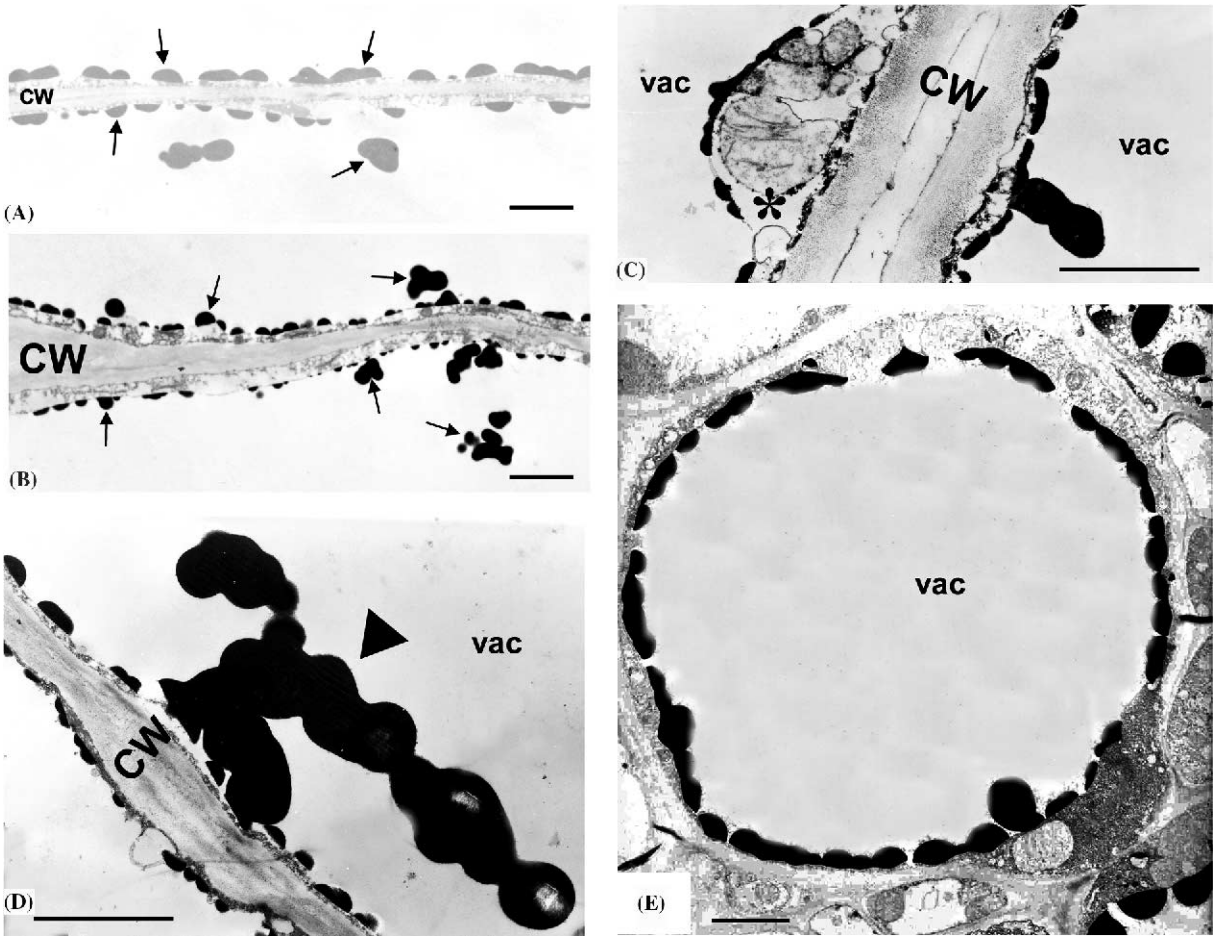

Figure 9. Ultrastructural features of tannin accumulations in strawberry fruit receptacle. (A) is a parenchymatic cell in which osmium tetroxide postfixation was omitted. (B) is an equivalent section postfixed in osmium, so that the osmiophilic nature of tannins (arrows) is observed. In (C) and (D) it is possible to observe that tannins are attached to the internal surface of the tonoplast. In (C) is shown a portion of the cytoplasm where some membrane organella is observed (asterisk). (D) shows a large structure (arrowheads) towards the vacuolar lumen resulting from tannin accumulation. (E) is a whole parenchymatic cell showing tannins in the luminal face of the tonoplast (cw = cell wall; vac = vacuolar lumen). Magnifications are: (A-D), bar = 4 μm; (E), bar = 8 μm.

In the present paper, we present the isolation and characterization of a strawberry fruit-specific gene encoding a protein which shows a clear identity and similarity with the HyPRP class B proteins of higher plants (Josè and Puigdomnèch, 1994).

The expression of the strawberry *Fahyprp* gene is fruit-specific and varies through the different fruit development and ripening stages. This expression pattern is similar to that observed in the pod dehiscence zone for the HyPRP of *Brassica napus* L. (Coupe *et al*., 1993). Thus, the transcripts corresponding to this gene (SAC51) were only detected in pods and accumulated in the dehiscence zones during the last stages of pod development, just before the dehiscence. However, a certain expression of this gene was observed during the first stages of pod development which clearly declined in the intermediate development stages (Coupe *et al*., 1993). Curiously, a similar cellulase activity pattern was also observed in *Brassica napus* pods and in strawberry fruit ripeness (Meakin and Roberts, 1990; Trainotti *et al*., 1999).

Additionally, the fruit ripening was accompanied by an approximate three fold increase in hydoxyproline content in grapes, suggesting that proteins containing hydroxyproline residues could play an important role in this process (Nunam *et al*., 1998).

It has been proposed that the auxins may be the primary hormones controlling strawberry fruit ripening (Perkins-Veazie, 1995). A gradual decline in the supply of auxin from achenes in the latter stages of growth has been associated with the basis of strawberry (Perkins-Veazie, 1995). We have shown that the strawberry *Fahyprp* gene expression is negatively regulated by auxins. A similar relationship between auxin and gene expression pattern has been described for other strawberry ripening-related genes (Trainotti *et al*., 1999; Aharoni *et al*., 2002; Blanco-Portales *et al*., 2002; Benitez-Burraco *et al*., 2003). Similarly, a *Catharanthus roseus* gene encoding a HyPRP was also shown to be induced in an auxin-free and sucrose-rich culture medium (Hotze *et al*., 1994). Also, it has been demonstrated that the expression of genes encoding HyPRPs from *Daucus carota* and soybean (ADR11) are negatively regulated, at transcriptional level, by auxins (Datta *et al*., 1993).

The results obtained by the "*in situ*" hybridization and immunolocalization experiments showed that the HyPRP protein is located in two different sites on strawberry fruits: namely in cells undergoing a lignification process and in parenchymatic cells of the receptacle of immature green fruits. It has been described that proline-rich proteins (PRPs) and hydroxy-proline-rich proteins are preferentially located in xylem cells under a process of lignification (Casab, 1998). A putative role for these of proteins as anchoring sites for lignin polymer through the interaction with the phenolic groups constituting the lignin was proposed (Casab, 1998). In coleoptiles of *Zea mays*, electron-microscopic immunogold labelling of epitopes recognized that lignin deposition was temporally and spatially correlated with the appearance of epitopes for proline-rich proteins, but not hydroxyproline-rich proteins (Müsel *et al*., 1997). Similarly, using anti-PRP2 antibodies, the localization of PRPs in lignified secondary walls as well as the secretion of the protein during the process of lignification has been described in differentiating protoxylem elements in etiolated bean and soybean hypocotyls (Ryser *et al*., 1997). These results support the hypothesis of Ye *et al*. (1991) that PRP localization is related to pattern of lignification. In this study a potential role for PRPs proteins as a scaffold for lignin deposition via their tyrosine groups followed by oxidative cross-linking of lignin monomers is proposed (Ryser *et al*., 1997).

The physiological role played by HyPRP proteins in strawberry fruit cells could be the anchorage of lignin polymer into the cell wall or on the plasma membrane. This conclusion can be drawn from two lines of evidence. First, the structural characteristics of the HyPRP protein, containing a putative carboxy-terminal transmembrane domain; and second, the co-immunolocalization of cinnamyl alcohol dehydrogenase (CAD), an enzyme directly involved in the biosynthesis of lignin (Blanco-Portales *et al*., 2002), and HyPRP protein in cells undergoing to lignification, such as immature xylem cells of vascular bundles on the schlerenchyma cell layers of the achenes (Blanco-Portales *et al*., 2002 and present paper). In this sense, it has been demonstrated that human salivary proline-rich proteins precipitate dietary polyphenols (Baxter *et al*., 1997). It has been proposed that condensed tannins reduce both water uptake and imbibition damage by solute leakin in seeds and, accordingly, may act as a mechanical barrier to prevent embryo injury

(Halloin, 1982; Bell *et al*., 1992; Kantar *et al*., 1996). Thus, the presence in the achenes of HyPRP protein associated to structures containing condensed tannins, as revealed by immunohistochemistry, could be related with a similar physiological role. Also, deposits of condensed tannins in vacuoles of seed endothelial cells have been recently reported in *Arabidopsis thaliana* (Devic *et al*., 1999; Debeaujon *et al*., 2001). In this sense, we observed a similar subcellular deposition pattern in parenchymatic cells of strawberry green fruits stages for both HyPRP protein and condensed tannins.

Immunolocalization of the HyPRP in parenchymatic cells of strawberry green fruits indicates that this protein is associated to spherules containing tannins, which are mainly located at the inner surface of the tonoplast. Previously, a decrease in the concentration of tannins along fruit ripening had been demonstrated in several fruits including strawberry (Perkins-Veazie, 1995; Boss *et al*., 1996; Morazzoni and Bombardelli, 1996; Harborne, 1997; Jaakola *et al*., 2002). Our histochemical studies have also shown the presence of tannin deposits in the vacuoles only in immature fruits at the first stages of development. The requirement or isolation of tannins within the cytoplasm is a reasonable strategy for the cell when the chemical properties of tannins are considered. Effectively, tannins are rich in hydrophobic aromatic rings with hydroxyl groups which can interact with biological molecules (particularly proteins) by hydrogen bonds and hydrophobic interaction. Thus, it is known that the tannins precipitate proteins and inhibit most enzyme reactions. The presence of free tannin molecules in the cytoplasm would precipitate structural and enzymatic proteins causing damage to the cell machinery.

It has been proposed that the concentration of tannins may play an important role in the defense mechanism in some plants (Costanbel, 1999, 2002). An example is the inhibition of the fungal extracellular hydrolytic enzymes that appear to be essential for disease development, making the plants nutritionally unavailable to most insects and fungi (Costanbel, 2002). The presence of tannins in immature fruits may be a genetically programmed strategy that could provide protection against to early feeding of the fruits (Harborne, 1997). Moreover, the vacuoles offer a larger storage space than cell walls, which is important to reach concentration large enough to be efficient in the protection against predators and pathogens (Klein *et al*., 2000).

Tannins have also been found to protect developing fruit tissues against fungal pathogens (Mercier, 1997). In strawberry, the fungal pathogen *Colletotrichum fragaria* is confined to a few cells beneath the infection site in resistant cultivars to anthracnose (as c.v. Apollo and Sequoia). This confinement is due to the cell walls thickening and the deposition of pectic material at the intracellular spaces of the cortex as well as accumulation of tannins in the parenchyma cells surrounding the site of infection (Milholland, 1982). Similarly, in the interaction between the strawberry fruit and its fungal pathogen *Botrytis cinerea*, the fungus remains quiescent in immature strawberry fruits after successful flower infection (Jersch, 1989). Further fungal growth occurs only when the fruits become mature and tannins content decreases. Thus, not suprisingly, the immature strawberry fruits are more resistant to *B. cinerea* than mature fruits. In this sense, the overall condensed tannin content of immature strawberry fruits is negatively correlated with the mycelial development on inoculated fruits (Jersch, 1989). These facts support the role of tannins on the incidence of the infection (Jersch, 1989). The pronounced deposition of condensed tannins in the receptacle corresponds to the restriction of the fungal development in such area after successful penetration. The condensed tannins could inactivate fungal extracellular hydrolytic enzymes that appear to be essential for disease development.

The presence of tannin deposits exclusively in parenchymatic cells of immature strawberry fruits could be related to such strategy. The HyPRP protein could play an anchoring role condensing tannins on the cell membrane of the vacuole in parenchymatic cells of fruits in green stages of development. However, no deposits were observed in the parenchyma cells of the receptacle of strawberry fruits undergoing ripening. In these stages of fruit-ripening a higher susceptibility to fungal pathogens is observed.

In summary, we have cloned and characterized a strawberry cDNA corresponding to a mRNA encoding a protein showing significant identity to hydroxyproline-rich proteins from other higher plants. The gene expression analysis, "*in situ*" hybridization, inmunolocalization and histoche-

mical experiments suggest that the strawberry *Fahyprp* gene is related to the fruit ripening process and possibly involved in the anchoring of polyphenols (lignin and condensed tannins) to cell membranes. To our knowledge, this is the first report assesing the phisyological role of HyPRP proteins in fruits of higher plants. Moreover, we demonstrate that the expression of the corresponding gene is fruit-specific and ripening-related.

Acknowledgements

This work has been supported by grant BIO2001-1958-C0-02 (Spain), FAIR. CT97-3005 and Junta de Andalucía (Grupo PAI CVI 278), Spain. The utilization of equipment of Instituto Andaluz de Biotecnología, Andalucía, Spain, and of SCAI of the University of Córdoba is acknowledged. The authors are grateful to J.J. Medina (C.I.F.A ''Las Torres-Tomejil'', Sevilla, Spain) for the support with the strawberry field cultivation.

References

Abrahams, S., Tanner, G.J., Larkin, P.J. and Ashton, R. 2002. Identification and biochemical characterization of mutants in the proanthocyanidin pathway in *Arabidopsis*. Plant Physiol 130: 561–576.

Aharoni, A. and O'Connell, A.P. 2002. Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. J. Exp. Bot. 53: 2073–2087.

Aharoni, A., Keizer, L.C.P., Van Den Broeck, H.C., Blanco-Portales, R., Muñoz-Blanco, J., Bois, G., Smit, P., De Vos, R.C.H. and O'Conell, A.P. 2002. Novel insight into vascular, stress, and auxin-dependent an –independent gene expression programs in strawberry, a non-climateric fruit. Plant Physiol. 129: 1019–1031.

Baker, S.S., Wilhelm, K.S. and Thomashow, M.F. 1994. The 5′-region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer col-, drought- and ABA-regulated gene expression. Plant Mol Biol 24: 701–713.

Baxter, N.J., Lille, T.H., Haslam, E. and Williamson, M.P. 1997. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochem. 36: 5566–5577.-

Bell, A.A., El-Zik, K.M. and Thaxton, P.M. 1992. Chemistry, biological significance and genetic control of proanthocyanidins in cotton (*Gossypium* spp). In: R.M. Hemingway and P.E. Lacks (Eds.) Plant Polyphenols: Plenum Press, New York, USA, pp. 571–595.

Benítez-Burraco, A., Blanco-Portales, R., Redondo-Nevado, J., Bellido, M.L., Moyano, E., Caballero, J.L. and Muñoz-Blanco, J. 2003. Cloning and characterization of two ripening-related strawberry (*Fragaria x ananassa* cv. Chandler) pectate lyase genes. J. Exp. Bot. 54: 633–645.

Blanco-Portales, R., Medina-Escobar, N., López-Ráez, J.A., González-Reyes, J.A., Villalba, J.M., Moyano, E., Caballero, J.L. and Muñoz-Blanco, J. 2002. Cloning, expression and immunolocalization pattern of a cinnamyl alcohol dehydrogenase gene from strawberry (*Fragaria x ananassa* cv. Chandler). J. Exp. Bot. 53: 1723 –1734.

Boss, P.K., Davies, C. and Robinson, S.P. 1996. Anthocyanin composition and anthocyanin pathway gene expression in grapevine sports differing in berry skin colour. Aust J. Grap Wine Res. 2: 163–170.

Busk, P.K. and Pages, M. 1998. Regulation of abscisic acid-induced transcription. Plant Mol. Biol. 37: 425–435.

Casab, G.J. 1998. Plant cell wall proteins. Ann. Rev. Physiol. Plant Mol. Biol. 49: 281–309.

Castonguay, Y., Laberge, S., Nadeau, P. and Vézina, L.P. 1994. A cold-induced gene from *Medicago sativa* encodes a bimodular protein similar to developmentally regulated proteins. Plant Mol. Biol. 24: 799–804.

Choi, D.W., Song, J.Y., Kwon, Y.M. and Kim, S.G. 1996. Characterization of a cDNA encoding a proline-rich 14 KDa protein in developing cortical cells of the roots of bean (*Phaseolus vulgaris*) seedlings. Plant Mol. Biol. 30: 973–982.

Clifford, M.N. 1974. Specificity of acidic phloroglucinol reagents. J. Chromatograph. 94: 321–324.

Coupe, S.A., Taylor, J.E., Isaac, P.G. and Roberts, J.A. 1993. Identification and characterization of a proline-rich mRNA that accumulates during pod development in oilseed rape (*Brassica napus* L.). Plant Mol. Biol. 23: 1223–1232.

Costanbel, C.P. 1999. A survey of hervibore-inducible defensive proteins and phytochemicals. In: A.A. Agrawaal, S. Tuzun and E. Bent (Eds.) Induced Plant Defenses Against Herbivores and Pathogens, APS Press, St Paul, USA, pp. 137–166.

Costanbel, C.P. 2002. Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant J. 32: 701–712.

Datta, N., LaFayette, P.R., Koner, P.A., Nagao, R.T. and Key, J.L. 1993. Isolation and characterization of three families of auxin down-regulated cDNA clones. Plant Mol. Biol. 21: 859–869.

Debeaujon, I., Peeters, A.J.M., Leon-Kloosterziel, K.M. and Koorneef, M. 2001. The TRANSPARENTA TESTA 12 gene on *Arabidopsis* encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell 13: 853–871.

Deutch, C.E. and Winicov, I. 1995. Post-transcriptional regulation of a salt-inducible alfalfa gene encoding a putative chimeric proline-rich cell wall protein. Plant Mol. Biol. 27: 411–418.

Devereux, J., Haeberli, P. and Smithies, O. 1984. A comprensive set of sequence analysis programs for the VAX. Nucl. Acids Res. 12: 387–395.

Devic, M., Guilleminot, J., Debeaujon, I., Bechtold, N., Bensaude, E., Koornneef, M., Pelletier, G. and Delseny, M. 1999. The *BANYULS* gene encodes a DFR-like protein and is a marker of early seed coat development. Plant J. 19: 387–398.

Fujita, T., Kouchi, H., Ichikawa, T. and Syono, K. 1994. Cloning of cDNAs for genes that are specifically or preferentially expressed during the development of tobacco genetic tumors. Plant J. 5: 645–654.

Gallie, D.R. 1993. Post-transcriptional regulation of gene expression in plants. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 44: 77–105.

Grundhöfer, P., Niemetz, R., Schilling, G. and Gross, G.G. 2001. Biosynthesis and subcellular distribution of hidrolyzable tannins. Phytochemical 57: 915–927.

Gutmann, M. and Feucht, W. 1991. A new method for selective localization of flavan-3-ols in plant tissues involving glycolmethacrylate embedding and microwave irradiation. Histochemistry 96: 83–86.

Halloin, J.M. 1982. Localization and changes in catechin and tannins during development and ripening of cottonseed. New Phytol. 90: 651–657.

Hattori, T., Terada, T. and Hamasuna, S. 1995. Regulation of the Osem gene by abscisic acid and the transcriptional activator VP1: analysis of cis-acting promoter elements required for regulation by abscisic acid and VP1. Plant J. 7: 913–925.

Hotze, M., Waitz, A. and Schröder, J. 1994. cDNA for a 14-Kilodalton Polypeptide from Madagascar Periwinkle (*Catharanthus roseus)*. Plant Physiol. 104: 1097–1098.

Jaakola, L., Maatta, K., Pirttila, A.M., Torronen, R., Karenlampi, S. and Hohtola, A. 2002. Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. Plant Physiol. 130: 729–739.

Jackson, D.P. 1991. In situ hybridization in plants. In: S.J. Gurr, M.J. Mc Pherson and D.J. Bowles (Eds.) Molecular Plant Pathology I: A practical approach, IRL Press, Oxford University Press, Oxford, pp. 163–174.

Jersch, S., Scherer, C., Huth, G. and Schlösser, E. 1989. Proanthocyanidins as basis for quiescence of *Botrytis cinerea* in inmature strawberry fruits. J. Plant Dis. Proct. 96: 365–378.

John, I., Wang, H., Held, B.M., Wurtele, E. and Colbert, J.T. 1992. An mRNA that specifically accumulates in maize roots delineates a novel subset of developing cortical cells. Plant Mol. Biol. 20: 821–831.

Josè-Estanyol, M., Ruiz-Avila, L. and Puigdomènech, P. 1992. A maize embryo-specific gene encodes a proline-rich and hydrophobic protein. Plant Cell 4: 413–423.

Josè, M. and Puigdomènech, P. 1994. Hybrid-Proline rich and related proteins. Current Topic in Mol. Genet. (Life Sci. Adv.) 2: 109–126.

Kader, F., Rovel, B., Girardin, M. and Metche, M. 1996. Fractionation and identification of the phenolic compounds of highbush blueberries (*Vaccinium corymbosum* L.). Food Chem 55: 35–40.

Kantar, F., Pilbeam, C.J. and Hebblethwaite, P.D. 1996. Effect of tannin content of faba bean (*Vicia faba*) seed on seed vigour, germination and field emergence. Ann. Appl. Biol. 128: 85–93.

Klein, M., Martinoia, E., Hoffmann-Thoma, G. and Weissenböck, G. 2000. A membrane-potential dependent ABC-like transporter mediates the vacuolar uptake ot rye flavone glucuronides: regulation of glucuronide uptake by glutathione and its conjugate. Plant J. 21: 289–304.

Lamhamedi, M.S., Chamberland, H. and Bernier, P.Y. 2000. Clonal variation in morphology, growth, physiology, anatomy and ultrastructure of container-grown white spruce somatic plants. Tree Physiol. 20: 869–880.

Lee, D., Meyer, K., Chapple, C. and Douglas, C.J. 1997. Down-regulation of 4-coumarate: CoA ligase (4CL) in *Arabidopsis*: effect on lignin composition and implications for the control of monolignol biosynthesis. Plant Cell 9: 1985–1998.

Li, Y.G., Tanner, G.J. and Larkin, P.J. 1996. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. J. Sci. Food Agric. 70: 89–101.

Manning, K. 1991. Isolation of nucleic acids from plants by differential solvent precipitation. Anal Biochem 195: 45–50.

Meakin, P.J. and Roberts, J.A. 1990. Dehiscence of fruit in oilseed rape (*Brassica napus* L.). I. Anatomy of pod dehiscence. J. Exp. Bot. 41: 995–1002.

Medina-Escobar, N., Cárdenas, J., Valpuesta, V., Muñoz-Blanco, J. and Caballero, J.L. 1997. Cloning and characterization of cDNAs from genes differentially expressed during the strawberry fruit ripening process by a MAST-PCR-SBDS method. Anal. Biochem. 248: 288–296.

Mercier, J. 1997. Role of phytoalexins and other antimicrobial compounds from fruits and vegetables in postharvest disease resistance. In: F. Tomas-Barberan (Eds.) Phytochemistry of Fruits and Vegetables, Oxford University Press, New York, pp. 221–241.

Millholland, R.D. 1982. Histopathology of strawberry infected with *Colletotrichum fragariae*. Phytopathology 72: 1434–1439.

Morazzoni, P. and Bombardellli, E. 1996. *Vaccinium myrtillus* L. Fitoterapia 67: 3–29.

Moyano, E., Portero-Robles, I., Medina-Escobar, N., Valpuesta, V., Muñoz-Blanco, J. and Caballero, J.L. 1998. A fruit-specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process. Plant Physiol. 117: 711–716.

Müsel, G., Schindler, T., Bergfeld, R., Ruel, K., Jacquet, G., Lapierre, C., Speth, V. and Schopfer, P. 1997. Structure and distribution of lignin in primary and secondary cell walls of maize coleoptiles analyzed by chemical immunological probes. Planta 201: 146–159.

Nunam, K.J., Sims, I.M., Bcic, A., Robinson, S.P. and Fincher, G.B. 1998. Changes in cell wall composition during grape berries. Plant Physiol. 118: 783–792.

Perkins-Veazie, P. 1995. Growth and ripening of strawberry fruit. Horticultural Reviews 17: 267–297.

Porankiewicz, A., Splund, J., Nilsson, B., Höglund, A.S. and Josefsson, L.G. 2000. Removal of cross reactive anti-carrier (keyhole limpet haemocyanin, KLH) antibodies from Rabbit serum using immobilized KLH. Life Sci. News 5: 16–17.

Ryser, U., Schorderet, M., Zhao, G.F., Studer, D., Ruel, K., Hauf, G. and Keller, B. 1997. Structural cell-wall proteins in protoxylem development: evidence for repair process mediated by a glycine-rich protein. Plant J. 12: 97–111.

Salts, Y., Kenigsburg, D., Wachs, R., Guissem, W. and Barg, R. 1992. DNA sequence of the tomato fruit expressed proline-rich protein gene TPRP-F1 reveals an intron within de 3′ untranslated transcript. Plant Mol. Biol. 18: 407–409.

Schneider, H. 1981. Plant Anatomy and General Botany. In: A.G. Clark (Eds.) Staining procedures, Ed 4a. Williams and Wilkins, Baltimore, pp. 315–339.

Trainotti, L., Spolaore, S., Pavanello, A., Baldan, B. and Casadoro, G. 1999. A novel E-type endo β-1,4-glucanase with a putative cellulose-binding domain is highly expressed in ripening strawberry fruits. Plant Mol Biol 40: 323–332.

Xu, Y., Buchholz, W.G., DeRose, R.T. and Hall, T.C. 1995. Characterization of a rice gene family encoding root-specific proteins. Plant Mol. Biol. 27: 237–248.

Yamaguchi-Shinozaki, K. and Shinozaki, K. 1994. A novel cis-acting element in a *Arabidopsis* gene is envolved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6: 251–264.