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Cloning and characterization of avocado fruit mRNAs and their expression during ripening and low-temperature storage

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Abstract

Differential screening of a cDNA library made from RNA extracted from avocado (*Persea americana* Mill cv. Hass) fruit stored at low temperature (7 °C) gave 23 cDNA clones grouped into 10 families, 6 of which showed increased expression during cold storage and normal ripening. Partial DNA sequencing was carried out for representative clones. Database searches found homologies with a polygalacturonase (PG), endochitinase, cysteine proteinase inhibitor and several stress-related proteins. No homologies were detected for clones from six families and their biological role remains to be elucidated. A full-length cDNA sequence for avocado PG was obtained and the predicted amino acid sequence compared with those from other PGs. mRNA encoding PG increased markedly during normal ripening, slightly later than mRNAs for cellulase and ethylene-forming enzyme (EFE). Low-temperature storage delayed ripening and retarded the appearance of mRNAs for enzymes known to be involved in cell wall metabolism and ethylene synthesis, such as cellulase, PG and EFE, and also other mRNAs of unknown function. The removal of ethylene from the atmosphere surrounding stored fruit delayed the appearance of the mRNAs encoding cellulase and PG more than the cold storage itself, although it hardly affected the expression of the EFE mRNA or the accumulation of mRNAs homologous to some other unidentified clones.

Introduction

Avocado is a tropical fruit with a climacteric pattern of ripening which is initiated after harvest. The time-lapse between harvesting and the preclimacteric minimum or the climacteric peak is a function of the state of maturity of the fruit and the conditions of storage, such as temperature

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X66426.

and the rate of air flow [6]. As with other climacteric fruit, avocado ripening is related to an increase in ethylene synthesis due to an increase in the activity of two enzymes, 1-aminocyclopropane-1-carboxylic acid synthase (ACC-synthase) and EFE [9]. Changes in the activities of several enzymes including cellulase, PG, pectinmethylesterase and acid phosphatase have been described during avocado ripening [5, 31, 45] and changes in the expression levels of a number of mRNAs have been demonstrated [15], including cellulase [16], cytochrome P-450 oxidase [7], EFE [39] and others of unknown function [15].

Recent studies have shown that the increase in ethylene production in ripening avocado is accompanied by a rise in EFE mRNA [39]. The expression of cellulase at the enzyme and gene level has been most widely studied owing to its importance in cell wall metabolism in avocado ripening. Cellulase is present at low levels in preclimacteric avocado fruit [32], is induced by ethylene [16] and its activity is suppressed in low O_2 atmospheres [31]. Fewer studies of PG gene expression have been undertaken, since the gene has not yet been isolated. PG is involved in cell wall metabolism, acting in cell wall polyuronide degradation during ripening [8]. It is known that PG activity is affected by low O_2 atmospheres to a lesser extent than cellulase activity and that PG is not induced by ethylene as much as cellulase [31].

Storage at low temperatures has been used to extend the post-harvest life of fruits, although temperatures below 10 °C may lead to chilling injury in tropical and subtropical fruits [38]. The critical temperature for avocado cv. Hass is believed to be about 5 °C, but long exposure at chilling temperatures is required before cells show signs of serious injury. The mechanism by which ripening is delayed at low temperatures has not yet been elucidated, although it could be related to a decrease in respiratory rate and general metabolic activity [38]. Alterations in protein profiles are associated with low temperature [30, 36]. New mRNAs are synthesized [29] and both stimulation and inhibition of gene expression have been noted at low temperatures in spinach leaves [25], alfalfa seedlings [40] and tomato fruit [47, 57]. Only a few cold-induced proteins have been cloned, however, including a thiol protease related to actinidin and papain from ripening tomatoes [47] and a low-temperature-induced gene from barley, suggested to be involved in a generalised stress response [22].

The aim of the work described in this paper was to analyse the effect of low temperatures, above and below the temperature for chilling injury, on mRNAs encoding enzymes involved in avocado ripening, to identify new cDNA clones for ripening-related mRNAs, and study the changes in their expression during normal ripening and low-temperature storage. Since ethylene has been implicated in the control of ripening process, studies of mRNA expression in fruit stored with ethylene removal were also conducted.

Materials and methods

Plant material

Frozen avocado (*Persea americana* Mill. cv. Hass) fruit samples were shipped from Instituto del Frío (Consejo Superior de Investigaciones Científicas, Madrid). Fruit were harvested from one commercial orchard in Southern Spain (Málaga) and 24 h after harvest the fruit were stored at 20 °C, 7 °C, 3 °C and at 7 °C and 3 °C in a low-ethylene atmosphere, obtained by keeping the fruit in sealed glass containers ventilated continuously with ethylene-free and carbon dioxide-free air at about 6 l/h, treated with 'Green Keeper' sachets (Promarket, Spain). Fruit samples were collected periodically and frozen in liquid nitrogen, as were samples of unripe freshly harvest avocados.

RNA extraction and poly(A)$^+$ purification

A method for large-scale isolation of total RNA from avocado fruit tissue was developed. 20 g

batches of fruit tissue were frozen in liquid nitrogen, ground to a fine powder using a coffee mill and homogenized in 80 ml of extraction buffer (containing 1% SDS, 30 mM EGTA, 5 mM DTT, 200 mM boric acid in water pH 9) previously heated to 100 °C. The solid debris was removed by centrifugation at 30000 × *g* for 30 min at 4 °C and the filtered aqueous phase partitioned twice against phenol/chloroform. Contaminating carbohydrates were removed by repeated batch washing in Sigmacell cellulose T-50 (Sigma). The solutes in the aqueous phase were precipitated with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol at −20 °C. The precipitated material was collected by centrifugation and the pellet suspended in a small volume of sterile distilled water. The RNA was then precipitated twice by the addition of 0.25 volume of 10 M LiCl and then ethanol-precipitated. Some RNA samples required further cleaning by binding to a hydroxylapatite column (BioRad) in 0.01 M sodium phosphate buffer pH 6.8, elution with 0.1 M sodium phosphate buffer pH 6.8 and precipitation with 6 M sodium acetate pH 5.6. Finally, the RNA samples were washed twice with 50 mM potassium acetate in 80% cold ethanol, once in 80% ethanol and once in 95% ethanol, dried and resuspended in sterile distilled water at a concentration of 5–10 μg/μl and stored at −70 °C.

Poly(A)$^+$ mRNA was purified from total RNA by chromatography on Oligo dT cellulose (Pharmacia) columns. The quality was tested by *in vitro* translation using a nuclease treated reticulocyte lysate from Promega (Promega, USA) according to the manufacturer's protocol.

Construction of a cDNA library from avocado fruits

An avocado fruit cDNA library was made from a poly(A)$^+$ mRNA population consisting of a mixture of equal amounts of poly(A)$^+$ mRNA isolated from RNA extracted from fruit after 25 and 56 days of storage at 7 °C using a λZAP-cDNA synthesis kit (Stratagene) according to the manufacturer's protocol. After digestion with *Xho* I and size fractionation, cDNA was directionally ligated into the Uni-ZAP XR vector and packaged using the Gigapack II gold packaging extract. The resulting library was maintained by freezing plate-amplified stocks in 15% DMSO at −70 °C. Small aliquots were maintained at 4 °C for differential screening experiments.

Differential screening of the library

Duplicate lifts to nylon membranes (Hybond N + from Amersham) were made from agar plates containing 30000–40000 individual recombinant plaques following the Amersham protocol. Labelled single-strand cDNA was used as probe. 500 g of poly(A)$^+$ mRNA was used as template for the reverse transcriptase enzyme in the presence of 1.85 MBq of ^{32}P-dCTP (110 TBq/mmol). One of the two identical filters was hybridized to the probe corresponding to the poly(A)$^+$ mRNA fraction of prestored fruit and the other to a similarly labelled probe corresponding to the poly(A)$^+$ mRNA from which the cDNA library was constructed (an equal mixture of RNA from 25 and 56 days of storage at 7 °C). The hybridization experiment was performed at 65 °C following the Amersham protocol for Hybond N + filters. After hybridization, the filters were washed in 0.5 × SSPE at 65 °C, exposed to X-ray film, and photographed. Plaques that showed appreciable differences (either an increase or decrease) in intensity of the autoradiograph signal between the two probes used, were identified as 'positives' and subsequently isolated and purified. *In vivo* excision of pBluescript SK- from Uni-ZAP XR vector was performed, with every 'positive' obtained after the second differential screening, following the manufacturer's instructions (Stratagene). The phagemid colonies obtained were then rescreened to confirm the differential expression. Glycerol stocks of 'positive' bacterial culture were made and stored at −70 °C.

Cross-hybridization by slot blot analysis was performed. The plasmids (10 μg) purified from the 'positive' colonies by the alkaline lysis method [46] were denatured and transferred to a nylon

membrane (Hybond N, Amersham) in a Bio Dot SF microfiltration apparatus (BioRad) following the manufacturer's instructions.

DNA sequencing and characterization of cDNA clones

The insert sizes of cloned cDNAs were determined by PCR using T3 and T7 primers and Taq DNA polymerase (NBL).

DNA clones were sequenced using the Pharmacia T7 Sequencing kit following the manufacturer's protocol. The sequence manipulation was carried out using the ESEE program [12], and the sequences obtained were sent to EMBL and GenBank DNA databases for homology searching. Provisional amino acid sequences from each clone, derived by translation of the cDNA strand corresponding to the mRNA (5′→3′ direction) in all three reading frames, were sent to the Swiss Prot EMBL databank, to confirm the nucleotide sequence homologies. If no homologies were found for the nucleotide sequence, possible homologies for the amino acid sequences of the protein were checked.

RNA blot hybridization with cDNA clones

Slot blot analysis was performed on all of the RNA samples extracted from avocado fruit tissue (prestored, stored at 3 °C and 7 °C, with and without the removal of ethylene, and normal ripening at 20 °C). The total RNA samples (10 μg) were denatured by the glyoxal method [46] and then transferred to a nylon membrane (Hybond-N from Amersham) in a Bio Dot SF microfiltration apparatus (BioRad) following the manufacturer's instructions.

The inserts from our cDNA library were isolated by digestion with the restriction enzymes *Eco* RI and *Xho* I followed by electrophoresis in 1% agarose gels in 1 × TAE buffer. After staining with ethidium bromide the inserts were purified from the agarose using the QIAEX gel extraction kit (Qiagen) following the manufacturer's instructions. cDNA inserts were also isolated from the plasmids of the avocado ripening-related clones pAVOe6 (cellulase) and pAVOe3 (avocado equivalent of tomato EFE clone [26]) supplied by Dr Rolf E. Christoffersen, University of California, Santa Barbara, after enzymatic digestion with *Pst* I. Purified cDNA inserts were labelled by nick translation (Boehringher Mannheim kit) except SBA15, which was labelled by incorporation of ^{32}P-dUTP into an RNA copy transcribed from the DNA template by the T7 RNA polymerase using the Riboprobe Gemini system (Promega). The Hybond-N filters were prehybridized and hybridized at 65 °C following the manufacturer's instructions and after hybridization the filters were washed in 0.5 × SSPE at 65 °C, exposed to X-ray film, and photographed.

Results

RNA extraction

While small amounts of tissue (2 to 4 g) could be successfully extracted from cv. Hass using the method described by Christoffersen *et al.* [15], the yield of RNA was low and insufficient for the later production of cDNA libraries. Extraction of larger amounts of tissue resulted in a copurification of excessive amounts of soluble carbohydrate and lipids that made subsequent purification difficult. We devised a novel and efficient protocol for the rapid extraction of pure RNA samples from 20 g samples of frozen fruit. Yields of RNA were between 80 to 180 μg per g fresh weight tissue. Electrophoresis of the samples in 1.5% agarose gels and subsequent staining with ethidium bromide showed discrete ribosomal bands under UV illumination, indicating the RNA to be un-degraded, with no detectable DNA or tRNA (data not shown).

cDNA library and insert size

The cDNA library produced with poly(A)$^+$ mRNA from fruit after storage at 7 °C contained 1.2×10^6 recombinants, with 2–5% of colonies

containing no inserts. The inserts from a random sample of the clones, ranged in size from 0.3 to 4.0 kb.

Library screening for clones encoding mRNAs affected by low temperature

After two rounds of differential screening and plaque purification, followed by *in vivo* excision we isolated 43 colonies with plasmids containing cDNA inserts homologous to mRNAs that changed in abundance during cold storage. mRNA homologous to 26 of these appeared or increased during cold storage at 7 °C. The other 17 decreased during cold storage, being more represented in prestored samples than after 25 or 56 days storage at 7 °C. We present here the result of the analysis of the 26 cDNA clones (SBA1–SBA26) that appeared to encode RNAs that accumulate during cold storage.

Characterization of cDNA clones

Two of the families obtained from the cross-hybridization analysis F1 (SBA1) and F4 (SBA4 and SBA12) had sequences which corresponded to different regions of 26S ribosomal RNA. The remainder 23 clones could be grouped into 10 families (designated F2, F3 etc, Table 1). Two clones SBA15 and SBA25 only had inserts of ca. 100 bp but the others were in the range 450–1800 bp.

The results obtained after total (F3 clones and SBA13, SBA15, SBA19 and SBA25) or partial (the remaining clones) sequencing from both 3′ and 5′ ends of at least one of the clones from every family and homology searches in the EMBL and GenBank DNA databases are summarized in Table 1. Except in the case of F3, in which we know that SBA3 and SBA24 are full-length clones, no further studies have been carried out on the remaining families.

Clones from four families, F3 (PG), F2 (proteinase inhibitor), F10 (thaumatin-like protein) and F18 (endochitinase), showed homology with sequences stored in the databases. cDNA clones SBA3, SBA22, SBA23, SBA24 (F3, Table 1) were found to have homology to tomato PG and were completely sequenced (Fig. 1). SBA3 and SBA24 were apparently full-length clones with an open-reading frame encoding a 462 amino acid polypeptide. The avocado PG showed 88.3% similarity (58% identity) by FASTA alignment [42] to the tomato polygalacturonase precursor protein (Fig. 2, accession number P05117, [23]) over a region of 409 amino acids and also showed a high similarity (80.4%, 40.2% identity) with the polygalacturonase from *Oenothera* pollen (accession number P24548, [11]). The overall similarity between the predicted tomato and *Oenothera* amino acid sequences and the avocado fruit polygalacturonase contrasts with the 60% similarity between the tomato sequence and that from maize pollen [44].

The nucleotide homology search of the whole inserts from SBA13 and SBA19 (F2) revealed 60.6% identity over 411 bp with orizacystatin-I, a cysteine proteinase inhibitor from rice (accession number J03469, [2]) and more than 90% similarity at the predicted amino acid sequence level with both the cysteine proteinase inhibitor-I and -II from rice (accession number P09229, [2] and P20907, [35] respectively) (Fig. 3).

The SBA10 (F10) nucleotide search showed homologies at the 3′ end with a thaumatin-like protein and some other stress-related proteins. These homologies, which in each case started at the same point and involved a 130 bp sequence, are: tobacco pathogenesis-related protein (accession number X12739, [41], 72.1% identity); tobacco E2 thaumatin-like protein (accession number X15223, [55], 70.5% identity); tomato NP24 salt-induced protein (accession number M21346, [33], 70.6% identity) and an *Atriplex* osmotin-like protein (accession number M84467, [13], 69.5% identity). The amino acid homology searches indicated that both the 3′ (80.9% similarity, 53.2% identity in a 47 amino acid sequence) and 5′ (95.5% similarity, 82.2% identity for a 45 amino acid sequence) ends match with the tobacco pathogenesis-related protein. The 5′ end amino acid sequence also showed a

Table 1. Characterization and expression of mRNAs homologous to avocado cDNA clones.

Family	Clone	Insert size	Base pairs sequenced (5′/3′)	Databank searches	Expression pattern of RNA
F2	SBA2	950		Cysteine proteinase inhibitor (oryzacystatin) [2]	Absent in prestored tissue. Increase during normal ripening. Delayed by 7 °C storage and even more at 3 °C. The amount is reduced in ethylene-free atmosphere. (Same pattern as F10, see Fig. 3)
	SBA5	650			
	SBA6	650			
	SBA7	650			
	SBA13	458	458*		
	SBA17	500			
	SBA19	458	458*		
F3	SBA3	1770	1770*	Polygalacturonase from tomato [25]	Absent in prestored tissue and 3 °C storage. Increases during normal ripening. Delayed by cold storage at 7 °C and even more in the absence of ethylene (see Fig. 3).
	SBA22	1196	1196*		
	SBA23	1420	1420*		
	SBA24	1770	1770*		
F8	SBA8	1800	140/140	No significant homologies	Not determined
F9	SBA9	950	370/300	No significant homologies	Absent in prestored tissue. Increases during cold storage but delayed with respect to normal ripening (see Fig. 3)
	SBA11	950	370/300		
	SBA16	650			
	SBA20	900			
F10	SBA10	900	232/320	Thaumatin-like protein [58]	Absent in prestored tissue. Increases during normal ripening. Delayed by 7 °C storage and even more at 3 °C storage. The amount is reduced to the same extent in ethylene-free atmosphere (see Fig. 3)
F14	SBA14	700	331/244	No significant homologies	Absent in prestored tissue and 3 °C storage. Increases in normal ripening. Delayed by 7 °C storage and even more in absence of ethylene (same pattern as F3, see Fig. 3)
	SBA21	685	316/244		
F15	SBA15	111	111*	No significant homologies	Present in every kind of tissue
F18	SBA18	1300	267/336	Endochitinase [56]	Absent in prestored tisue. Increases during normal ripening. Delayed by 7 °C storage and even more at 3 °C. Delayed more at 7 °C in ethylene-free atmosphere. (Same pattern as F10, Fig. 3 except that appears after 56 days in ethylene-free atmosphere at 7 °C)
F25	SBA25	114	114*	No significant homologies	Not determined
F26	SBA26	1300	220/215	No significant homologies	Not determined

* Whole insert sequenced.

high degree of homology with an α-amylase/trypsin inhibitor from maize (accession number P13867, [43]) (87.8% similarity, 63.3% identity).

The cDNA clone SBA18 (F18) showed different homologies at the 3′ and 5′ ends. The 5′ end showed 94.5% identity for a sequence of 217 nucleotides with mitochondrial 26S ribosomal RNA from different plants. The 3′ end showed homology with endochitinases from different organisms, including rice (accession number X54367, [28], 71.8% identity for a sequence of 201 nucleotides) and barley (accession number X15349, [53], 68.4% identity for a sequence of 206 nucleotides). For dot blot experiments with this clone, hybridization was carried out with an 800 bp 3′ *Eco* RI/*Xho* I fragment from this insert.

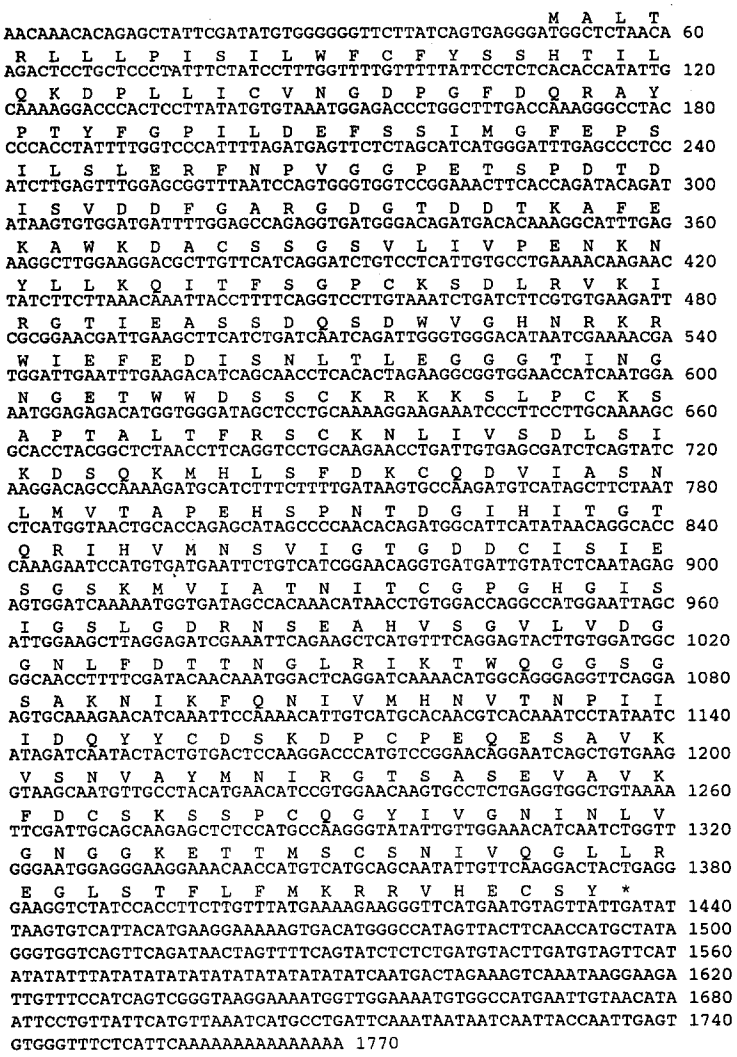

Fig. 1. Nucleotide sequence of SBA24, the polygalacturonase cDNA clone of avocado and predicted amino acid sequence of avocado polygalacturonase.

Effect of low-temperature storage and ethylene removal on mRNA expression

Slot blot hybridizations of total RNA extracted from the different avocado tissues were carried out using purified cDNA inserts as probes. mRNA homologous to SBA3, encoding PG was not present in prestored tissue, appeared after 6 days at 20 °C and increased markedly after 9 days (Fig. 4A). At 3 °C PG mRNA was either undetectable or present at only a very low level, with or without ethylene. Cold storage at 7 °C delayed its appearance and diminished the levels, appearing first after 25 days at 7 °C, and increasing to the end of storage period (62 days). The removal of ethylene from the atmosphere at 7 °C further delayed the appearance of the PG mRNA, which was only detected after 56 days storage. Only one other family (F14) showed the same pattern of expression (data not shown).

In contrast, cellulase mRNA was present in prestored tissue at a very low level and started to accumulate after 4 days at 20 °C, rising rapidly up to 6 days of storage and then increased more slowly up to 10 days (Fig. 4B). Storage at 7 °C

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pgavo                          MALTRLLLPISILWFCFYSSHTILQKDPLLICVNGDPG   38

pgtom                                      MVIQRNSILLLIIIFASSISTCRSNV   28

pgavo  FDQRAYPTYFGPILD-EFS-SIMGFEPSI-LSLERFNPVGGPETSPDTDISVDDFGARGD   95
       :|:: :   :: ||: ||: :: :: : :  ::|: | ::  :::  : |:| :|||:||
pgtom  IDDNLFKQVYDNILEQEFAHDFQAYLSYLSKNIESNNNIDKVDKNGIKVINVLSFGAKGD   86

pgavo  GTD-DTKAFEKAWKDACSSGSV--LIVPENKNYLLKQITFSGPCKSDLRVKIRGTIEASS  152
       |:: |: |||:||::|||| :   ::||:|||||||||||||||:|:::||| |::|||
pgtom  GKTYDNIAFEQAWNEACSSRTPVQFVVPKNKNYLLKQITFSGPCRSSISVKIFGSLEASS  146

pgavo  DQSDWVGHNRKRWIEFEDISNLTLEGGGTINGNGETWWDSSCKRKKSLPCKSAPTALTFR  212
       : ||:  ::|: ||:|::: ||:::|||||||||::|| |||| :||||||:|||||||:
pgtom  KISDY--KDRRLWIAFDSVQNLVVGGGGTINGNGQVWWPSSCKINKSLPCRDAPTALTFW  204

pgavo  SCKNLIVSDLSIKDSQKMHLSFDKCQDVIASNLMVTAPEHSPNTDGIHITGTQRIHVMNS  272
       :|||| |::|: |::|::|::|::| :|:|||||::|:::|||||:|:::|| |:: ::
pgtom  NCKNLKVNNLKSKNAQQIHIKFESCTNVVASNLMINASAKSPNTDGVHVSNTQYIQISDT  264

pgavo  VIGTGDDCISIESGSKMVIATNITCGPGHGISIGSLGDRNSEAHVSGVLVDGGNLFDTTN  332
       :|||||||||| ||| | |||||||||||||||||: ||||:||:| |:::::::::|
pgtom  IIGTGDDCISIVSGSQNVQATNITCGPGHGISIGSLGSGNSEAYVSNVTVNEAKIIGAEN  324

pgavo  GLRIKTWQGGSGSAKNIKFQNIVMHNVTNPIIIDQYYCDSKDPCPEQESAVKVSNVAYMN  392
       |:|||||||||| |:|||| |: |::|: |||||| ||| : :|| :| |||:|:||:| |
pgtom  GVRIKTWQGGSGQASNIKFLNVEMQDVKYPIIIDQNYCDRVEPCIQQFSAVQVKNVVYEN  384

pgavo  IRGTSASEVAVKFDCSKSSPCQGYIVGNINLVGNGGKETTMSCSNIVQGLLREGLSTFLF  452
       |:||||::||:|||||:: ||:| |::|||||||:|| :: :|:|:  :   :: :
pgtom  IKGTSATKVAIKFDCSTNFPCEGIIMENINLVGESGKPSEATCKNVHFNNAEHVTPHCTS  444

pgavo  MKRRVHECSY     462
       :: : :|: :
pgtom  LEISEDEALLYNY  457
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Fig. 2. Avocado and tomato polygalacturonase amino acid sequence homology by FASTA alignment. pgavo, avocado polygalacturonase; pgtom, tomato polygalacturonase; |, identical amino acid; :, conservative substitution.

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                                        10        20        30
SBA13                                ETEELARFAVQEHNKKANTRLEFSRVVKAKEQVVAGTMY
                                     :|||||| |||||||: |||:::|::|:|||||||:|
Oryza-I  MSSDGGPVLGGVEPVGNENDLHLVDLARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLY
                10        20        30        40        50        60

         40        50        60        70        80
SBA13    YITLEVVEAGQKKIYEAKVWVKLWENFKELQEFKLSGIAHPPLLMLNA *
         |:|:|| |:::||:|||||| | | :|||||||
Oryza-I  YFTIEVKEGDAKKLYEAKVWEKPWMDFKELQEFKPVDASANA
                70        80        90       100
```

Fig. 3. Homology found in the predicted amino acid sequence between the avocado SBA13 clone (F2) and the cysteine proteinase inhibitor oryzacystatin-I (oryza-I). The 5 amino acid sequence involved in the activity of the inhibitor is boxed; *, first stop in avocado cysteine-like protein sequence.

delayed and diminished cellulase mRNA production. Up to 5 days there was a basal level similar to that in prestored tissue, increasing slowly up to 62 days to a level 10 times less than after 6 days at 20 °C storage. In tissue stored at 3 °C the increase in cellulase mRNA was only seen in the sample corresponding to 25 days of storage. The removal of ethylene at 7 °C storage caused a delay in cellulase mRNA accumulation until 56 days of storage and it was barely detectable in tissue stored at 3 °C without ethylene.

Similar results were obtained when we hybridized RNA from avocado tissues ripening at 20 °C with the purified insert of the EFE clone (pAVOe3, avocado equivalent to the tomato EFE clone) as the probe (Fig. 4C). However there were

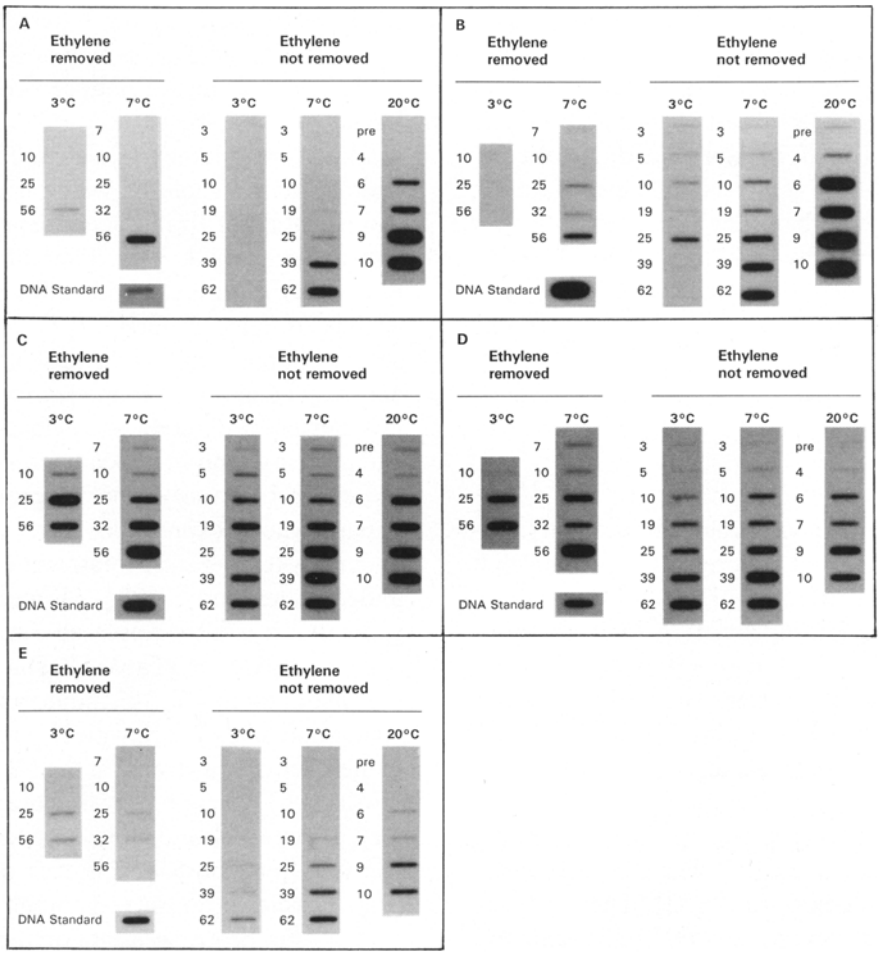

Fig. 4. Expression of mRNAs homologous to cDNA clones during normal ripening and storage of avocado fruit at low temperatures with and without ethylene removal. A. Clone SBA3 (PG). B. pAVOe6 (cellulase). C. pAVOe3 (EFE). D. Clone SBA9. E. Clone SBA10 (thaumatin-like protein). Pre, prestored tissue; numbers refer to days of storage. DNA standards at the bottom left of each panel were 0.1 ng of the corresponding plasmid, except in E (clone SBA10) where 1 ng of plasmid was used.

some differences during cold storage. At 7 °C the EFE mRNA accumulation was delayed with respect to normal ripening, increasing after 10 days and reaching similar or even greater levels than those for normal ripening at 20 °C. During the storage at 3 °C (below the critical temperature for chilling injury) EFE mRNA was present, but in a smaller amount than at 7 °C. Ethylene removal further reduced the accumulation of the EFE mRNAs at 7 °C. At 3 °C, however, the accumulation of EFE RNA was not delayed further and the amount was slightly greater when ethylene was removed from the atmosphere.

The expression of the mRNAs homologous to other clones from the library are summarized in Table 1. Four different patterns were found. SBA15 (F15) showed hybridization with RNA from every kind of tissue studied (data not shown). F9 clones had a particular pattern of expression, not shared by any of the other clones obtained from our library (Fig. 4D) but quite similar to, although delayed from, that of the pAVOe3 (EFE) clone (Fig. 4C). Although no homologies were found in the databases, this mRNA may be involved in the ripening process, being regulated in a similar way to the avocado EFE. Clone SBA10, which has homology to a thaumatin-like protein, showed a pattern of expression similar to

the families F2 (cysteine proteinase inhibitor) and F18 (endochitinase) (Fig. 4E). RNA accumulation rose during normal ripening after 6 days at 20 °C but to a lesser extent than cellulase, PG and EFE mRNAs. 7 °C storage delayed the accumulation of these mRNAs and this was more dramatic at 3 °C, when they appeared only after 25 days and in a lower amount than at 7 °C. Removing ethylene from the atmosphere surrounding the stored fruits significantly reduced the levels of these mRNAs.

Discussion

To analyze the avocado cold storage cDNA library, we carried out differential screening that would allow us to find cDNA clones whose mRNAs increase during long term cold storage at 7 °C. These included some delayed ripening-related mRNAs which were shown by sequence analysis to encode PG, a cysteine proteinase inhibitor, an endochitinase and thaumatin-like protein (Table 1).

PG activity increases during ripening of avocado [5] and is believed to cleave cell wall pectin chains in ripening fruits [52]. The complete sequence of an avocado fruit cDNA encoding PG was obtained (Fig. 1), which is apparently full length and shows a high degree of sequence identity to tomato fruit PG, which is synthesised as a pre-protein [23]. The deduced amino acid sequence of the avocado pre-PG is 5 amino acids longer than that of tomato, whose mature protein starts at amino acid position 72 [23]. There are a few similarities in the first 26 amino acids of the pre-protein sequence, including a charged residue at position 5, followed by a cluster of hydrophobic amino acids (Fig. 2). In tomato this region is removed by ER processing [21]. There is 64% similarity in the region 27–71, which in tomato is removed to form the mature protein, located in the cell wall [23]. For the remaining part of the amino acid sequence, the overall similarity to the tomato PG protein is 88.3% (58% identity). Three possible glycosylation sites have been found in contrast with the four in tomato PG [23]. There are fewer matches in the C-terminal region, the last 13 amino acids of which are also believed to be removed from the mature protein in tomato [49].

Clones from family F2 showed homology with oryzacystatin (Table 1), which is a cysteine proteinase inhibitor from the endosperm of developing rice grains [1]. Two different oryzacystatins have been described, with different specificities against cysteine proteinases (I is more effective against papain and II against cathepsin) [35], and may be involved in the regulation of proteolysis caused by different proteinases. The cystatin-like protein found in avocado could play a role in the control of the action of proteases such as papain whose expression is induced in tomato under low temperature treatment [47] and in peas under water stress [24]. However our results show that it is also expressed during normal ripening in avocados (Table 1). The oryzacystatins are small proteins (I, 102 amino acids [2], and II, 105 amino acids [35]), with two regions involved in their activity: a glycine at the NH_2-terminal end (position 5) whose removal decreases the inhibitory activity [3] and the sequence Gln-Val-Val-Ala-Gly (position 53–57 in rice oryzacystatin I) (Fig. 3) which is conserved among all of the members of this protease inhibitor family and is the primary region of interaction with the cysteine proteinase and mainly involved in the inhibitory activity [4]. The avocado cDNA clone encoding a protein with homology to cystatin described here also has this Gln-Val-Val-Ala-Gly sequence (Fig. 3).

Clone SBA10 is related to thaumatin as well as to some stress-related proteins, such as osmotin [51], a salt-induced protein [33] and pathogenesis-related proteins [18] as well as proteinase inhibitors [43]. Thaumatin is a sweet protein present in the fruits of the tropical plant *Thaumatococcus daniellii* [54]. Not all thaumatin-like proteins taste sweet, however, and their expression under stress situations may be due to a more general function [18]. Singh *et al.* [51] proposed that it could be linked to general defence response; to osmotic adaptation, facilitating accumulation or compartmentation of solutes or as

a storage protein whose accumulation is linked to reduced growth and stress adaptation.

The different homologies between the 3′ (endochitinase) and 5′ (mitochondrial RNA) ends of the SBA18 clone suggest that it could be due to a mixture of two different cDNA clones. SBA18 contains an internal *Eco* RI site, 500 bp from the 5′ end, supporting this suggestion. The expression pattern of the endochitinase-like mRNA that hybridizes to the 3′ fragment was similar to that of the cysteine proteinase inhibitor and the thaumatin-like protein. Endochitinases are generally involved in plant defence mechanisms [48] and their synthesis is sometimes induced by ethylene [10]. However several endochitinases may have other functions apart from their role in the defence response [50] as for example in somatic embryo development [19]. The expression of mRNAs for a thaumatin-like protein, cysteine proteinase inhibitor, and endochitinase during normal ripening (Fig. 4E, Table 1) indicates that they could be induced by ethylene, since a burst of ethylene synthesis is also involved in the induction of pathogen-related proteins in other organs. This is supported by the reduced expression of these mRNAs in ethylene-free atmospheres. It is possible, however, that the thaumatin-like protein also plays a role in the sweetening of avocado fruit during ripening.

Awad and Young [5] found cellulase activity in avocado fruit immediately after harvest which is consistent with the presence of mRNA encoding cellulase in prestored tissue (Fig. 4B). In avocado, cellulase activity rather than PG appears closely related to the climacteric rise and plays a critical role during ripening, associated with disruption of the cell wall, by the cleavage of the cross-bridges between cellulose microfibrills and xyloglucan [27]. The cellulase mRNA that accumulates during normal ripening is believed to be transcribed from a single ripening-related gene [14]. The delay in the appearance of the RNA encoding cellulase at 7 °C and 3 °C, may be related to the delay in the production of ethylene during storage at 7 °C and almost complete inhibition at 3 °C (C. Merodio, unpublished).

During avocado ripening, there is also a dramatic increase in PG activity [5, 31]. PG activity is absent in preclimacteric fruits [32] and only detectable from the 7th day of ripening [5]. The increase in the expression of the mRNA encoding the PG enzyme after 6 days at 20 °C (Fig. 4A) is consistent with these observations and, as in peach and tomato, PG mRNA is absent in preclimacteric fruits [20, 37]. A similar observation has been made by Laties, cited by Cass *et al.* [14]. The fact that the increase in the mRNA encoding cellulase (Fig. 4B) precedes the increase of the mRNA encoding PG (Fig. 4A) during normal ripening and cold storage is consistent with the rise in cellulase activity and some softening occurring prior to any detectable PG activity [5].

During storage of avocado at low temperatures, the expression of several mRNAs was strongly reduced (cellulase) or totally inhibited (PG) whilst the EFE and also the F9 mRNAs showed a marked expression which was not decreased by the removal of ethylene. These observations could indicate that the EFE and the unknown product of the F9 family are involved in fruit ripening and are also induced by cold storage. It is well known that a cold induction mechanism for ethylene synthesis exists in pears [34], cucumbers [56] and some citrus fruits [17] and that this probably involves an accumulation of at least ACC synthase mRNA [34]. Furthermore, EFE mRNA, but not ethylene synthesis, is strongly induced in tomato at low temperature [57]. A similar situation exists in avocado, since although EFE mRNA is induced by low temperature (Fig. 4C) ethylene synthesis is inhibited (C. Merodio, unpublished results). It is possible that the accumulation of some of the EFE mRNA is under the regulation of a low-temperature-inducible gene promoter. There are known to be at least three EFE genes in tomato, which are regulated by different developmental and environmental conditions (Hamilton, Bouzayen and Grierson, unpublished).

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