Isolating and Characterizing Genes Differentially Expressed Early in Apple Fruit Development

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ABSTRACT. A cDNA library was constructed from apples (*Malus × domestica* Borkh.) harvested 1 week after anthesis (WAA). Four cDNA clones differentially hybridized to cDNAs from fruit picked 1 WAA versus cDNAs from fruit harvested 8 WAA and flower buds. The mRNAs corresponding to these cDNA clones—pAFD101 through 104—accumulated in the early stages of apple development, with highest levels at 2 to 4 WAA. The mRNA of pAFD104 was detected only in fruit tissue, while mRNAs for the other three clones were expressed at various levels in a range of tissues. Sequence analysis showed that pAFD101 had high homology to genes encoding the translation elongation factor 1 α (EF-1 α); pAFD102 had homology to the β -subunit of translation initiation factor 2 (eIF-2 β); pAFD104 showed strong similarity to a proline-rich protein and to auxin down-regulated genes; while pAFD103 had no significant homology to any sequences in the databases. Genomic Southern hybridization showed that genes corresponding to all four cDNAs were representatives of small gene families present in the apple genome.

Fruit development is a complex process, starting with development and fertilization of the ovary, followed by growth of the fruit by cell division and expansion, and finally ending with fruit ripening. Each of these developmental stages affects fruit yield and final fruit quality, by influencing factors such as size, shape, color, flavor, aroma, texture and nutrient content. In recent years, there have been extensive studies on genes related to fruit ripening. In the case of the tomato (*Lycopersicon esculentum* Mill.), these studies have led to genetically modified fruit with improved storage life and keeping qualities (Fray and Grierson, 1993; Gray et al., 1994).

In contrast, relatively little attention has been paid to genes expressed early in fruit development, an important stage involving cell division and expansion, ovary and embryo development, and seed formation (Gillaspay et al., 1993). mRNAs expressed early in fruit development have been identified in tomato (e.g., Tiemen and Handa, 1996), peach (*Prunus persica* L.) (Callahan et al., 1993), and kiwifruit (*Actinidia deliciosa* A. Chev.) (Ledger and Gardner, 1994). Among the genes so far identified are elongation factor 1 α (eEF-1 α , Pokalsky et al., 1989), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR, Narita and Gruissem, 1989), 2A11 (Pear et al., 1989), a proline-rich protein (TPRP-F1) (Salts et al., 1991), threonine deaminase (Tiemen and Handa, 1996), a metallothionein-like protein, and the β subunit of mitochondrial ATP synthase (Ledger and Gardner, 1994).

In most cases, expression of these genes is not restricted to young fruit. For example, eEF-1 α mRNA levels are high in most

actively dividing tissues. In only a few instances (2A11, eEF-1 α) have techniques such as in situ hybridization or promoter fusions to reporter genes been used to determine expression patterns more accurately. Thus, the biological role of these genes in early fruit development has not yet been determined. Further research is needed to identify genes with the potential to control early fruit development and to understand the mechanism of early fruit development at the molecular level. This research should ultimately lead to the ability to improve fruit quality by genetically altering developmental processes.

There has been a considerable amount of physiological research on fruit development in apple, a major fruit crop grown worldwide. The development of apples follows a sigmoidal growth curve resulting from rapid cell division and expansion in the ovary and receptacle tissue immediately after flowering. About 4 weeks after anthesis (WAA), most of the cell division is completed and the remaining increase in fruit size is primarily due to cell expansion (Jackson, 1986; Percy et al., 1996; Smith, 1950). Although there are several studies reporting the isolation of ripening-related genes, such as ACC oxidase (Dong et al., 1992), polygalacturonase (Atkinson, 1994), and ACC synthase (Lay-Yee and Knighton, 1995), there have been no reports on genes involved in early apple development.

The goal of this research is to isolate and characterize genes expressed early in the development of apples. By gaining an understanding of the regulation of gene expression during the early stages of fruit development, we aim to improve fruit quality by genetic manipulation. Here we report on the isolation of four cDNAs corresponding to mRNAs that accumulate during early apple development. The expression patterns of these four cDNAs were characterized during fruit development and in different tissues.

Materials and Methods

PLANT MATERIAL. 'Granny Smith' apple (*Malus × domestica*) tissues were collected from trees grown at the Kumeu Research Orchard near Auckland, New Zealand. Apple flowers were tagged at full bloom and tissue was harvested as follows: flower buds at

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preanthesis, open flowers with ovary, petals at anthesis, and fruit at 1, 2, 4, 6, 8, and 12 WAA. To examine gene expression in different parts of the fruit, apples at 4 WAA were cut into three parts: the core including seeds, carpels, and some flesh tissue; the middle cortex containing only flesh tissue; and outer skin tissue with some flesh. Leaves were collected at very young, expanding, and expanded stages from shoots on the apple trees. Roots were collected from tissue-cultured plantlets. Plant material was immediately frozen in liquid nitrogen and stored at -80°C . The longitudinal length and transverse diameters of fruit at each developmental stage were measured at the time of sampling.

NUCLEIC ACID ISOLATION. RNA was isolated using a modification of the procedure described by López-Gómez and Gómez-Lim (1992). Frozen apple material was ground with a mortar and pestle in liquid nitrogen, transferred to lysis buffer [2% SDS, 50 mM EDTA and 1 M Tris base with the pH adjusted to 7.5 with 1 M boric acid, containing 10% (w/v) insoluble polyvinylpyrrolidone and 1% β -mercaptoethanol], and then homogenized in a polytron at full speed for 1 min at room temperature. The homogenate was briefly vortexed with 0.1 volumes of 5 M potassium acetate and 0.25 volumes of cold absolute ethanol, then extracted sequentially with chloroform, 1 phenol : 1 chloroform, and chloroform. The nucleic acids were precipitated with 1 volume of isopropanol at room temperature for 1 h, recovered by centrifugation at $3000 g_n$ for 10 min, and resuspended in water treated with dimethyldicarbonate (DMDC). RNA was selectively precipitated with LiCl (3 M final concentration) at 4°C overnight and was collected by centrifugation ($12,000 g_n$, 30 min at 4°C). The RNA was then resuspended in DMDC-treated water and precipitated with absolute ethanol overnight at -20°C . After centrifugation ($12,000 g_n$, 30 min at 4°C) the RNA was washed twice with 70% ethanol and resuspended in DMDC-treated water. RNA concentration was estimated from A_{260} measurement and by gel electrophoresis. Poly(A)⁺ RNA was isolated from the total RNA by passage through oligo(dT) cellulose columns (mRNA Purification Kit, Pharmacia).

DNA was extracted from expanding apple leaves following the protocol of Dellaporta et al. (1983).

cDNA LIBRARY CONSTRUCTION AND DIFFERENTIAL SCREENING. A cDNA library was constructed from poly(A)⁺ RNA isolated from fruit harvested 1 WAA. cDNA was synthesized using the SuperScript System, ligated to the λ phage vector ZipLox (Gibco BRL), and packaged using the Packagene System (Promega).

Clones of interest were identified by differential plaque hybridization. Triplicate plaque lifts were performed using nylon filters (NEN Research Products). The filters were prehybridized at 65°C in 1% SDS, 1 M NaCl, 10% (w/v) dextran sulfate, and $50 \text{ mg}\cdot\text{L}^{-1}$ denatured salmon sperm DNA for 3 to 6 h, then hybridized at 65°C overnight in the same solution with the addition of ^{32}P -labelled cDNA. cDNA synthesized from poly(A)⁺ RNA isolated from flower buds and

fruit harvested 1 WAA and 8 WAA was ^{32}P -labelled using a random primer kit (Gibco BRL). After hybridization, the filters were washed twice for 5 min in $2\times$ SSC at room temperature and twice for 30 min in $2\times$ SSC, 1% SDS at 65°C .

NORTHERN AND SOUTHERN BLOT ANALYSIS. Northern blots were prepared by separating 10 or $20 \mu\text{g}$ of total RNA per sample on 1% sodium phosphate or 1% formaldehyde agarose gels. RNA was transferred to nylon membranes (Hybond-N⁺, Amersham) by capillary transfer with 0.05 M NaOH. Filters were prehybridized at 65°C for 3 to 5 h in $5\times$ SSC, $5\times$ Denhardt's solution, 0.5% SDS, and $50 \text{ mg}\cdot\text{L}^{-1}$ denatured salmon sperm DNA, then hybridized in the same solution with the addition of ^{32}P -labelled cDNA at 65°C overnight. Gel-purified fragments from the cDNA clones pAFD101, -102, -103, or -104 were labelled by random priming. After hybridization the membranes were washed twice for 10 min in $2\times$ SSC, 0.1% SDS at room temperature and twice for 15 min in $1\times$ SSC, 0.1% SDS at 65°C . The membranes were stripped of probe according to the manufacturer's directions and reprobed. RNA molecular weight markers (Gibco BRL) were used to estimate mRNA size. RNA loading per lane was estimated by hybridization with an 18S rRNA gene from crab apple (*Malus*) (Simon and Weeden, 1992).

For Southern blot analysis, $20 \mu\text{g}$ of apple leaf DNA was digested with each of four different restriction enzymes, electrophoresed on agarose gels and transferred to Hybond-N⁺ membranes in 0.4 M NaOH according to the manufacturer's instructions. Hybridization and washing conditions were as for northern blots, except that the final wash was in $0.1\times$ SSC, 0.1% SDS at 65°C . A 1-kb DNA ladder (Gibco BRL) was used as a molecular weight marker.

DNA SEQUENCE ANALYSIS. The differential clones were excised from the λ ZipLox vector as pSPORT1 plasmids, as described by the manufacturer (Gibco BRL). Appropriate restriction fragments of the cDNA inserts were subcloned into pSPORT1 or pUC19 so that both strands could be sequenced completely. Sequencing

Fig. 1. 'Granny Smith' apple fruit development. Photographs of typical samples are shown. The graph shows transverse diameter and longitudinal length of fruit as measured at the indicated number of the weeks after anthesis (WAA). Values are means of 10 to 28 samples. The developmental phases (from Smith, 1950; Jackson, 1986 and Percy et al. 1996) are indicated above the graph.

Table 1. Characteristics of pAFD cDNA clones. Insert size was determined by sequence analysis and includes poly(A) residues. mRNA size was estimated from northern blots. The number in parentheses for pAFD104 represents the size of a second, less abundant transcript. Copy number was estimated by Southern hybridization. Homologies were identified by BLAST searches of the database. EF-1 α = elongation factor 1 α , eIF-2 β = translation initiation factor 2 β , PRP = proline-rich protein, and ADR = auxin down-regulated gene. The sequences have been deposited in the GenBank database under the accession numbers shown.

Clone	Insert size (bp)	mRNA size (bases)	Copy no.	Homology	Accession no.
pAFD101	748	1900	2-5	EF-1 α	U80268
pAFD102	1135	1500	3-5	eIF-2 β	U80269
pAFD103	1015	1700	3-4	none	U80270
pAFD104	554	1800 (1200)	2-4	PRP and ADR	U80271

reactions were carried out on an ABICatalyst Robotic Workstation and electrophoresed on a 373A DNA sequencer. Sequence data was analyzed using the Wisconsin Genetics Computer Group (GCG) sequence analysis software package version 8.1 and database searches used the BLAST programs (Altschul et al., 1990).

Results

SEQUENCE OF APPLE FRUIT DEVELOPMENT. The developmental stages and morphological changes of apples are shown in Fig. 1. After pollination and fruit set, the ovary and receptacle tissues of apples underwent a rapid period of growth from one to six WAA, followed by a second more gradual period of growth that continued until the fruit were ready for commercial harvest (24 WAA). Differences in the rate of transverse and longitudinal growth over the latter, more gradual period of increase resulted in a shape change during this period.

ISOLATION OF DIFFERENTIAL cDNA CLONES. A cDNA library was constructed from apples at 1 WAA. To isolate clones preferentially expressed in early fruit development, triplicate plaque lifts were screened with cDNA probes from fruit at 1 or 8 WAA or from flower buds. Clones that showed a good hybridization signal to the 1-week fruit cDNA probe, but no or weaker signal to the cDNA probes from 8 WAA and flower buds were selected as differential clones. After two rounds of screening, plasmid DNA from putative differential clones was purified and further screened with the same three probes used in the original selection. Finally, clones of interest were used as probes in northern analysis. Four clones

(pAFD101 through 104) were found to be differentially expressed in the early stages of apple development. The characteristics of the four pAFD cDNA clones, including the insert size and their corresponding mRNA size, copy numbers, and homologies, are summarized in Table 1.

EXPRESSION PATTERNS OF DIFFERENTIAL cDNAs. Probes were hybridized to a northern blot containing RNA isolated from fruit harvested 1 to 12 WAA to determine more detailed expression patterns of pAFD101 through 104 during apple development. The mRNAs hybridizing to all four cDNA clones preferentially accumulated in the early stages of fruit development, with the highest levels at 2 and 4 WAA (Fig. 2).

Figure 3A shows the results of hybridizing the four clones to RNA from four different organs: flower buds, open flowers at anthesis, leaves, and fruit at 4 WAA. pAFD101 mRNA was expressed to moderate levels in all three nonfruit organs; pAFD102 mRNA was found in flower buds and open flowers (Fig. 3A) and

Fig. 2. Expression of pAFD mRNAs during apple fruit development. Total RNA ($\approx 10 \mu\text{g}$ per lane) from fruit at 1 to 12 weeks after anthesis (WAA) was hybridized with labelled insert from the pAFD clone indicated. Hybridization with 18S rRNA was used to estimate RNA loadings.

Fig. 3. Expression of pAFD mRNAs in different organs and tissues. Hybridization with an 18S rRNA was used to estimate RNA loadings. (A) Total RNA ($\approx 10 \mu\text{g}$ per lane) from different organs [B = flower buds, O = open flowers, L = leaves, F = fruit harvested 4 weeks after anthesis (WAA)] was hybridized with labelled insert from the pAFD clone indicated. (B) The inserts of pAFD101 and pAFD104 were hybridized to total RNA ($20 \mu\text{g}$ per lane) isolated from different organs (B = flower buds, O = open flowers, P = petals, R = roots); leaf developmental stages (L₁ = very young leaves, L₂ = expanding leaves, L₃ = expanded leaves); and fruit tissues from fruit harvested 4 WAA (C = core, M = middle cortex, S = skin). (C) Longitudinal section of apple fruit at 4 WAA. The lines indicate the tissue used for northern blot analysis.

at a lower level in leaves (visible on a long exposure, data not shown); pAFD103 mRNA was present in open flowers (Fig. 3A) and at a lower level in leaves (long exposure data not shown); while the expression of pAFD104 mRNA was detected only in fruit.

RNA from other organs, including petals, roots, and leaves at different developmental stages, was used for northern hybridization at low stringency (2× SSC, 0.1% SDS, 65 °C) to confirm the fruit-specific expression of pAFD104. The result (Fig. 3B) showed that there was no signal in these organs, indicating that the level of hybridizing mRNA was very low and that the expression was fruit specific. In this low stringency hybridization, a second smaller band (pAFD104b, mRNA size ≈1200 nt compared to ≈1800 nt for the larger band, pAFD104a) was observed. The smaller band had a different pattern of hybridization, indicating that it is transcribed primarily in open flowers. A lower level of transcript was found in flower buds and petals, but there was no band in roots and leaves including very young, expanding, and fully expanded leaves. In contrast, pAFD101 was expressed in all organs examined except roots, and strongest expression was found in very young leaves.

Figure 3B (last three lanes) also shows hybridization of pAFD104 and pAFD101 to RNA from three different fruit tissues at 4 WAA.

pAFD104a and pAFD101 were expressed in core (which included seeds, carpels, and some flesh tissues), in middle cortex (flesh tissue only), and at a low level in skin. pAFD104b was most highly expressed in middle cortex. These results indicate that transcripts of pAFD104 (a and b) and pAFD101 occur in apple flesh tissue and that they may also be expressed in the core.

SEQUENCE ANALYSIS OF FOUR cDNA CLONES. All four cDNAs were completely sequenced, and the sequences compared to those in the database. pAFD101, with an insert of 748 base pairs (bp), was found to be similar to other plant genes encoding translation elongation factor 1α (EF-1α). It is a partial cDNA, missing ≈300 amino acids at the amino terminal. The amount of identity between the pAFD101 polypeptide of 143 amino acids and the equivalent regions of other EF-1α proteins is 94.4% for tomato (Pokalsky et al., 1989), 97.2% for tobacco (*Nicotiana tabacum* L.) (Ursin et al., 1991), 96.5% for *Arabidopsis thaliana* (Axelos et al., 1989), 93.7% for barley (*Hordeum vulgare* L.) (Sutto and Kenefick, 1994), and 95.8% for wheat (*Triticum aestivum* L.) (Metz et al., 1992).

pAFD102 contains an insert of 1135 bp, and the deduced amino acid sequence is similar to the β subunit of translation initiation factor 2 (eIF-2β) from human (Pathak et al., 1988), rabbit

(*Oryctolagus cuniculus* Linn.) (Price et al., 1993), *Drosophila melanogaster* (Ye and Cavener, 1994), and yeast (*Saccharomyces cerevisiae* Rafn.) (Donahue et al., 1988). It also has a high similarity to a partial *Arabidopsis* cDNA from an expressed sequence tags (accession number Z18133). Alignment and comparison of these eIF-2β amino acid sequences shows that the carboxy terminal region of the protein is highly conserved while the amino terminal is much less conserved (Fig. 4). The identity between the 163 amino acid C-terminus of pAFD102 and other eIF-2β genes was 61% for human and rabbit and 58% for fruitfly. There are three Lys repeats present in the N-

Arabidopsis						
pAFD102						
human	MSGDEMIF	DPTMSKKKK	KKKPFMLDEE	GDTQTEETQP	SETKEVEPEP	TEDKDLEADE
rabbit	MSGDEMIF	DPTMSKKKK	KKKPFMLDEE	GDAQTEETQP	SETKEVEPEP	TEDKDVEADE
fruitfly	MDAEDGF	DPTLLKKKK	KKTTFDLDA	LGLE.....		DDTKKEDPDQ
yeast	MSSDLAELGF	DPALKKKKK				..TKKVIPDD
Arabidopsis						
pAFD102	MADDNQNEVK	DEVVADIAPF	DPTKKKKKKE	VVIQDITDDS	VGKLAEKAEA	CTASEGQEST
human	EDTRKKDASD	DLDDLNFNFQ	KKKKKKTKKI	FDIDEAEEGV	KDLKIES..D	VQEPTEPEDD
rabbit	EDSRKKDASD	DLDDLNFNFQ	KKKKKKTKKI	FDIDEAEEGV	KDLKIEN..D	VQEPAPEDD
fruitfly	EASAEGGAAA	EEDNLDLESE	GKKKKKKKKP	FNMDIEAAI	PSFGGDDVAA	SEEPPEEEIN
yeast	FDAAVNGKEN	GSGDDLFAGL	KKKKKKSKSV	SADAEAEKEP	TDDIAEALG	
Arabidopsis						
pAFD102	FAGLKKKKKK	PIETSIILNEE	SGDAVEDLNE	RTGEDEEGEG	IVLETPSYF	WEGSDRDYTY
human	LDIMLGNNKK	KKKNVKFPDE	DEILEKDEAL	EDEDNKKDDG	ISFSNQTGPA	WAGSERDYTY
rabbit	LDIMLGNNKK	KKKNVKFPDE	DEILEKDEAL	EEDSKKDDG	ISFSNQTGPA	WAGSERDYTY
fruitfly	LDMDFSMAKK	KKKSKK.KEL	DELFAQQADD	DKSEDKEND	EDNSST	WFGSDRDYTY
yeast	.ELSLKKKKK	KTKDSSVDAF	EKELAKAGLD	NVDAESKEG	TPSANS	SIQQEVGLPV
Arabidopsis						
pAFD102	DELLGRVFN	LRENNPEL.A	GDRRTVMRP	POVLREGTKK	TVFVNFMDLG	KTMHRQPDHV
human	EELLDRVFTI	LRENNPDL.A	GDRRTVMRP	POVLREGTKK	AVFVNFMDLG	KTMHRQPDHV
rabbit	EELLNRVFN	MREKNPDMVA	GEKKKFVMKP	POVVRVGTKK	TSFVNFTDIC	KLLHRQPKHL
fruitfly	EELLNRVFN	MREKNPDMVA	GEKKKFVMKP	POVVRVGTKK	TSFVNFTDIC	KLLHRQPKHL
yeast	DELLKRVFEI	ILDKNPDMAA	GRKPKFVMRP	POVLRVGTKK	TSEANFMDIA	KTLHRLPKHL
	SELLSRFENI	LRTNNPELAG	DRSGPKFRIP	PPVCLRDGKK	TIFSNIQDIA	EKLHRSPEHL
Arabidopsis						
pAFD102	MQYLLAELGT	SGSLDGOORL	VVKGRFAPKN	FEGILRRYIT	DYVICLGCKS	PDTILXKE..
human	MAFLLAELGT	SGSLDGOORL	VVKGRFAPKN	FEGILRRYVN	EYVICLGCS	PDTILSKE..
rabbit	LAFLLAELGT	SGSIDGNNQL	VIKGRFQKQ	IENVLRRYIK	EYVTCHTCRS	PDTILQKD..
fruitfly	LDLFLAELGT	SGSIDGNNQL	VIKGRFQKQ	IENVLRRYIK	EYVTCHTCRS	PDTILQKD..
yeast	IQYLFALGT	SGSMDGNQOL	IIKGRFQPKQ	IENVLRRYIK	EYVTCHTCRS	PETILQKD..
	IQYLFALGT	SGSVDGQKRL	VIKGRFQSKQ	MENVLRRYIL	EYVTCKTCKS	INTELKREQS
Arabidopsis						
pAFD102	NXLFFLRCEK	CGSQRSV...				
human	NRLEFFLRCEK	CGSGRSVAPN	KAGFMARVGR	RNACT*		
rabbit	IRLYFLQCEK	CHSRCSVASI	KTGFQAVTGK	RAQLRAKAN*		
fruitfly	TRLYFLQCEK	CHSRCSVASI	KTGFQAVTGK	RAQLRAKAN*		
yeast	TRLFFLOCES	CGSRCSVASI	KSGFQAVTGK	RAAIRAATT*		
	NRLFFMVCKS	CGSTRSVSSI	KTGFQAVTGK	RRRM*		

Fig. 4. A comparison of the amino acid sequence of pAFD102 with that of other eIF-2β proteins found in *Arabidopsis* (GenBank accession no. Z18133), human (M29536), rabbit (P41035), *Drosophila* (L19197), and yeast (S29369). Stop codons are indicated by asterisks. The conserved zinc finger motif is underlined.

Fig. 5. Southern blot analysis of apple genomic DNA. Total genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), *Hind*III (H) or *Xba*I (X). After electrophoresis, DNA was transferred to a nylon membrane and hybridized with the cDNA clone insert indicated. The size of the hybridizing bands can be estimated using the scale (in kb) shown to the left.

terminal in other species, but only two in pAFD102, possibly because it is a partial cDNA (22 amino acids missed in N-terminal). In the C-terminal there is a putative C₂-C₂ type zinc-finger-like motif, with a structure of Cys-X₂-Cys-X₁₇-Cys-X₂-Cys that is very similar to that found in other eukaryotic species (Fig. 4).

pAFD103 has a 1015-bp insert showing no significant similarity to sequences in the database at either nucleotide or amino acid levels. This cDNA is also likely to be incomplete as the clone hybridizes to a 1700-bp mRNA and there was no obvious in-frame start codon (ATG) in the longest open reading frame. The function of pAFD103 is not yet known.

AFD104 is a partial cDNA containing an insert of 547 bp. It encodes the C-terminal portion of a gene, which has similarity to proline-rich proteins (PRP) from other plants. The amount of identity between the pAFD104 polypeptide of 75 amino acids and the equivalent regions of other PRP is 80% for tomato (Salts et al., 1991), 85.3% for alfalfa (*Medicago sativa* L.) (accession number L37017), 84% for dodder (*Cuscuta reflexa* L.) (Subramaniam et al., 1994) and 70.7% for maize (*Zea mays* L.) (Jose-Estanyol et al., 1992). The C-terminal region of pAFD104 also showed similarity to a C-terminal partial soybean (*Glycine max* L.) auxin down-regulated gene, ADR11 (Datta et al., 1993). For the C-terminal domain, the identity is 62.7% between the pAFD104 and ADR11.

GENOMIC BLOT ANALYSIS. Each cDNA clone was hybridized to Granny Smith genomic DNA digested with *Bam*HI, *Eco*RI, *Hind*III or *Xba*I to determine the approximate number of related genes in the apple genome. One or two major bands and two to six minor bands were obtained in each digest after washing at high stringency for each cDNA clone (Fig. 5). The strong bands were expected to represent the genomic sequence corresponding to the cDNA clones and the weak bands may represent related genes. These results indicated that genes corresponding to the four cDNAs were present in the apple genome at low copy number and that they are representatives of small gene families.

Discussion

The increase in volume associated with growth of fruit is usually attributed to a combination of cell division and cell

expansion (Gillaspy et al., 1993; Jackson, 1986). Previous research into apple development suggested that fruit growth over the first 4 weeks resulted primarily from cell division, with the subsequent increase being primarily cell enlargement (Jackson, 1986). Recently, Percy et al. (1996) measured cell division in 'Braeburn' apples using flow cytometry. Their results showed that the major cell division phase occurred in the first 3 to 4 WAA. The number of cells undergoing division was lowest at 8 WAA followed by a small increase of cell division activity. For 'Granny Smith', our results showed a basic change in the rate of growth at 6 weeks. It is possible that the change from primarily cell division to primarily cell enlargement may occur at this time in this variety.

To isolate cDNAs for genes expressed predominantly during early fruit development, we performed differential hybridization between the flower buds (before fruit set), 1-week-old fruit (that were expected to be undergoing cell division), and 8-week-old fruit (presumed to be undergoing cell expansion). Northern hybridizations showed that all four cDNAs represent genes expressed during the early stages of fruit development. Transcripts of pAFD104 were not found in leaves, petals, or flower buds, suggesting that this gene may have a fruit-specific expression pattern.

Sequence analysis of pAFD101 showed that it had homology to the elongation factor 1 α gene from other plants. EF-1 α plays an essential role in protein synthesis (Merrick, 1992; Riis et al., 1990) where it facilitates the GTP-dependent binding of aminoacyl-tRNA to the A site of the ribosome during the elongation phase of translation. In addition, EF-1 α is involved in various other cellular processes and has to be regarded as a multifunctional protein (Durso and Cyr, 1994). For example, it has been identified as a microtubule severing protein that may participate in the rearrangement of microtubules during the cell cycle (Marchesi and Ngo, 1993; Shiina et al., 1994). We have shown that expression of EF-1 α was high in young leaves and young fruit but was not present in roots. Similar expression patterns were found for EF-1 α in tomato by Pokalsky et al., (1989), who previously isolated EF-1 α as a gene differentially expressed in young leaves and green fruit. Southern analysis of pAFD101 showed that genes encoding EF-1 α constitute a small gene family in apple, similar to tomato (Shewmaker et al., 1990) and maize (Berberich et al., 1995).

pAFD102 encodes translation initiation factor 2 β (eIF-2 β), which in mammalian systems functions in the early steps of protein synthesis by forming a ternary complex with GTP and initiator tRNA (Pain, 1986; Pathak et al., 1988). There is a zinc finger motif in the C-terminus of eIF-2 β and mutational analysis of yeast eIF-2 β indicated that the zinc finger domain was required for translation start-site selection during the scanning process (Donahue et al., 1988). The cysteine residues and the intervening amino acids of this zinc finger motif are essential for eIF-2 β function in *in vivo* translation initiation (Castilho-Valavicius et al., 1992). It is not known, however, if a similar regulatory mechanism exists in plants. The strong conservation of the polylysine block and zinc finger motif among human, animal, and plant proteins implies that these structures are functionally important.

pAFD104 encodes a proline-rich protein. The expression of an 1800-bp hybridizing transcript was high in young apple fruit harvested 2 to 4 WAA but decreased during later stages of fruit

development. The mRNA level of a tomato proline-rich protein gene (*TPRP-F1*) was also high in immature fruit and low in mature green and ripe fruit. The transcript was not found in other tissues (Salts et al., 1991). In other plant species, *PRP* also shows stage-specific expression, e.g., during early embryogenesis in maize (Jose-Estanyol and Ruiz-Avila, 1992), haustoria development in dodder (Subramaniam et al., 1994), and early fiber development in cotton (*Gossypium hirsutum* L.) (John and Keller, 1995). These expression patterns suggest that the function of *PRP* is related to cell division and expansion. In fact, *PRP* has been identified as a cell wall protein with potential functions in cell wall architecture (Gillaspy et al., 1993; John and Keller, 1995). In early developmental stages, a high rate of synthesis of cell-wall materials is required to maintain cell division and significant cell expansion.

Under low stringency, the pAFD104 insert hybridized in northern blots to a second faint band that was smaller than expected for a *PRP*. This 1200 nt transcript showed a different expression pattern to the main band. These results indicated that there was a second mRNA in apples that hybridized to the pAFD104 probe. Sequence analysis showed the cDNA of pAFD104 to be similar to an auxin down-regulated (*ADR11*) gene from soybean. The mRNA size of *ADR11* was below 1110 bp (Datta et al., 1993) so that the 1200 nt hybridizing band may represent an apple homolog of the *ADR* gene. In soybean, *ADR11* was expressed at a high level in elongating hypocotyls and at a low level in the primary leaves. The *ADR11* sequences are highly similar to other *PRP* sequences at the C-terminus, and may represent a subfamily of *PRPs*.

The results in this paper represent an initial molecular description of early fruit development in apple. The genes and their activities should help us to understand the mechanisms of molecular regulation in this important, early stage of fruit development. These cDNAs could be used for over expression or co-suppression studies in transform plants to determine their effects on fruit development. pAFD104, which showed fruit-specific expression, is a good candidate gene to isolate promoter sequences specific to early fruit development.

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