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Cloning, expression and immunolocalization pattern of a cinnamyl alcohol dehydrogenase gene from strawberry (*Fragaria*×*ananassa* cv. Chandler)[1]

R. Blanco-Portales[2], N. Medina-Escobar[2], J. A. López-Ráez[2], J. A. González-Reyes[3], J. M. Villalba[3], E. Moyano[2], J. L. Caballero[2] and J. Muñoz-Blanco[2,4]

[2] *Departamento de Bioquímica y Biología Molecular, Edificio C-6, Campus Universitario de Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain*

[3] *Departamento de Biología Celular, Edificio C-6, Campus Universitario de Rabanales, Universidad de Córdoba, 14071 Córdoba, Spain*

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Abstract

Cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) catalyses the conversion of *p*-hydroxy-cinnamaldehydes to the corresponding alcohols and is considered a key enzyme in lignin biosynthesis. By a differential screening of a strawberry (*Fragaria*× *ananassa* cv. Chandler) fruit specific subtractive cDNA library, a full-length clone corresponding to a *cad* gene was isolated (*Fxacad1*). Northern blot and quantitative real time PCR studies indicated that the strawberry *Fxacad1* gene is expressed in fruits, runners, leaves, and flowers but not in roots. In addition, the gene presented a differential expression in fruits along the ripening process. Moreover, by screening of a strawberry genomic library a *cad* gene was isolated (*Fxacad2*). Similar to that found in other *cad* genes from higher plants, this strawberry *cad* gene is structured in five exons and four introns. Southern blot analyses suggest that, probably, a small *cad* gene family exists in strawberry. RT-PCR studies indicated that only the *Fxacad1* gene was expressed in all the fruit ripening stages and vegetative tissues analysed. The *Fxacad1* cDNA was expressed in *E. coli* cells and the corresponding protein was used to raise antibodies against the strawberry CAD polypeptide. The antibodies obtained were used for immunolocalization studies. The results showed that the CAD polypeptide was localized in lignifying cells of all the tissues examined (achenes, fruit receptacles, runners, leaves, pedicels, and flowers). Additionally, the cDNA was also expressed in yeast *(Pichia pastoris)* as an extracellular protein. The recombinant protein showed activity with the characteristic substrates of CAD enzymes from angiosperms, indicating that the gene cloned corresponds to a CAD protein.

Key words: Cinnamyl alcohol dehydrogenase, fruit growth, fruit ripening, strawberry.

Introduction

As in most non-climacteric fruits, the development of the strawberry receptacle (pseudo-fruit) appears as a continuous process encompassing overlapping phases of cell division, expansion and senescence (Perkins-Veazie, 1995). Several criteria may be used to characterize the onset of the ripening process. One of them is the production and accumulation of anthocyanin, which is asssociated with a decrease in the content of chlorophyll and carotenoid pigment of the fruit. It is thought that fruit ripening begins when the first signs of red pigmentation are observed (turning stage) after fruit elongation has been completed (Perkins-Veazie, 1995). Moreover, as fruit ripens, the achenes (a combination of seed and ovary tissue) undergo a strong lignification of their thick pericarp (Perkins-Veazie, 1995).

[1] The nucleotide sequence data reported will appear in the EMBL and GenBank databases under the accession number U63534 for the cDNA and AF320110 for the CAD gene.

[4] To whom correspondence should be addressed. Fax: +10 34 957211079. E-mail: bb1mublj@uco.es

Lignin biosynthesis is probably controlled by two signal-transduction pathways. One is involved in the development of vascular tissue and the other in plant defence responses (Walter, 1992; Mitchell and Barber, 1994). Lignin is deposited mainly in cell walls of supporting and conducting tissues, such as fibres and tracheary elements. The mechanical rigidity of lignin strengthens these tissues so that tracheary elements can endure the negative pressure generated from transpiration without collapse of the tissue.

Lignin is derived from dehydrogenative polymerization of 'monolignols' (hydroxycinnamyl alcohols). A cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) catalyses the second reductive step of the lignin committed branch, leading to the hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl and sinapyl alcohols, the monomeric precursors of lignin. Thus, CAD activity is directly involved in lignification and the enzyme has been purified from the xylem tissue of a number of tree species (Goffner *et al*., 1998; O'Malley *et al*., 1992). However, CAD activity has also been reported in apparently non-lignified tissues (O'Malley *et al*., 1992), and monolignols are also used for the synthesis of non-lignin products such as lignans (Lewis and Yamamoto, 1990) and surface polymers such as cutin (Holloway, 1982) and suberin (Kolattukudy, 1981).

CAD cDNAs have been isolated from angiosperms (Knight *et al*., 1992; Hibino *et al*., 1993; Grima-Pettenati *et al*., 1993) as well as from gymnosperms (O'Malley *et al*., 1992; Galliano *et al*., 1993). The eucalyptus and spruce cDNAs have been unambiguously proven to encode CAD by functional studies ((Grima-Pettenati *et al*., 1993; Galliano *et al*., 1993). CAD cDNAs share extensive nucleotide sequence homology (approximately 80% identity among all published angiosperm sequences, and 70% between angiosperms and gymnosperms) suggesting that the *cad* gene has been conserved during evolution. Moreover, sequence analysis of CAD has shown that it belongs to the long chain zinc-containing alcohol dehydrogenase family. Northern experiments performed in several higher plants species, have shown a high level of *cad* gene expression in stems (Knight *et al*., 1992; Grima-Pettenati *et al*., 1993; Hibino *et al*., 1993) and especially in tissue undergoing active lignification (e.g. xylem) (Grima-Pettenati *et al*., 1993).

The *cad* gene has been characterized in tobacco (Walter *et al*., 1994*), Eucalyptus botryoides* (Hibino *et al*., 1994) and *Eucalyptus gunii* (Feuillet *et al*., 1994). Studies with transgenic plants expressing CAD promoter–GUS constructions have shown a high GUS activity in roots followed by stems and leaves (Feuillet *et al*., 1994; Walter *et al*., 1994). In eucalyptus, histochemical staining for GUS activity indicated a strong expression in the vascular tissue of stems, roots, leaves, and petioles, mainly in parenchyma cells located between the xylem conducting elements. After secondary growth has occurred, GUS activity was localized to xylem ray cells and parenchyma cells surrounding the phloem fibres. This expression pattern suggests the export of lignin precursors from their site of synthesis towards their sites of assembly, and supports the concept of cell co-operation in lignin biosynthesis (Feuillet *et al*., 1995). These results were confirmed by *in situ* hybridization experiments (Hawkins *et al*., 1997). Similarly, cytochemical studies in *Phaseolus vulgaris*, *Vicia faba* and *Pisum sativum* demonstrated that the CAD enzyme was present in developing xylem elements and also in the epidermal and subepidermal layers of both roots and shoots, suggesting that several distinct forms of cinnamyl alcohol dehydrogenase could play different roles according to the cell types in which the corresponding gene is expressed (Baudraco *et al*., 1993). Recently, the cell-specific localization of the eucalyptus CAD2 promoter expression has been investigated. The promoter was active in young xylem, developing phloem fibres and chambered parenchyma cells around phloem, supporting a relevant role for CAD2 in lignification (Samaj *et al*., 1998).

Heavy lignification of the achenes is one of the most undesirable traits of the strawberry fruit. In order to determine the role that the cinnamyl alcohol dehydrogenase gene can play in this process, a comprehensive study of the molecular and expression characteristics of this gene during strawberry fruit growth and ripening has been performed. The present study shows the cloning and characterization of a strawberry *Fxacad1* gene differentially expressed during the strawberry fruit ripening process that is also expressed in vegetative tissues. The structural features of a strawberry *Fxacad1* gene are also shown. Immunolocalization, Northern and quantitative real time PCR analysis allowed a relationship between the CAD spatio-temporal expression pattern and the strawberry lignifying process to be proposed.

Materials and methods

Plant material

The strawberry fruit (*Fragaria×ananassa* cv. 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). Fruits and other tissues were immediately frozen in liquid nitrogen after harvesting.

RNA isolation

Total RNAs from a pool of six or seven strawberry fruits at different stages of fruit development and from roots, leaves, flowers, and runners tissues were isolated according to Manning (1991), and the

remaining carbohydrates removed by passing the total RNA fraction through a cellulose column (Medina-Escobar *et al*., 1997*a*).

Strawberry CAD cDNA and gene cloning

The cloning and isolation of a full length strawberry cDNA (*Fxacad1*) was performed by differential screening of a strawberry cDNA subtractive library according (Medina-Escobar *et al*., 1997*a*).

The cloning and isolation of a strawberry CAD genomic clone (*Fxacad2*) was performed by plaque hybridization screening of about 1.5×10^5 pfu of a λ-FIX strawberry genomic library (*Fragaria×ananassa* cv. Chandler), using the isolated strawberry *Fxacad1* cDNA as a radioactive probe. Filters were prehybridized and hybridized at 65 °C in hybridization solution: 5× SSC, 5× Denhardt's, 200 μg ml^{-1} salmon sperm, and 0.5% SDS. After hybridization, filters were washed (twice) for 15 min at room temperature, in a 2× SSC, 0.5% SDS solution. Afterwards, the filters were washed twice for 15 min, at 65 °C, in a 0.2× SSC, 0.1% SDS solution. Four positive clones were isolated after three rounds of plaque purification. Restriction mapping allowed the conclusion that they all were overlapping clones. In addition, restriction analysis allowed appropriate subclones to be obtained for sequencing.

RNA blot analysis

To investigate the differential expression of this gene during the strawberry ripening process, the strawberry *Fxacad1* cDNA was used as the template for a radioactive probe in RNA analysis. Single-stranded probes were PCR labelled with ^{32}P-dCTP to a specific activity of approximately 108 cpm μg^{-1}. A cDNA corresponding to 18 S ribosomal RNA was used as the radioactive probe to control equal loading of RNA samples.

Twenty micrograms of total RNA per sample were routinely used for the Northern analysis. The blots were prehybridized at 65 °C for 1 h, in 15 ml of hybridization solution (0.25 M NaH_2PO_4, 7% (w/v) SDS and 0.1 mM EDTA). Afterwards, the probe was added to the same solution and hybridization was carried out at 65 °C for 14–16 h. Filters were washed (twice) for 15 min, at 65 °C, in 100 ml of 0.2× SSC, 0.1% (w/v) SDS, and then exposed to X-ray film, at –70 °C for 24–48 h.

Auxin treatments

Achenes of two sets of G2-stage strawberry fruits were carefully removed from the growing plant using the tip of a scalpel blade. One set of de-achened fruits was treated with the synthetic auxin 1-naphthaleneacetic acid (NAA) using a lanolin paste containing 1 mM NAA in DMSO 1% (v/v). The other set of de-achened fruits (control group) was treated with the same paste but without NAA. Fruit samples were harvested at 0, 24, 48, 72 h, and 96 h after treatment, immediately frozen in liquid nitrogen and then stored at –80 °C.

RT-PCR experiments and quantitative real time-PCR (QRT-PCR)

Studies related to the differential expression between strawberry *Fxacad1* and *Fxacad2* genes during fruit growth and ripening, leaves, runners and 5 d de-achened fruits were carried out by RT-PCR. Briefly, a specific common primer corresponding to the untranslated 5′ end of both CAD transcribed sequences (5′-AGGAATTCTCCCCACATTTTACTACTC-3′) was used in conjunction with a common 3′ primer (5′-AGGAATTCTTTC-TTCATTGCTCCAGTC-3′). Total RNA was extracted from both receptacle (de-achened fruit) and achenes corresponding to different stages of fruit growth and ripening and from leaves, runners and 5 d de-achened fruits. One microgram of total RNA was DNase I treated (Pharmacia) and the resulting RNA was retrotranscribed using Superscript II (Invitrogen) reverse retrotranscriptase and the common 3′ 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 recommended by the manufacturer (Gibco-BRL).

The amplified products were transferred to membranes (Southern-blot) and hybridized as described previously. Moreover, the amplified cDNA fragments were gel purified and subcloned into pGEM-T Easy vectors (Promega). For each fruit growth and ripening stages (both receptacle and achenes), 5 d de-achened fruits and also for leaves and runners tissues, four subclones from each RT-PCR amplified cDNA fragment were sequenced.

For the quantitative real time-PCR (QRT-PCR) measures of gene expression the iCycler (BioRad) system was used. The PCR reactions consisted of 24 μl of a mixture containing in 1× PCR buffer: 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 3 μl SYBR Green I (1:15 000 diluted), 3 μl of transcribed cDNA, and 0.5 U of Taq polymerase. For QRT-PCR, the cycling programme was as described above.

In QRT-PCR analysis, quantification is based on Ct values. The Ct (threshold cycle) is a measurement taken during the exponential phase of amplification when limiting reagents and small differences in starting reagent amount have not yet influenced the PCR efficiency. Ct is defined as the cycle at which fluorescence is first detectable above background and is inversely proportional to the log of the initial copy number. In this system, each 10-fold difference in initial copy number produced a 3.2 cycle difference in Ct. Each reaction was done in 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) *tubulin* gene. The efficiency of each QRT-PCR was also calculated. All these values were used to determine the increases in gene expression.

DNA extraction and Southern blot analysis

Strawberry genomic DNA was extracted as follows: young leaves were kept in distilled water, for 2 d, in the dark at 4 °C. Afterwards, 2 g of leaves were ground under liquid nitrogen into a fine powder and gently resuspended in 25 ml of a hot (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). β-mercaptoethanol, SDS and PVP were added just before use. The mixture was incubated in a rotatory oven at 65 °C for 1 h with gentle rotation. Then, 8 ml of 3 M potassium acetate, pH 4.8, were added and the resulting mix was incubated on 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, and 2 vols of chilled ethanol were added. Genomic DNA was extracted with a microcapillar, washed 2–3 times in fresh chilled ethanol and dried at room temperature.

Genomic DNA (2 μg) was digested with the restriction enzymes *BamH*I, *Bgl*II, *Eco*RI, and *Hind*III, fractionated on 0.7% (w:v) agarose gels and then alkaline transferred to Hybond-N+ membranes. The blot was hybridized using the *Fxacad1* cDNA as a radioactive probe, with a specific activity of 10^8 cpm μg^{-1}. Hybridization and washing conditions were as described above for Northern blot experiments.

DNA sequencing and computer analysis

DNA was sequenced by the dideoxy-chain termination method using an Applied Biosystems automatic sequencer. For the *Fxacad1* cDNA the putative positives clones in λZAP II phage (Stratagene) were excised into pBluescript SK(–) clones according to the

manufacturer's conditions. For *Fxacad2*, the insert contained in λFIX phage was subcloned into pBluescript KS(+). The corresponding clones (genomic and cDNA) were deleted with *Exo* III and sequenced, in both strands. The DNA sequences were analysed for ORFs using codon preference (UWGCG) (Devereux *et al*., 1984). Amino acid sequences were analysed using programs from the UWGCG package (2000). Sequences were compared with the GenBank and EMBL Nucleic Acid databases and the PIR and SWISPROT databases using BESTFIT, DOTPLOT and PRETTYBOX.

Expression in E. coli *and* Pichia pastoris *cells and antibody production*

To obtain the construct producing the strawberry protein corresponding to the full length *Fxacad1* cDNA, an oligonucleotide with the sequence 5′ GAGCGGGATCCCATGGCTATCGAGCAAGAACACCGCAAG 3′, containing the start sequence of the *Fxacad1* cDNA coding region and carrying a *BamH*I site, and a T3 oligonucleotide primer from the pBluescript KS(+) vector, downstream of the cDNA coding region, were used to amplify the entire FxaCAD1 coding sequence by PCR. The PCR product was digested with *BamH*I and *Pst*I restriction enzymes and cloned 'in frame' into the *BamH*I and *Pst*I sites of the pQE51 (Quiagen) vector producing the pQE51–CAD plasmid. This pQE51–CAD plasmid was introduced into *E. coli* host strains BL21 and SURE. The transformed bacterial cells were grown at 37 °C in Luria–Bertani medium containing 1 mM $ZnCl_2$ and 50 μg ml^{-1} ampicillin until they reached an A_{600} of 0.8–1.0. The cells were cooled to 15 °C and 1 mM isopropyl β-D-thiogalactoside (IPTG) was added to induce the CAD synthesis. The bacteria were grown for another 16 h at different temperatures and then harvested by centrifugation. A pQE51 plasmid without DNA insert was used as a control, and treated in parallel in the same way. Preparation of crude extracts from *E. coli* transformants was performed as follows (Logeman *et al*., 1997): the cells were resuspended in lysis buffer (20 mM TRIS–HCl, pH 7.5; 10% glycerol; 5 mM DTT; 0.1% desoxycholate, DOC; 1 mM phenylmethylsulphonyl fluoride; 1 mM EDTA; 5 μg ml^{-1} leupeptin). Lysozyme was added to a final concentration of 2 mg ml^{-1}, and the cells were incubated at 4 °C for about 1 h. Afterwards, the cells were disrupted in a French-Press (1.200 psi) twice. Cell debris was pelleted by centrifugation and the supernatant was tested for protein induction. For the analysis of total bacterial proteins, aliquots were pelleted in a microcentrifuge, boiled in SDS-lysis buffer (0.1 M, TRIS–HCl, pH 6.8; 1.6% glycerol; 0.008% bromophenol blue; 4 mM EDTA; 10 mM DTT; 3% SDS) for 3 min at 95 °C and loaded on a 12% SDS–polyacrylamide gel. Proteins were visualized by Coomassie blue staining. Protein concentrations were determined spectrophotometrically by the Bradford assay.

The recombinant protein contained in the inclusion bodies was purified by gel electrophoresis in 12% SDS–polyacrylamide gel. The band containing the recombinant FxaCAD1 protein was carefully excised, and the gel slice was used directly as the antigen source for polyclonal antibody production in white rabbits.

To express the *Fxacad1* gene in yeast, the corresponding cDNA was subcloned in frame into the *Not* I site of the pPIC9–MCS vector (Invitrogen) producing the FxaCAD1–pPIC9 plasmid. The new plasmid was digested and linearized with *Stu*I and used to transform by electroporation *Pichia pastoris* GS115 yeast cells, according to the manufacturer's protocol. The recombinant yeast cells were selected and grown according to the manufacturer's protocols. The recombinant FxaCAD1 protein was induced by the continuous addition of methanol (0.5%) for 52 h and following the manufacture's protocol. The presence of recombinant FxaCAD1 protein in the yeast cell culture medium was tested by Western blot procedures using the polyclonal anti-FxaCAD1 antibody (1/500 dilution) previously obtained.

Measurement of CAD activity

The induced recombinant yeast cells were harvested by centrifugation at 5000 *g*, 10 min, 0–4 °C. Then, the culture medium containing the FxaCAD1 recombinant protein was concentrated and dialysed against enzyme storage buffer: Tris–HCl 100 mM, pH 7.5; 5% ethylene glycol; 5 mM DTT, and 50% glycerol.

The activity of the FxaCAD1 enzyme was determined by measuring the decrease in A_{340} due to the production of cinnamyl alcohols at 30 °C according to Somssich *et al*. (1996). The final reaction mixture (1 ml) contained: 34 μM coniferyl aldehyde (or another cinnamyl aldehyde), 0.2 mM NADPH, 100 mM sodium phosphate (pH 6.5), and 500 μl of the enzyme preparation.

Immunolocalization of the strawberry CAD protein and structural studies

For the cytolocalization of the FxaCAD1 polypeptide, tissue sections were prepared as follows: small portions of strawberry fruits, and several vascularized organs (including runners, pedicels, leaves, and flowers), were fixed in ethanol–acetic acid (3:1 v/v), dehydrated through an ethanol–tertiary butanol series, and embedded in Paraplast Plus (Sherwood Med. Co., St Louis, MO). 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 structural studies, sections were stained with $ZnCl_2$–Safranin-Orange G–tannic acid–NH_4–$Fe(SO_4)_2$ as described by Schneider (1981). For immunolocalization purposes, sections were blocked with 2% non-fat dried milk in TBS and immunological detection was then performed using a primary anti-strawberry FxaCAD1 polyclonal antiserum diluted 1/25, 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 for 15–30 min. The sections were dehydrated through graded ethanols, cleared in xylene and mounted in Entellan New (Merck). An Olympus AH-2 (Japan) photomicroscope was utilized for sample visualization and photography.

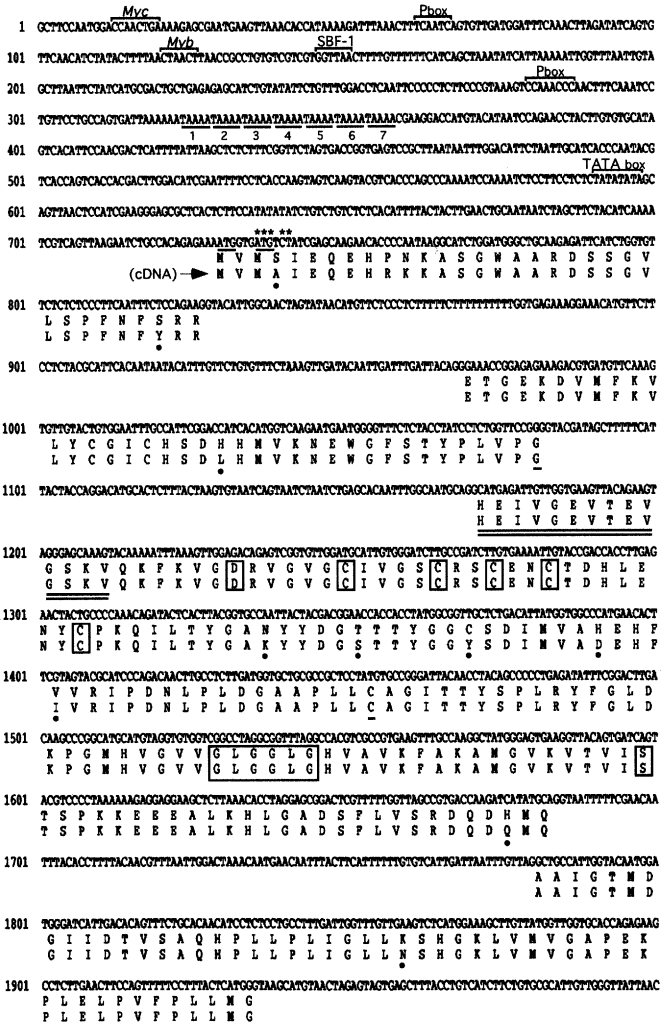
To perform a quantitative study of anti-FxaCAD1 immunostained cells, transversal sections from fruits at the G1, G2 and G3 stages were obtained and the number of positive stained cells per vascular bundle were scored. About 50 vascular bundles were scored for each stage.

Lignin staining

Lignified structures were visualized using the phloroglucinol/HCl test (Weisner reagent). Slices of strawberry fruits were incubated in a solution of 1% phloroglucinol in 70% ethanol until they were totally cleared. Slices were then mounted on slides and a few drops of concentrated HCl were added. The slices of fruit were covered with a coverslip after lignified structures appeared pink-red (about 5 min later), since the colour faded in about 30 min, micrographs were taken immediately (Clifford, 1974).

Results

The *Fxacad1* cDNA sequence is 1401 bp long with one major open reading frame of 360 amino acids extending from nucleotide 112 to 1188 (Fig. 1). The cDNA has a 5′ untranslated region of 111 bases and a 3′ untranslated region of 224 bases including 16 A residues of the poly-(A^+) tail. A putative consensus plant polyadenylation site AATAAA is found in this region and 5′ upstream of this polyadenylation site, a stop codon sequence TTAAA that agrees with the stop codon sequence preferentially utilized



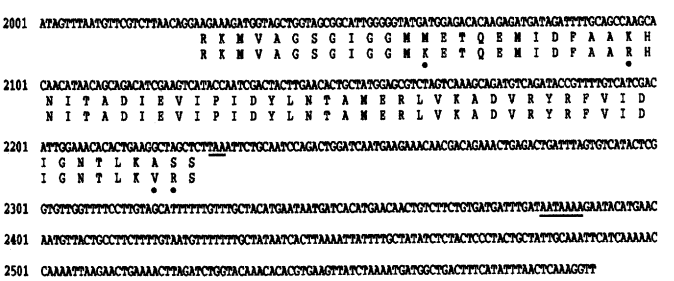


Fig. 1. Nucleotide sequence of the strawberry *Fxacad2* gene. The deduced CAD proteins both from the genomic clone and the isolated *Fxacad1* cDNA are also shown. Several putative binding sites are found in the promoter: *Myc* (Murre *et al.*, 1989), *P box* (Sablowski *et al.*, 1994; Douglas *et al.*, 1996), *Myb* (Sablowski *et al.*, 1994; Feuillet *et al.*, 1995), *SBF-1* (Feuillet *et al.*, 1995), and AT-regions (numbered 1–7, Martínez-García and Quail, 1999). Putative start codons, stop codon and the polyadenylation site are underlined. Asterisks indicate nucleotides matching the consensus start codon found in plants (Gallie, 1993). Dots indicate differences between both deduced proteins. The predicted $NAD^+/NADP^+$ binding consensus motif and the serine residues related to the selection of $NADP^+$ as coenzyme for CAD enzymatic activity found in other CAD proteins from higher plants are boxed. The cysteine residues identified as structural ligands are also boxed. The predicted zinc binding amino acid consensus sequences are double-underlined.

by higher plants, can also be found (Gallie, 1993). The predicted molecular mass of the cDNA-deduced FxaCAD1 protein is 39 367 Da with a *pI* of 6.92. No signal peptide is observed at the N-terminal amino acid sequence. As in the case of *Stylosanthes humilis*, *Arabidopsis thaliana*, *Picea abies*, and *Medicago sativa* a SKL motif, putatively involved in peroxisomal targeting (Gould *et al.*, 1988), is absent in the strawberry CAD protein. However, this motif is conserved in CAD proteins from *Nicotiana tabacum*, *Populus deltoides*, *Eucalyptus gunnii*, *Aralia cordata*, and *Pinus taeda*.

The comparison of the strawberry *Fxacad1* cDNA and protein sequence with the sequences present in the GenBank and EMBL DNA databases and the PIR and SWISPROT databases, revealed a significant identity with other CAD proteins previously cloned that ranged between 57% and 71% and between 53% and 77% at the nucleotide and amino acid levels, respectively. This high degree of consensus between the strawberry CAD amino acid sequence and other sequences corresponding to CAD enzymes from higher plants (Fig. 2), strongly suggests that the isolated strawberry cDNA corresponds to an mRNA encoding a CAD enzyme. Moreover, the results of enzymatic activity (see below) corresponding to the recombinant enzyme, indicates clearly that the cDNA cloned is undoubtedly a CAD enzyme.

By screening a strawberry (*Fragaria*×*annassa* cv. Chandler) genomic library, using the isolated *Fxacad1* cDNA as a probe, a 14 kb genomic clone containing a complete ORF corresponding to a putative strawberry *cad* gene (*Fxacad2*) has been isolated. The *Fxacad2* genomic sequence is presented in Fig. 1. By comparison with the isolated *Fxacad1* cDNA, the gene is structured in five exons and four introns. This molecular structure is identical to that found in other *cad* genes from higher plants (Walter *et al.*, 1994; Baucher *et al.*, 1995; Feuillet *et al.*, 1995; Somers *et al.*, 1995). However, a *cad* gene containing five introns has also been described in Norway spruce (Schubert *et al.*, 1998). The exon–intron junctions of the strawberry *Fxacad2* gene obey the rule AG/GT

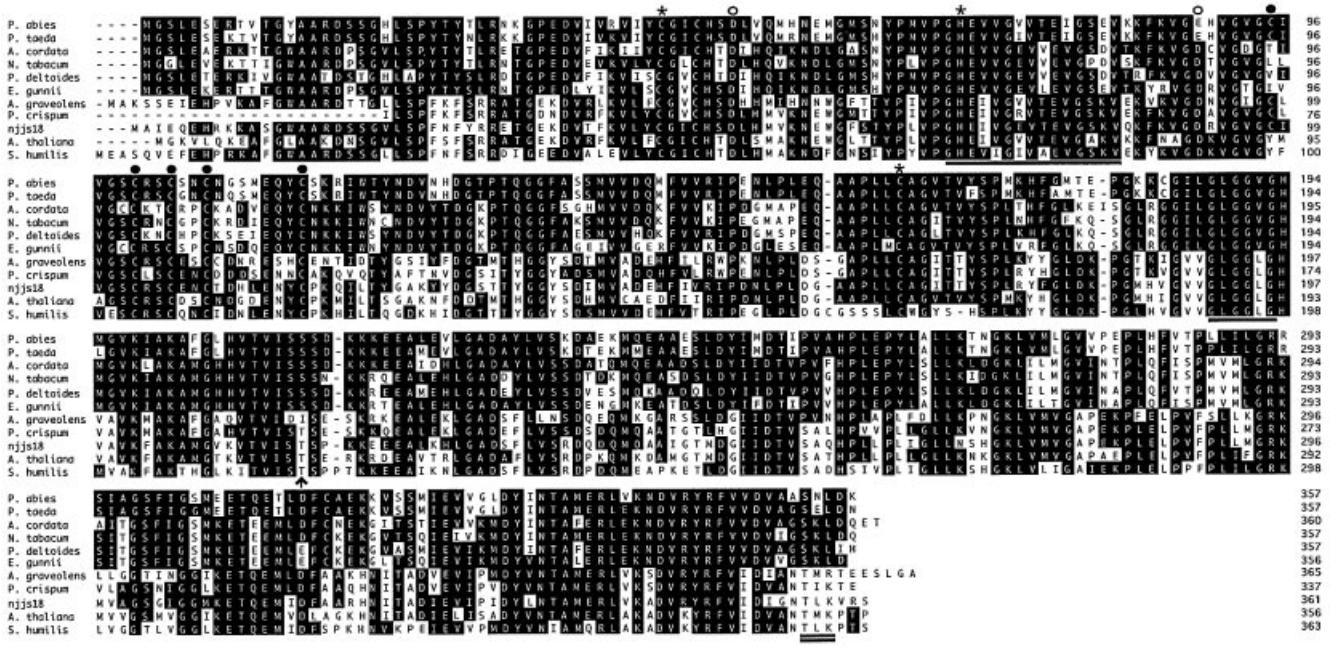


Fig. 2. Comparison of the amino acid sequence of strawberry CAD deduced protein versus amino acid sequence of higher plants zinc-containing alcohol dehydrogenases using the Prettybox program. Consensus amino acids are in black boxes. Conserved amino acids are shaded. The zinc conserved motif and the $NAD^+/NADP^+$ motif is underlined. Asterisk (*) indicates the His-72 and the Cys-47 and Cys-164 residues conserved in all ADHs. Dots indicate the cysteine residues identified as structural ligands in other ADH genes. Arrow indicates the Thr-215 replaced residue in the strawberry putative *cad* gene and also in *P. crispum* and *S. humilis* genes. Open dots indicate the acidic residues involved in binding interactions. The SKL motif, only present in CAD proteins, is double-underlined. The accession numbers are Q08350 for *Picea abies* (P. abies); P41637 for *Pinus taeda* (P. taeda); P42495 for *Aralia cordata* (A. cordata); P30359 for *Nicotiana tabacum* (N. tabacum); P31657 for *Populus deltoides* (P. deltoides); P31655 for *Eucalyptus gunnii* (E. gunnii); U24561 for *Apium graveolens* (A. graveolens); X67817 for *Petroselinum crispum* (P. crispum); U63534 for *Fragaria×ananassa* (njjs18; Fxacad1); X67816 for *Arabidopsis thaliana* (A. thaliana); L36456 for *Stylosanthes humilis* (S. humilis).

found for higher plant genes (Breathnach and Chambon, 1981), and, as for higher plant genes, the introns are relatively AT-rich in comparison with the coding regions. Minor changes in the amino acid sequence could be observed in the deduced protein when compared with that of *Fxacad1* cDNA, which supports the presence of a small *cad* gene family in strawberry. Thus, all the structural characteristics present in this FxaCAD2 protein are identical to that found in FxaCAD1 protein.

Expression of the recombinant protein in E. coli *and* Pichia pastoris *cells*

IPTG induced the expression of the strawberry *Fxacad1* cDNA in *E. coli* at various temperatures ranging from 25–37 °C, resulting in the exclusive accumulation of the FxaCAD1 protein in inclusion bodies, as analysed by SDS–PAGE (data not shown). It has been indicated that this problem could be partly avoided by decreasing the incubation temperature to 15 °C before the addition of IPTG and/or reducing the IPTG concentration to 0.1 mM (Lauvergeat *et al*., 1995). Additionally, cells were grown in the presence of sorbitol (660 mM) and betaine (2.5 mM) to avoid accumulation or storage of protein in inclusion bodies (Blackwell and Horgan, 1991). Resting cells and cells in the stationary phase of growth were also used for induction conditions. However, the recombinant FxaCAD1 protein produced from the pQE51-sCAD plasmid always remained associated with the inclusion bodies. Additionally, no enzymatic activity could be detected in supernatants of disrupted cells. However, the recombinant FxaCAD1 enzyme expressed in *Pichia pastoris* cells had high activity with the characteristic substrates of the angiosperm CAD enzymes. Thus, the high activity observed with cinnamaldehyde (100% activity; *V*max of 0.62 μmol min^{-1} mg^{-1} protein), coniferaldehyde (51.2%) and sinapaldehyde (64.3%), and the low activity detected with other benzylaldehydes (benzaldehyde, 2-methoxy-benzaldehyde, 3-methoxy-benzaldehyde, activity lower than 10%), indicates that FxaCAD1 is a cinnamyl alcohol dehydrogenase.

Gene expression studies

The spatial and temporal expression pattern of the strawberry *cad* gene has been studied. As shown in Fig. 3, the CAD transcript is 1.4 kb, which suggests that the *Fxacad1* cDNA clone is a full-length cDNA. The gene is expressed in fruit, runners, leaves, and flowers, but not in roots. Besides, *Fxacad1* gene exhibits a differential

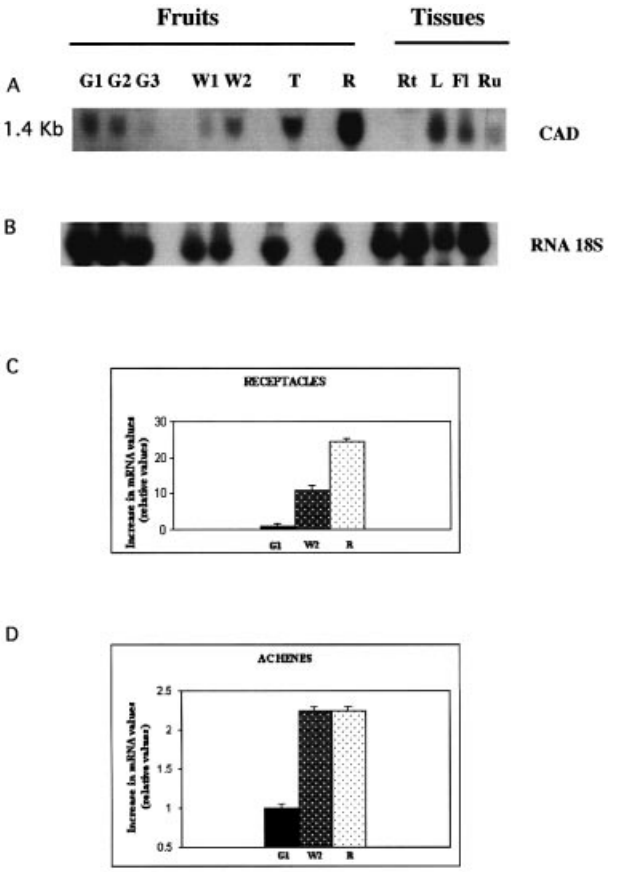


Fig. 3. Developmental and spatial expression of the putative strawberry *cad* gene. (A, B) Northern analysis of 20 μg of total RNA isolated from pooled samples of G1, G2, G3, W1, W2, T, and R strawberry fruit ripening stages and from roots (Rt), leaves (L), flowers (F), and runners (Ru). Size of hybridizing transcript is indicated in kb. (A) Northern blot hybridized with a single-stranded ^{32}P-labelled *Fxacad1* cDNA probe. (B) Northern blot hybridized with a double-stranded ^{32}P-labelled 18S ribosomal probe. (C, D) Expression studies by QRT-PCR from receptacle and achenes corresponding to G1, W2 and R stages. Quantification is based on Ct values as described in Materials and methods. Each QRT-PCR reaction were normalized using the Ct value corresponding to a strawberry *tubulin* gene.

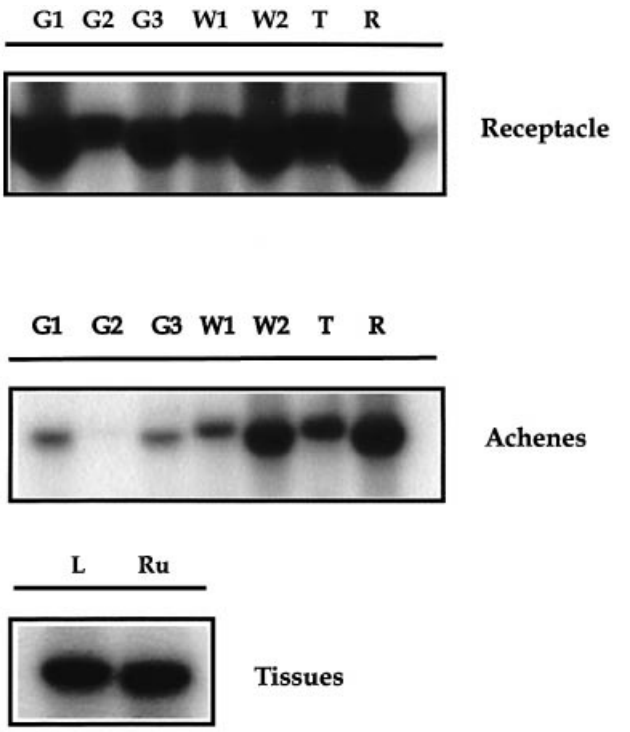


Fig. 4. Southern blot of amplified fragments by RT-PCR studies. Total RNA extracted from the receptacle and achenes corresponding to different stages of fruit growth and ripening (G1, G2, G3, W1, W2, T, and R stages) and from vegetative tissues (L, leaves and Ru, runners). The blot was hybridized with a double-stranded ^{32}P-labelled *Fxacad1* cDNA probe. RT-PCR studies were carried out as described in Materials and methods.

expression pattern during the strawberry fruit ripening process with a clear decrease of the expression during fruit elongation followed by an increase during the ripening process. The maximum level of transcripts of the gene was observed in full-ripe red stage of fruit development (Fig. 3A). This pattern of expression is consistent with a role of the strawberry FxaCAD1 enzyme in the lignification of vascular elements during fruit development, as proposed for CAD activity in higher plants (Walter, 1992). As it is shown in Fig. 3C and D, QRT-PCR studies shown that the increase in the levels of *Fxacad1* mRNA during fruit ripening was mainly due to the increase in *Fxacad1* mRNA in the receptacle.

In order to ascertain if both *cad* genes (*Fxacad1* and *Fxacad2*) are expressed in different fruit (receptacle and achene) and vegetative (leaves and runners) tissues, specific expression studies have been performed by RT-PCR (Fig. 4). The amplified cDNA fragments were subcloned and four independent clones were sequenced in each case. Only sequences identical to that of the corresponding sequence of the *Fxacad1* gene were found (data not shown). These results strongly support that, in strawberry fruit, the *Fxacad1* gene is predominantly expressed. As shown in Fig. 4, the gene is expressed along all the stages of fruit growth and elongation, both in achene and receptacle tissues. In runners and leaves, the results were identical to those found in strawberry fruit, thus showing that only the *Fxacad1* gene is expressed in all the tissues studied.

Some strawberry fruit-specific genes have been shown to be under the control of auxins ((Medina-Escobar *et al*., 1997*a*, *b*; Moyano *et al*., 1998; Manning, 1998). In order to study the effect of auxins on *Fxacad1* gene expression, gene expression analysis was performed on de-achened green fruit (G2 stage). A clear increase in gene expression was detected in fruits after removing the achenes (Fig. 5). However, contrary to the expression of other strawberry genes (Medina-Escobar *et al*., 1997*a*, *b*; Moyano *et al*., 1998; Manning, 1998), this increase was not reversed by

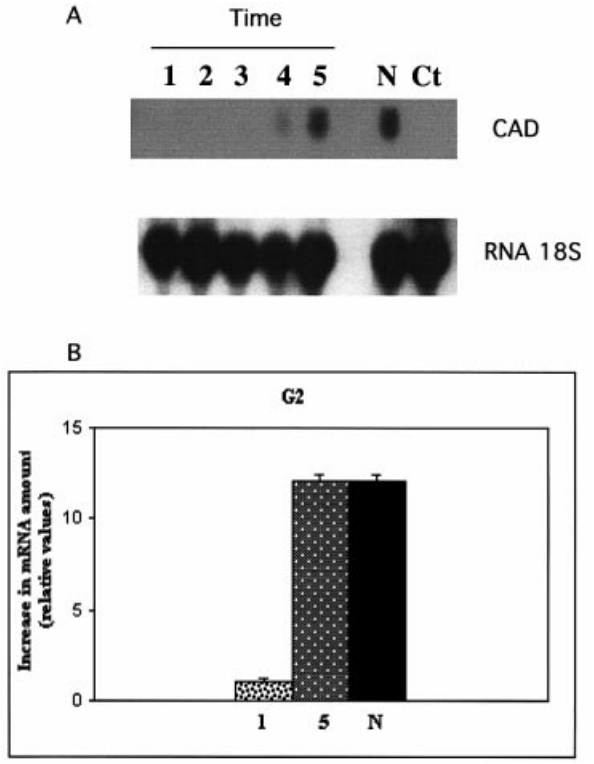


Fig. 5. Effects of removing achenes and treatment with auxins on the *cad* gene expression. (A) Northern analysis of 20 μg of total RNA isolated of G2-stage strawberry fruit after removing the achenes: (1) 0 h; (2) 24 h; (3) 48 h; (4) 72 h; (5) 96 h; (N) 96 h G2-stage strawberry de-achened fruit treated with NAA; (Ct) control fruits, G2-stage strawberry fruit with achenes. Blot was hybridized with a double-stranded ^{32}P-labelled *Fxacad1* cDNA probe. The amount of RNA loaded on the gel was controlled by hybridization of the same blot with a ribosomal probe as in Fig. 3. (B) Expression studies by QRT-PCR. 1, 5 and N as described in (A). Quantification is carried out as in Fig. 3.

the external application of auxins (NAA). Similar RT-PCR studies, as described above, were also carried out in de-achened fruit receptacles (after 5 d of removing the achenes). The sequences found were identical to that of the *Fxacad*1 gene (data not shown).

Southern blot analysis

By using the same probe previously used in the Northern blot studies, several DNA fragments have been detected that hybridized on Southern blots of genomic strawberry DNA digested with *BamH*I, *Bgl*II, *Eco*RI, and *Hind*III (Fig. 6). A minimum of five fragments, except for the *Hind*III lane, with size over 2 kb were detected. However, no restriction sites for *BamH*I, *Bgl*II, and *Eco*RI, were found either in the isolated strawberry *Fxacad1* cDNA sequence or in the *Fxacad2* genomic sequence, and only a restriction site for *Hind*III was present. These results suggest that a small *cad* gene family exists in strawberry.

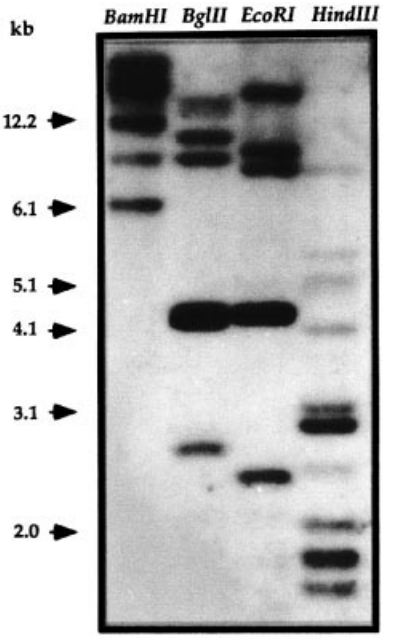


Fig. 6. Southern blot of *Fragaria*×*ananassa* cv. Chandler genomic DNA (2 μg) digested with several restriction enzymes and hybridized with ^{32}P-labelled *Fxacad1* cDNA probe.

Immunolocalization studies

Control experiments verified the specificity of the histochemical reaction, no staining being observed in sections incubated with the pre-immune antiserum (Fig. 7G). As expected, the FxaCAD polypeptide was localized in lignifying tissue in all samples tested (fruit receptacle, achenes, runners, leaves, pedicels, flowers) (Fig. 7). Stained cells corresponding to immature xylem were detected in vascular bundles of flowers and fruits. However, little or no staining was observed in most fully mature tracheary elements (compare Fig. 7I and J, Q and R). Furthermore, the FxaCAD polypeptide was also localized in the sclerenchyma cell layers of the achenes (Fig. 7A, C, E). These cell layers are under progressive lignification during achene development as shown by lignin staining with phloroglucinol (Clifford, 1974) (Fig. 7B, D, F, H), indicating a close relationship between strawberry FxaCAD protein localization and the lignification processes.

In this way, the FxaCAD immunostaining pattern was apparently related to the maturation degree of the achenes. In immature achenes, the FxaCAD polypeptide was first localized in the apical zones of the schlerenchyma cell layer (i.e. opposite the site of the fruit receptacle), and then progressed to the receptacle as the achene matures (Fig. 7A, C, E). Interestingly, the expression level in the apical zones gradually decreased as the immunostaining progressed to the receptacle. The detection of lignin by the phloroglucinol staining technique showed a similar distribution pattern to that of FxaCAD polypeptide. Thus, lignification

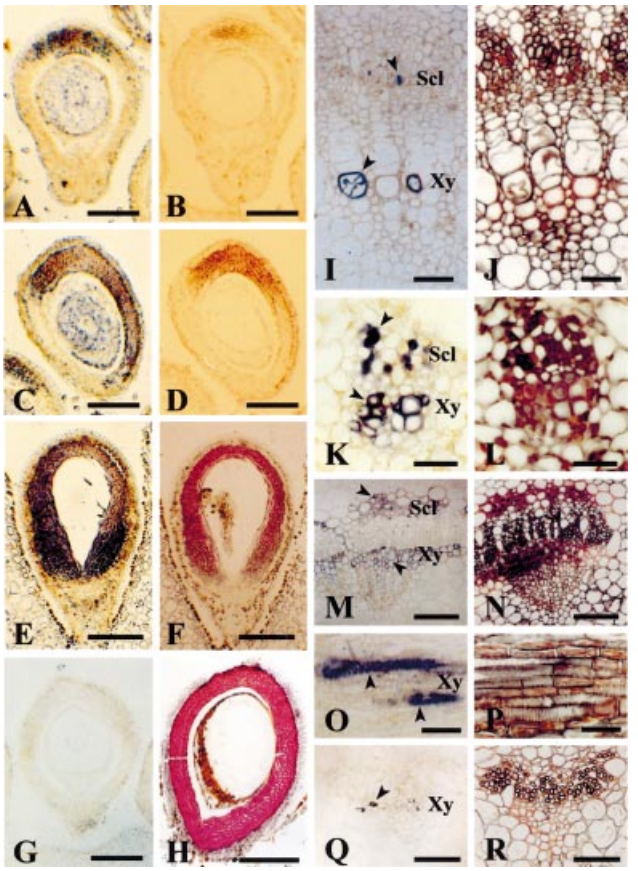


Fig. 7. Immunohistochemical localization of CAD polypeptide in paraplast sections of various strawberry organs. (A, C, E) CAD expression; (B, D, F, H) lignification in achenes of strawberry during fruit ripening using an immunohistochemical technique and the phloroglucinol stain, respectively (see Materials and methods). (G) An achene stained with pre-immune serum as a control. In runners (I) and leaves (K) the reaction is found in phloem-associated sclerenchyma (Scl) and early tracheary elements of xylem (Xy). In pedicels (M) the reaction is found in sclerenchyma (Scl) surrounding the phloem. In both flowers (O) and fruits (Q) inmunopositive cells are found only in tracheary elements of xylem (Xy). (J, L, N, P, R) The structural features of each organ as visualized after staining with ZnCl–Safranin-Orange G. (O, P) Longitudinal sections, while the remaining pictures represent cross-sections of the respective organ. Arrowheads show positively stained cells. Magnifications are as follows: (A–H) bar=250 μm; (I, J, P) bar=50 μm; (K, L) bar=25 μm; (M, N) bar=200 μm; (Q, R) bar=100 μm.

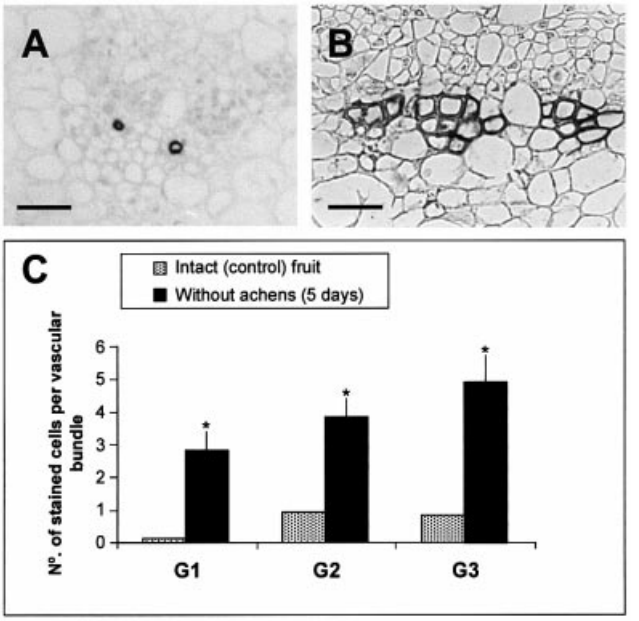


Fig. 8. Increase in the cell CAD protein in de-achened strawberry fruits. Fruits were de-achened at different growth stages and kept on the plant for 5 d after removing the achenes. Afterwards, de-achened and control fruits were studied by immunolocalization. (A, B) Immunolocalization of the CAD polypeptide in vascular bundles: control (A) and de-achened fruits (B) (bar=25 μm). (C) Diagram comparing intact and de-achened fruits at different stages of ripening (mean numbers ±standard error of inmunostained cell per xylem bundle).

apparently starts at the apical zone of the achene and gradually progresses to the receptacle. However, lignin deposition seems to occur once FxaCAD expression ceases (Fig. 7B, D, F, H). Consequently, in the schlerenchyma cell layers of the achene, lignification is preceded by an increase in CAD-expression, and progresses from the apical zone of the organ to the junction with the fruit receptacle.

In runners that had initiated secondary growth and leaves, staining with anti-FxaCAD antiserum was localized in the regions of the developing xylem and phloem-associated sclerenchyma fibres where lignification had started (Fig. 7I, K). A similar pattern of reaction was observed in pedicels. In this case, schelerenchyma fibres, which were also positive for FxaCAD-staining, were located on the external side of the vascular bundles (Fig. 7M). However, in fruit receptacles and flowers, anti-FxaCAD antiserum was found only in xylem vessels (Fig. 7O, Q).

Consistent with the increase in *Fxacad1* mRNA expression in de-achened fruits (see above), an increase in the number of stained cells with the anti-FxaCAD antibody in the vascular bundles of G1, G2 and G3 de-achened fruits compared with vascular bundles of normal fruits, was found (Fig. 8). These results indicate a close relationship between the observed increase in the expression of the strawberry *cad* gene after removing the achenes of the fruit, and the increase of the FxaCAD protein presence in vascular bundles of these fruits. Additionally, histochemical studies revealed the presence of lignin in the same cell types that showed immunostaining. This also supports a clear relationship between strawberry FxaCAD protein and lignification.

Discussion

For the first time in fruits, a strawberry cDNA clone (*Fxacad1*) complementary to an mRNA that encodes a

CAD enzyme has been studied. In addition, a strawberry *cad* gene (*Fxacad2*) has been cloned. Moreover, the spatial and temporal expression pattern of the strawberry *Fxacad1* gene in different organs of the plant and through the fruit ripening process has also been studied and supports a role for this FxaCAD1 enzyme in lignification.

The presence, in the strawberry FxaCAD1 deduced protein, of consensus motifs conserved in other CAD proteins of higher plants is in agreement with a CAD role for the deduced FxaCAD1 strawberry protein. Thus, the consensus amino acid sequence GHEXXGXXXXXGXXV found between residues 69–83 indicates that the strawberry CAD protein can be classified as a zinc-containing alcohol dehydrogenase (Vallee and Aulds, 1990; Knight *et al*., 1992; Galliano *et al*., 1993; Grima-Pettenati *et al*., 1993; Hibino *et al*., 1993). A His-70 adjacent to Gly-69 residue that could be involved as a second ligand of the catalytic zinc atom is also present in this consensus sequence (Grima-Pettenati *et al*., 1993). The Cys-164, conserved in all CADs, is probably related to a zinc binding site (Vallee and Aulds, 1990; Knight *et al*., 1992; Galliano *et al*., 1993; Grima-Pettenati *et al*., 1993; Hibino *et al*., 1993). Moreover, there is a GXGXXG consensus sequence, in the strawberry protein, which is also present in all CAD proteins previously studied. This sequence has been proposed as an NADP+ binding domain and is usually located, as in the strawberry protein, between the amino acid residues 189–194 (Knight *et al*., 1992; Galliano *et al*., 1993; Grima-Pettenati *et al*., 1993). Cysteines 96, 101, 104, 107, and 115 have been identified as structural ligands (Vallee and Aulds, 1990; Galliano *et al*., 1993; Grima-Pettenati *et al*., 1993). Also, the As*p*-90 would be putatively involved in the coenzyme binding (Vallee and Aulds, 1990; Galliano *et al*., 1993; Grima-Pettenati *et al*., 1993). The residue Ser-212 conserved in higher plants CAD, has been suggested to be related to the selection of $NADP^+$ as a coenzyme for CAD enzymatic activity, thus determining the cofactor specificity of CAD enzymes and it is also present in the strawberry CAD protein (Vallee and Aulds, 1990; Knight *et al*., 1992; Grima-Pettenati *et al*., 1993). All these structural characteristics are also present in FxaCAD2 deduced protein and indicate that both *Fxacad* genes are likely to code for cinnamyl alcohol dehydrogenase proteins. Moreover, the expression of the *Fxacad1* cDNA in *Pichia pastoris* cells produced a recombinant protein that showed activity with characteristic substrates of CAD enzymes from angiosperms, clearly indicating that the strawberry *Fxacad1* cDNA codes for a cinnamyl alcohol dehydrogenase enzyme.

The immunodetection experiments showed that, in all tissues studied, the strawberry CAD polypeptide was exclusively localized in cells undergoing lignification, such as xylem and phloem-associated sclerenchyma cells. In poplar, *cad* gene expression has been shown in vascular cambium/differentiating xylem as well as in ray cells of parenchyma and in lignifying cells in the bark (phloem fibre cells, sclereids and periderm). Besides, CAD promoter–GUS constructions have revealed the same sites of expression (Feuillet *et al*., 1995; Hawkins *et al*., 1997). Moreover, also in poplar, a eucalyptus CAD promoter fused to a GUS gene reporter drove the expression of the reporter gene in xylem ray and parenchyma cells surrounding the lignified phloem and sclerenchyma fibres. This suggests that parenchyma cells expressing CAD may provide lignin precursors to the adjacent lignified elements (vessels and fibres) (Feuillet *et al*., 1995). Thus, a close relationship between *cad* gene expression, CAD activity and the lignification process has been observed. Consequently, it was suggested that, in the strawberry plant and fruit, the FxaCAD enzyme could be involved in lignification processes related to both vasculature development and achene maturation. In this sense, it has been shown that in strawberry fruit, the peroxidase activity (the enzyme following CAD in the lignin biosynthesis pathway) is mainly localized in the concentric array of the vascular bundles, in the vascular connections with the seeds and in the epidermal cells, which could suggest a possible role in the lignification of xylem vessels of the strawberry receptacle (López-Serrano and Ros-Barceló, 1995).

Southern blot analysis has shown that more than one *cad* gene exists in strawberry. Accordingly, a genomic *cad* gene (*Fxacad2*) has been isolated whose deduced protein presents only minor amino acid changes within its sequence compared with the *Fxacad1* cDNA.

The data on gene expression determined by QRT-PCR showed that the increase in *Fxacad1* gene expression during the stages of fruit ripening is mainly due to a clear increase in the expression of this gene in receptacle tissue, though expression in achene tissue is also observed.

Thus, the increase in gene expression along the ripening stages of the fruit could be related to the development of the vascular tissue of the receptacle as the fruit size is increasing. Furthermore, the immunolocalization and the QRT-PCR data suggest a relationship of the strawberry FxaCAD1 enzyme with the lignification process of the achene as it matures and vegetative tissues. Thus, the mature achene contains a lignified and relatively thick pericarp (Perkins-Veazie, 1995).

It has been proposed that auxin stimulates the expansion of the receptacle and inhibits ripening in strawberry fruit (Perkins-Veazie, 1995). Moreover, an induction pattern of mRNA populations in inmature de-achened fruit was found to be reversed by the application of the synthetic auxin NAA to de-achened fruit receptacles (Perkins-Veazie, 1995). Also, a large increase in gene expression after the removal of achenes has been shown for several fruit-specific, ripening-related strawberry genes. These increases were partially reversed by NAA application to de-achened receptacles, supporting a relationship between

ripening-induced gene expression and the absence of auxin (Medina-Escobar *et al*., 1997*a*, *b*; Moyano *et al*., 1998; Manning, 1998). Here, however, although the strawberry *Fxacad* gene expression was largely increased in de-achened fruits, it was not reversed by the application of external auxin (Fig. 5).

The *eli3* genes, a novel type of pathogen induced benzyl alcohol dehydrogenases, BADs, have been recently described in several higher plants (Trezzini *et al*., 1993; Somssich *et al*., 1996). The *eli3* genes are strongly activated both in elicitor-treated cells and in fungal infection sites, but not in lignifying tissue (Schmelzer *et al*., 1989). These enzymes exhibit high amino acid sequence similarity with CADs from various higher plants. While these BADs efficiently converted several cinnamyl aldehydes, using NADPH as the co-substrate, one characteristic substrate of most previously analysed angiosperms CADs, sinapaldehyde, was not reduced (Logeman *et al*., 1997; Somssich *et al*., 1996). Moreover, coniferyl aldehyde, another characteristic substrate of CADs, was only poorly converted by BADs. Thus, both substrate specificity and the pathogen induction pattern of BADs suggest that these enzymes are not involved in the lignification process. Thus, a close association with local defence gene expression at pathogen infection sites and genetically determined disease resistance for BAD enzymes has been demonstrated (Logeman *et al*., 1997). However, although the strawberry *cad* genes sequences show a high sequence homology with that of the *eli3* genes, the cellular localization of strawberry CAD and the fact that the strawberry *cad* gene can use sinapaldehyde and coniferyl aldehyde as substrates and is not induced by salicylic acid, a known inductor of *eli 3* genes (data not shown), refutes the suggestion that the gene cloned could be an *eli*-like gene.

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