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Gene Expression in the Pulp of Ripening Bananas[1]

Two-Dimensional Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of in Vitro Translation Products and cDNA Cloning of 25 Different Ripening-Related mRNAs

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mRNA was extracted from the pulp and peel of preclimacteric (d 0) bananas (*Musa* AAA group, cv Grand Nain) and those exposed to ethylene gas for 24 h and stored in air alone for a further 1 (d 2) and 4 d (d 5). Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of in vitro translation products from the pulp and peel of these fruits revealed significant up-regulation of numerous transcripts during ripening. The majority of the changes were initiated by d 2, with the level of these messages increasing during the remainder of the ripening period. Pulp tissue from d 2 was used for the construction of a cDNA library. This library was differentially screened for ripening-related clones using cDNA from d-0 and d-2 pulp by a novel microtiter plate method. In the primary screen 250 up- and down-regulated clones were isolated. Of these, 59 differentially expressed clones were obtained from the secondary screen. All of these cDNAs were partially sequenced and grouped into families after database searches. Twenty-five nonredundant groups of pulp clones were identified. These encoded enzymes were involved in ethylene biosynthesis, respiration, starch metabolism, cell wall degradation, and several other key metabolic events. We describe the analysis of these clones and their possible involvement in ripening.

Bananas (*Musa* spp.) are a globally important fruit crop. They are not only a popular dessert fruit, but represent a vital carbohydrate staple in the tropics, with as many as 100 million people subsisting on bananas and plantains as their main energy source (Rowe, 1981). The cultivated dessert banana is commonly triploid, parthenocarpic, and belongs to the *Musa* AAA genome group, e.g. Cavendish subtypes (Simmonds and Stover, 1987). Bananas are monocots and have climacteric fruits with ripening regulated by ethylene. Numerous biochemical changes occur during the ripening of banana fruit, including major alterations in carbohydrate composition, cell wall disassembly, synthesis of volatile compounds, changes in phenolic constituents, and degradation of chlorophyll in the peel (Seymour, 1993, and refs. therein).

The triploid and parthenocarpic nature of the banana crop has hampered conventional methods of breeding for improved characteristics, despite an urgent need to improve disease resistance in commercial cultivars. Also, an enormous pool of genetic resources for enhancing postharvest characteristics of the fruit has remained untapped. The physiology of the banana fruit has been the subject of intensive research during the last 70 years (Marriott, 1980). This has led to well-established recommendations for the level of ethylene required to initiate ripening and to maintain optimum storage conditions. The biochemical basis of the physiological changes occurring during ripening has, however, proved more difficult to characterize. The high phenolic and starch content of banana fruit has made protein extraction and quantitative measurement of enzyme activities difficult.

Molecular approaches now provide the key not only for identifying ripening-related genes, but also for their manipulation for banana improvement. The latter was made possible by recent work reporting the genetic transformation of bananas by *Agrobacterium tumefaciens* (May et al., 1995) and particle bombardment (Sági et al., 1995). Automated sequencing technology and the large number of nucleic acid sequences available in the databases make this a timely moment to begin identifying the molecular basis of changes associated with the ripening of one of the world's major food crops. This study and work by Clendennen and May (1997) describe the isolation of more than 30 nonredundant, ripening-related clones from banana pulp.

MATERIALS AND METHODS

Unripe preclimacteric bananas (*Musa* AAA group, cv Grand Nain) were obtained from a commercial source in the United Kingdom. The bananas were initiated to ripen by exposure to exogenous ethylene (100 μL L^{-1} for 24 h at 20°C) and then held in humidified air alone at 20°C for

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Abbreviations: ACO, ACC oxidase; IFR, isoflavonoid reductase.

another 7 d. Unripe (prior to ethylene treatment) and ripening fruits were analyzed for ethylene production, total soluble solids in the pulp, and chlorophyll content in the peel, as described below. The pulp and peel tissue of selected fruits was frozen in liquid nitrogen for RNA extraction.

Measurement of Ethylene Production, Pulp Total Soluble Solids, and Peel Chlorophyll Content

Individual banana fruits were sealed in 1-L gastight jars at 20°C, and a 1-mL sample of gas was removed after 1 h for GC determination of ethylene content (Ward et al., 1978). The pulp and peel tissue of selected fruits was then frozen in liquid nitrogen and stored at −70°C. Total soluble solids of a crushed sample of fruit pulp were measured using a refractometer (Atago PR-1, Jencons Scientific, Leighton Buzzard, UK). Peel chlorophyll was determined on frozen tissue following the protocol of Hiscox and Israelstam (1979).

Extraction of Total RNA

Total RNA was extracted from banana pulp and peel tissue following the method described by Chang et al. (1993) for tissues with high concentrations of polyphenols and polysaccharides.

In Vitro Translation and Two-Dimensional SDS-PAGE

For the synthesis of L-[^{35}S]Met-labeled polypeptides, 10 μg of total RNA was used in a rabbit reticulocyte translation system (GIBCO-BRL) containing 925 kBq of L-[^{35}S]Met (Amersham) in a reaction volume of 30 μL. The reactions were undertaken at 30°C and were terminated after 2 h by the addition of 3 μL of RNase A (1 mg mL^{-1}; Sigma). The TCA-precipitable counts were determined in 2-μL aliquots of the reaction mixture by scintillation counting. The labeled polypeptides were separated by two-dimensional SDS-PAGE using a Protean II apparatus (Bio-Rad) according to the instructions of the manufacturer and based on the methods of O'Farrell (1975) and Manning (1994), with modifications.

For the first dimension the IEF gel contained 1% of carrier ampholytes at pH 3 to 10 (Biolyte, Bio-Rad), 9% of carrier ampholites at pH 6 to 8 (Sigma), and piperazine diacrylamide (Bio-Rad) in place of *N,N'*-methylene-bisacrylamide. Unincorporated radioactivity in the translation mixtures was removed by acetone precipitation. Approximately 120,000 cpm of incorporated ^{35}S dissolved in sample buffer was loaded onto the IEF gels. The second-dimension SDS-PAGE was carried out using slab gels (16 × 16 cm) polymerized with 12.5% (w/v) acrylamide/0.1% (w/v) piperazine diacrylamide in the separating gel and 4% (w/v) acrylamide/0.04% (w/v) piperazine diacrylamide in the stacking gel.

pI values were calibrated using a kit for IEF separations (Merck, Poole, UK). Molecular weight protein standards (14,000–200,000) labeled with ^{14}C were from GIBCO-BRL. Gels were fixed and impregnated with Amplify (Amersham) according to the manufacturer's instructions. The dried gels were exposed to preflashed Hyperfilm MP (Amersham) at −70°C. The exposure times for gels prepared in different runs were adjusted to give similar total radioactive exposures.

Poly(A)$^+$ mRNA Isolation and cDNA Library Construction

mRNA was isolated from total RNA by oligo(dT)-cellulose chromatography (Bantle et al., 1976). The first and second strands of the cDNAs were synthesized from the poly(A)$^+$ RNA using a commercial cDNA synthesis kit (ZAP-Express Gold Cloning kit, Stratagene). Double-stranded cDNAs were cloned into the vector, packaged, and mixed with plating bacteria to determine the titer for library screening, following the manufacturer's instructions. The ripening banana pulp cDNA library was prepared with an efficiency of 1.66 × 10^6 plaque-forming units per microgram of cDNA. In a sample of clones, the sizes of the inserts in the library ranged from 0.3 to 10 kb, with a mean insert size of 1.47 kb.

Differential Screening

The unamplified cDNA library from ripening banana pulp was differentially screened using cDNA from unripe (green, untreated with ethylene) and ripening (24 h after ethylene treatment) banana pulp tissue. Approximately 2000 plaques were plated at low density (about 500 plaques per 15-cm-diameter plate), and duplicate plaque lifts were made onto Hybond-N nylon filters (Amersham) according to the manufacturer's instructions. One filter was hybridized to cDNA from green fruit labeled with [^{32}P]dCTP, and the duplicate filter was hybridized to radiolabeled cDNA from ripening (d 2) fruit (probe size ranged from 500 bp to more than 2 kb). Hybridizations were carried out at 65°C in 5× SSPE, 0.01% SDS, containing 0.25% skim milk. For the primary screen 1 μg mL^{-1} poly(A) oligomer was added.

Posthybridization washes at 65°C consisted of two 20-min washes with 6× SSC, followed by two 20-min washes with 3× SSC (all solutions contained 0.1% SDS). Plaques hybridizing preferentially with ripening or unripe radiolabeled cDNA probes were picked and replated for a second round of selection. A novel technique for differential screening of a large number of clones was used. Aliquots of an enriched phage suspension were placed into a microtiter plate well. Using a microtiter replica apparatus (Denley Instruments Ltd., Billingshurst, UK), the phage suspensions were dotted (96 clones/plate) on a freshly plated lawn of strain XL-1-Blue MRF' *Escherichia coli* (Stratagene).

After an overnight incubation at 37°C, the plates were treated as for the primary screening. Duplicate filters were made from each plate and hybridized with radiolabeled cDNA as described above. Hybridization was carried out at 65°C as for the primary screen. Posthybridization washes were carried out at 65°C and involved two 20-min washes with 3× SSC, followed by two 20-min washes with 2× SSC, and a final 20-min wash with 0.5× SSC (all solutions contained 0.1% SDS). Ripening-related clones selected from the secondary screen were in vivo excised from the ZAP-Express vector into the pBK-CMV phagemid vector using

an interference-resistant helper phage (ExAssist, Stratagene), according to the manufacturer's protocol.

Sequencing and Characterization of the Ripening-Related cDNA Clones

From the 250 plaques isolated in the primary screen, 59 putative ripening-related clones were obtained as a result of the secondary screen. These 59 clones were partially sequenced from their 5′ ends using a dye-terminator cycle-sequencing ready-reaction kit (Prism, Applied Biosystems) with DNA polymerase FS (AmpliTaq, Applied Biosystems) with forward primers specific for the pBK-CMV vector. Approximately 600 to 700 bp of good-quality sequence was obtained from each clone. The sequences were edited to remove any vector sequence and compared with all known DNA sequences in the GenEMBL database using software (version 8) and the FASTA, TFASTA, and BLASTN programs from the Genetics Computer Group (Madison, WI). Nonredundant partial sequences from the banana clones have been submitted to the database; the accession numbers are shown in Table I.

Northern Analysis

Total RNA samples (10 μg) from d-0 and d-2 fruit pulp were separated on 1.5% formaldehyde-agarose gels and transferred to nylon membranes (Magnacharge, Genetic Research Instrumentation Ltd., Felsted Dunmow, UK) by capillary blotting. Prehybridization and hybridization steps were conducted in 5× Denhardt's solution, 5× SSPE, 1% SDS, and 100 μg mL^{-1} denatured salmon-sperm DNA at 65°C, and the membrane was probed with a radiolabeled insert of a chosen cDNA clone. Following hybridization the filters were washed twice with 3× SSPE, 0.1% SDS for 20 min at 65°C, and then twice with 0.3× SSPE, 0.1% SDS for 20 min at 65°C. The membranes were then exposed to film for 48 h.

RESULTS

Banana ripening was initiated by exposure to ethylene at 100 μL L^{-1} for 24 h. A climacteric rise in endogenous ethylene production was observed during ripening, and an increase in total soluble solids in the ripening pulp was accompanied by a decrease in chlorophyll content in the peel (Fig. 1).

Two-Dimensional SDS-PAGE of in Vitro Translation Products from Unripe and Ripening Banana Peel and Pulp

The changing patterns of translation products in the peel and pulp of ripening bananas are shown in Figure 2. A significant number of translation products was present in both unripe peel and pulp, and several spots on the gels appear to be common to both tissues, e.g. those marked 1 to 8. After ethylene treatment for 24 h and another 24 h in humidified air alone (d 2), both peel and pulp tissues

Table I. *Characterization of up- (pBAN UU) and down-regulated (pBAN UD) cDNA clones from ripening banana pulp*

Clone	No. of Clones	Transcript Size	Database Accession No. for Banana Clone	Putative Identity	Related Sequence and Accession No.[a]	Identity/bp Overlap	
		kb				%	*bp*
pBAN UU10	2	1.3	Z93121	ACO	Apple M81794	68	794
pBAN UU21	1	1.4	Z93113	Transcriptional activator	Maize L13454	54	311
pBAN UU32	2	1.9	Z93112	β-Amylase	Sweet potato D01022	54	789
pBAN UU40	1	1.8	Z93111	No significant homology			
pBAN UU43	2	1.8	Z93110	2A11-Related clone	Tomato X13743	54	396
pBAN UU55	2	1.5	Z93109	*O*-Methyltransferase	Maize L14063	60	223
pBAN UU70	1	0.8	Z93108	Root-related protein	Rice L27208	64	420
pBAN UU80	1	1.3	Z93107	No significant homology			
pBAN UU84	6	1.8	Z93106	Pectate lyase	Tomato X55193	66	736
pBAN UU90	2	1.7	Z93105	Glutamate decarboxylase	Petunia L16797	73	735
pBAN UU91	1	3.0	Z93104	Aconitase	Pumpkin D29629	76	766
pBAN UU93	2	2.1	Z93103	Heat-shock protein	Tomato X54030	76	771
pBAN UU96	1	1.4	Z93127	Expressed sequence tag	Arabidopsis H36910	69	452
pBAN UU103	1	2.1	Z93120	Cell wall invertase	Maize U17695	66	567
pBAN UU104	4	1.5	Z93119	IFR	Arabidopsis Z49777	61	696
pBAN UU116	2	1.4	Z93118	Polyubiquitin	Rice X76064	82	625
pBAN UU129	1	1.8	Z93117	β-Glucosidase	Barley L41869[b]	60	575
pBAN UU130	1	1.5	Z93116	No significant homology			
pBAN UU131	2	1.7	Z93115	*S*-Adenosyl homocysteine hydrolase	Wheat L11872	80	699
pBAN UU136	2	1.3	Z93114	β-1,3-Glucanase	Barley M91814	60	800
pBAN UD39	1	0.9	Z93125	Antifungal protein	Maize U06831	69	517
pBAN UD66	6	2.1	Z93124	Granule-bound starch synthase	Cassava X74160	64	669
pBAN UD75	2	1.1	Z93123	Wali 7	Wheat L28008	74	703
pBAN UD93	4	1.3	Z93122	Chitinase	Cowpea X88801	62	701
pBAN UD120	2	0.9	Z93126	Extensin	Almond X65718	57	576

[a] Most similar sequence as identified by FASTA search. [b] Compared with gene sequence edited to remove introns.

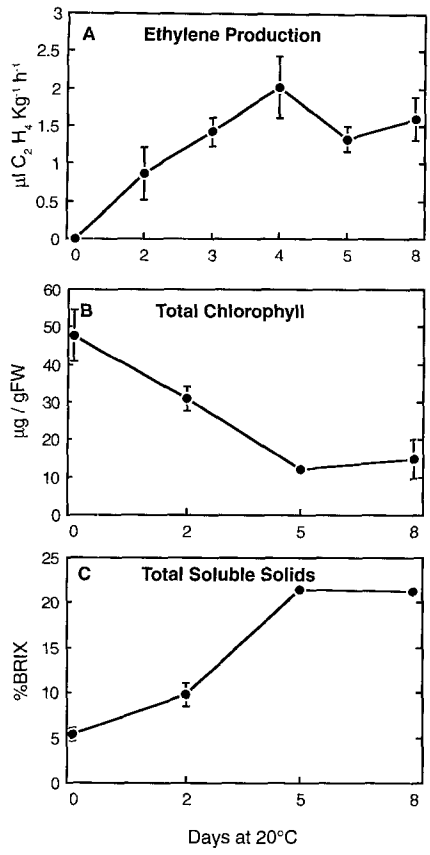

Figure 1. Changes in ethylene production (A), chlorophyll content in the peel (B), and total soluble solids in the pulp (C) during ripening of banana fruits. The fruits were initiated to ripen by exposure to ethylene at 100 μL L^{-1} for 24 h at 20°C, and then held in air alone at 20°C under high humidity. Fruits were sampled 2, 5, and 8 d after the start of the experiment. Each point represents the mean of three fruits.

showed an increase in labeling of products represented by spots labeled 2 to 5 in both tissues. Some messages were clearly down-regulated, such as the spots labeled 6, 13, and 22 in peel and those labeled 12, 13, 16, and 17 in pulp (Fig. 2). A few translation products were absent from green fruit and appeared during ripening; some were present only in peel or pulp. In peel tissue these included translation products labeled 17 to 19, which were not present in unripe or ripe pulp. Translation products that appeared exclusively at the onset of ripening in the pulp were more difficult to detect. Figure 2 shows that much of the ripening-related pattern of translation products in both tissues was established by 24 h after the ethylene treatment. After this time there was mainly an increase in the abundance of translation products already present.

Screening of the Pulp cDNA Library and Isolation of Clones

In the initial round of screening 250 plaques were picked and both up- and down-regulated clones were apparent. The secondary screen employed a novel microtiter plate technique (see "Materials and Methods") and 59 clones were picked. The inserts were then cloned into pBK-CMV and all 59 clones were partially sequenced from their 5' ends by the dideoxynucleotide method. Each clone was then analyzed by comparison with sequences on nucleic acid databases as described in "Materials and Methods." Of the 59 clones from the secondary screen, there were 25 nonredundant classes of sequence. Putative identities could be assigned to the majority of the clones, and the data for nucleotide comparisons are summarized in Table I. Comparisons between the banana clones and those from other species were also made at the amino acid level, and the putative identities were consistent with those from the nucleotide data.

Assignment of Putative Identities to Individual Clones

Northern-blot analysis demonstrated that clones isolated in the differential screen could be grouped into two classes, pulp up-regulated (pBAN UU) and pulp down-regulated (pBAN UD), according to the pattern of expression of their transcripts (Fig. 3). Each clone was assigned a name, and details relating to its putative identity, number of identical clones, transcript size, and database accession number are summarized in Table I.

Up-Regulated Messages

The majority of our cDNA clones were shown by northern-blot analysis to be up-regulated after the initiation of ripening with ethylene; these data are summarized in Figure 3. Clones pBAN UU21, -104, -116, and -131 showed little apparent change in levels of expression between d 0 and d 2. However, dot blots from a wider range of ripening stages indicated that these messages were up-regulated in ripening pulp, reaching maximum levels of expression a few days later as the peel fully reached a yellow color (data not shown).

A banana ACO homolog (pBAN UU10) was isolated. The partial sequence obtained from this clone appears to be identical to that of an ACO clone from banana obtained by López-Gómez, et al. (1997), accession no. X91076, and is closely related to other ACO sequences (Table I). A single clone for aconitase, pBAN UU91, was recovered from the library. Sequence translation of 600 bp from the banana clone and comparison of the notional translation product with pumpkin (*Cucurbita pepo*; D29629) and Arabidopsis (X82839) aconitase amino acid sequences revealed a high degree of homology and the conserved cluster of Cys's likely to be involved in the action of aconitase (Hayashi et al., 1995). The pumpkin and Arabidopsis aconitase clones were obtained by immunoscreening cDNA libraries with antibodies raised to an active aconitase enzyme (Hayashi et al., 1995; Peyret et al., 1995).

Starch accounts for 20% to 25% of the fresh weight of bananas, and several clones sharing homology with enzymes involved in carbohydrate metabolism were isolated. These included pBAN UU32, which had sequence homology to β-amylase, and pBAN UU103, which shared identity with plant cell wall-bound invertase cDNA clones from a

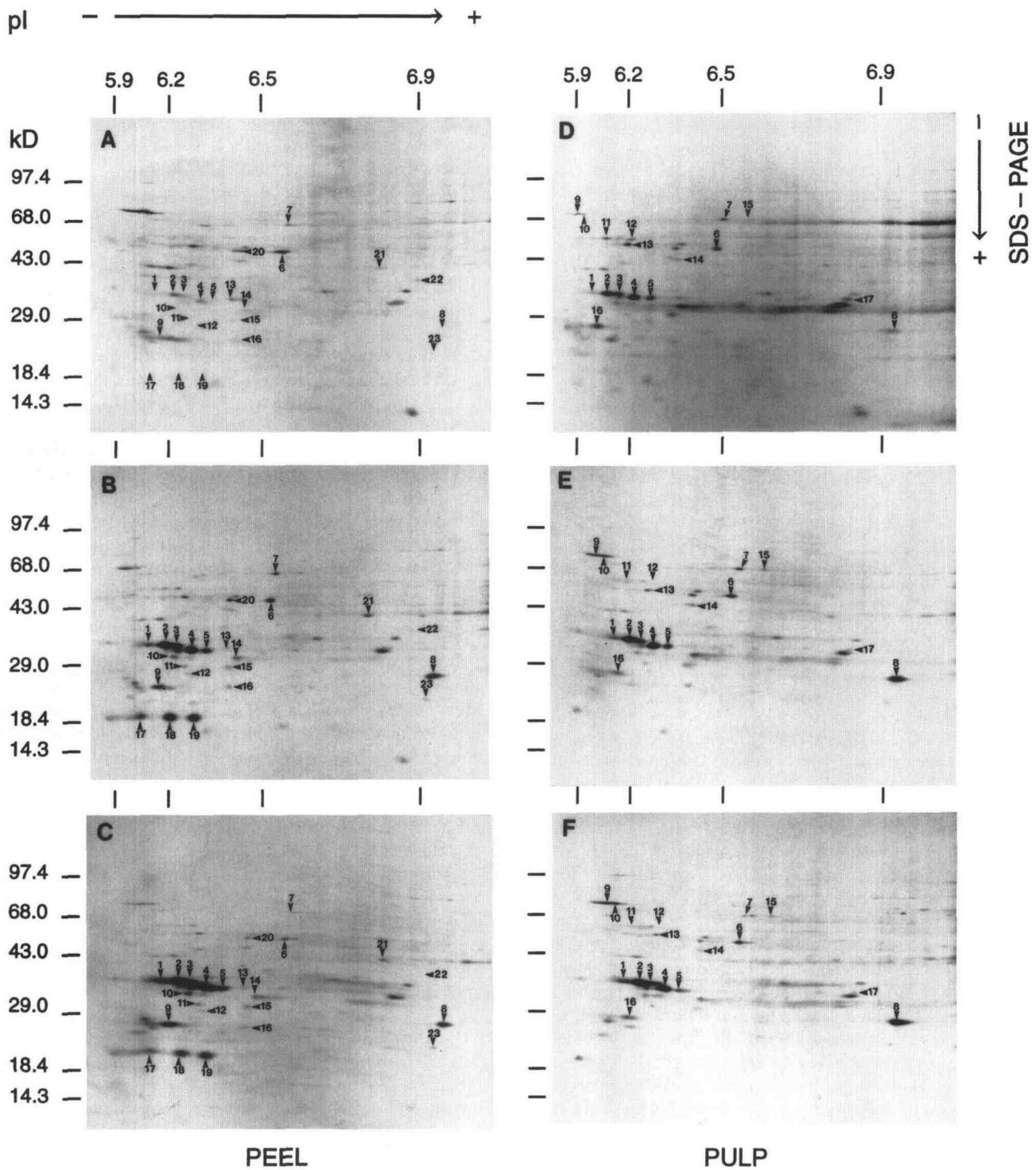

Figure 2. Two-dimensional gel-electrophoresis fluorographs of in vitro translation products from ripening banana peel and pulp tissue. Translation products are shown from unripe fruit tissue (A and D), and from tissue 24 h (B and E) and 4 d (C and F) after ethylene treatment. Numbered arrows denote spots that change in intensity during ripening. Spots labeled 1 to 8 appear to be common to both peel and pulp tissue.

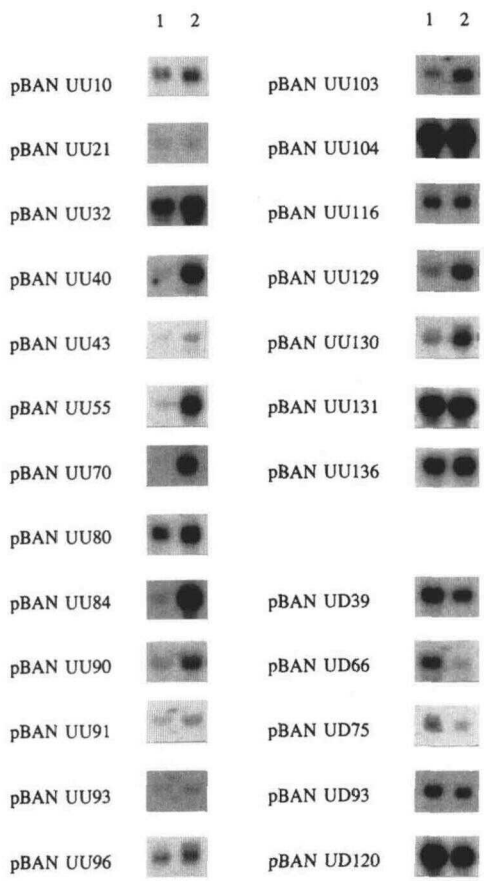

Figure 3. Northern-blot analysis of ripening-related clones from banana pulp. Lanes 1, Unripe (d 0) pulp tissue; lanes 2, pulp tissue 24 h after treatment with ethylene (d 2). Each lane contained 10 μg of total RNA.

number of species including maize (*Zea mays*) (Table I). The translated sequence of pBAN UU103 contains the conserved invertase peptide domain NDPNG (Weber et al., 1995).

Messages encoding enzymes likely to be involved in cell wall degradation were also isolated as up-regulated clones. Pectate lyase homologs were the most abundant ripening-related clones obtained from the pulp library (Table I). There were insufficient sequence data available to determine whether all of the clones represented the same or closely related sequences. However, the sequence of pBAN UU84 shares homology with a putative pectate lyase cDNA from tomato (*Lycopersicon esculentum*) (Budelier et al., 1990), and the banana clone appears to have the conserved regions (including the substrate-binding sites) characteristic of microbial pectate lyases (Turcich et al., 1993).

Other putative wall-degrading enzymes included clones encoding β-1,3-glucanase and β-glucosidase homologs (Table I). Initial FASTA searches indicated that clone pBAN UU129 was the most homologous to a β-glucosidase sequence from white clover (*Trifolium repens* L.; 61% identity in a 517-bp overlap; accession no. X56733; Oxtoby et al., 1991). However, comparison with a barley (*Hordeum vulgare*) β-glucosidase gene sequence modified to remove introns indicated a similar degree of identity (60% identity in a 575-bp overlap; accession no. L41869; Leah et al., 1995).

Sequences normally associated with defense responses in plants were also isolated. Clone pBAN UU104 showed homology with IFR-like sequences from Arabidopsis (Table I). A comparison of the putative translation product from the banana clone with IFR-related amino acid sequences from Arabidopsis (Z49777), potato (*Solanum tuberosum*; X92075), and alfalfa (*Medicago sativa*; X58078) showed the presence of residues proposed to interact with NAD(H) or NADP(H) (Hibi et al., 1994). pBAN UU93, a clone with homology to 70-kD heat-shock proteins, was also obtained.

Several other clones were isolated that could be given putative functions with some confidence. Clone pBAN UU90 showed significant homology with glutamate decarboxylase sequences from petunia (*Petunia* spp.) (Table I) and tomato (74% identity in a 690-bp overlap; accession no. X80840; Gallego et al., 1995). The petunia clone has been expressed in *E. coli* and shown to have glutamate decarboxylase activity, suggesting that this is indeed the functional activity of pBAN UU90. Clone pBAN UU131 was identified as an *S*-adenosyl homocysteine hydrolase. Clone pBAN UU116 was identified as a polyubiquitin, and clone pBAN UU70 had sequence homology with root-related proteins from rice (*Oryza sativa*) and maize (John et al., 1992; Xu et al., 1995) (Table I).

Several other cDNA clones with transcripts up-regulated during ripening were isolated, but homologies to sequences in the database were either less convincing or absent. Clone pBAN UU55 showed homology with an *O*-methyltransferase sequence from maize (Held et al., 1993). Clone pBAN UU43 appeared to be related to the 2A11 tomato fruit-specific cDNA (Pear et al., 1989), and pBAN UU21 shared homology with a transcriptional activator for anthocyanin biosynthesis from maize. Clone pBAN UU96 was closely related to an expressed sequence tag from Arabidopsis, but its function is unknown. There were three clones, pBAN UU40, -80, and -130, that had no significant sequence matches in the database (Table I).

Down-Regulated Messages

The differential screen was performed against plaques from a ripening pulp cDNA library. It was therefore designed to identify clones that were up-regulated during ripening. However, some down-regulated messages were also identified by northern-blot analysis (Fig. 3). Clone pBAN UD66 showed homology to granule-bound starch synthases. The banana sequence showed the highly conserved Gly-rich region, which contained the KTGGL substrate-binding site found in all granule-bound starch synthase clones (data not shown). Another abundant message that is down-regulated during ripening encodes a chitinase (Table I). The cDNA screening also revealed clones with significant sequence homology to *wali7* (pBAN UD75), a gene induced in wheat (*Triticum aestivum*) roots by exposure to Al (Richards et al., 1994). A clone that shared homology with an antifungal protein (pBAN UD39) was also iso-

lated and was down-regulated during ripening. Clone pBAN UD120 was related to an extensin (Table I).

DISCUSSION

Numerous biochemical and molecular events are responsible for the ripening of fleshy fruits. Two-dimensional SDS-PAGE gels of in vitro translation products from banana peel and pulp showed significant up-regulation of a wide variety of transcripts within 24 h after exposure to exogenous ethylene. In many cases the same translation products appeared to be present in both unripe and ripe fruit tissue, but their expression was greatly enhanced during ripening. The generation of a pulp cDNA library from fruit 24 h after exposure to ethylene (d 2) and differential screening of this library permitted the isolation of a large number of ripening-related cDNA clones. Many of these are likely to be involved in key metabolic pathways, including ethylene biosynthesis, respiration, carbohydrate metabolism, and cell wall degradation, and are reported here for the first time as being up- or down-regulated during fruit ripening. Also, to our knowledge, this paper and the work of Clendennen and May (1997) are the first studies of ripening-related gene expression in the fleshy fruit of a monocot.

A climacteric increase in respiration accompanies ripening in banana fruits, and it might be expected that messages encoding enzymes involved in respiration would be isolated. Clendennen and May (1997) did not obtain any of these transcripts in their differential screen, which may reflect differences in the stages of ripening examined by each laboratory. The current study yielded a single clone encoding the enzyme aconitase, which participates in the TCA cycle and can also be involved in the glyoxylate cycle. The factors regulating the climacteric increase in respiration and the role of this increase in ripening are unclear. These data suggest, however, that alterations in the level of aconitase activity are a component of these changes. Relatively few plant aconitase genes have been cloned (Hayashi et al., 1995; Peyret et al., 1995), but in Arabidopsis aconitase is up-regulated during a number of developmental events, including seed and pollen maturation and seed germination.

Starch metabolism and sugar accumulation are among the most apparent changes that occur in ripening bananas. Biochemical studies on banana have not established the main route for starch degradation, e.g. hydrolytic or phosphorolytic (see Seymour, 1993, and refs. therein), although the evidence suggests that starch breakdown in bananas is phosphorolytic (Hill and ap Rees, 1994). In the present study several of the sequences isolated encode proteins likely to be involved in starch metabolism. Granule-bound starch synthase was down-regulated during ripening, consistent with the change from starch biosynthesis during fruit filling to starch mobilization, whereas β-amylase was up-regulated during ripening. The latter enzyme catalyzes the liberation of maltose from the nonreducing end of 1,4-α-D-glucans, and β-amylase activity in banana has been reported to increase before the onset of the respiratory peak and parallel to starch degradation (Garcia and Lajolo, 1988).

Other starch-degrading activities that have been measured in ripening bananas include α-amylase, α-1,6-glucosidase, and phosphorylase (Garcia and Lajolo, 1988), but no cDNA clones for these enzymes were positively identified in our screen. The cell wall-bound invertase cloned in the current work is ripening-related and likely to be directly involved in the carbon flow from starch to Glc and Fru. However, the key invertase activity in ripening fruit is usually associated with soluble forms of the enzyme (Elliot et al., 1993).

Cell wall disassembly is a very apparent feature of ripening in fleshy fruit. In contrast to tomato and other climacteric fruit studied thus far, clones with homology to pectate lyases were particularly abundant in banana pulp. These enzymes are common in plant pathogenic bacteria that cause soft-rot diseases and they catalyze the depolymerization of the cell wall pectic polymers via degradation of the polyuronide backbone. Several plant sequences with homology to pectate lyases have been reported, including those from pollen grains in tomato (Wing et al., 1989), tobacco (Rogers et al., 1992), and maize (Turcich et al., 1993), and from stylar tissue in tomato and tobacco (Budelier et al., 1990).

Little is known about the occurrence of pectate lyase activity in fruits, and as far as we are aware, the substrate specificity of the published clones from other plant tissues has not been confirmed. The banana cDNA clone pBAN UU84 and the other plant sequences, however, do contain conserved regions corresponding to the reported substrate-binding region for pectate lyases (Turcich et al., 1993). The abundance of these pectate lyase-like clones in banana suggests a significant role for this activity in wall disassembly during ripening in the pulp.

Other clones that may be involved in cell wall disassembly are those encoding a putative β-1,3-glucanase and β-glucosidase. β-1,3-Glucanase is often regarded as a pathogenesis-related protein, being triggered in response to infection and having antifungal activity. However, β-1,3-glucanases are also expressed under other conditions. In cereals they act to degrade endosperm walls to facilitate starch mobilization during germination (Stone and Clarke, 1992; Simmons, 1994), and reporter gene studies with a β-1,3-glucanase promoter have indicated that the enzyme facilitates protrusion of the radicle through the endosperm in tobacco seeds (Vögeli-Lange et al., 1994).

The protein encoded by the banana β-glucosidase-like sequence may also function as a wall-degrading enzyme, an idea supported by its sequence similarity to a β-glucosidase from barley that appears to play a role in degrading endosperm cell wall polysaccharides during germination (Leah et al., 1995). Perhaps the function of these enzymes is analogous to their role in developing seeds, facilitating starch mobilization by cell wall disassembly in the pulp.

The extensin-like sequence encoded by pBAN UD120 is down-regulated in bananas during ripening and this may reflect biochemical events designed to weaken/change the structure of the pulp cell walls. We believe this to be the first report of ripening-related changes in the expression of a gene that may encode a cell wall structural protein.

Clendennen and May (1997) discovered abundant transcripts that encode proteins normally associated with plant defense responses in the pulp of ripening bananas. These included pathogenesis-related proteins such as chitinase, endochitinase, β-1,3-glucanase, and thaumatin, and stress-related messages with homologies to metallothioneins and ascorbate peroxidase. Many similar clones were isolated in the present study.

The thaumatin-like clone pBAN UD39, which showed a decrease in expression during ripening, has homology with an antifungal protein from maize (Malehorn et al., 1994). In cherry (*Prunus avium* L.), however, a thaumatin-like protein increases as the fruit ripens, but in preliminary studies it had no antifungal activity (Fils-Lycaon et al., 1996). Several of the clones expressed in ripening banana pulp have also been reported from ripening tomatoes, including heat-shock proteins, components of the ubiquitin pathway, and glutamate decarboxylase (Picton et al., 1993; Gray et al., 1994; Gallego et al., 1995). Induction of IFR gene expression has not been previously reported in ripening fruit, but recent findings indicate that an IFR-like sequence from Arabidopsis can confer resistance to oxidative stress in yeast (Babiychuk et al., 1995).

The biochemical functions of many of the cDNAs described in the present paper remain unknown, e.g. pBAN UU70, which is related to root-specific proteins. The rapid rate at which plant genes are being isolated, however, suggests that these sequences may soon be assigned a putative identity/function by database comparisons.

The cDNA clones described here and by Clendennen and May (1997) provide a basis for understanding the sequence of biochemical events involved in banana ripening. Work is now under way to characterize the pattern of expression of a variety of these clones, not only to relate their expression to specific ripening-related biochemical events, but also to provide information to allow the isolation of useful promoters.

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