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Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits

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Abstract. The ripening of strawberry (*Fragaria ananassa* Duch.), a non-climacteric fruit, is a complex developmental process that involves many changes in gene expression. To understand how these changes relate to the biochemistry and composition of the fruit the specific genes involved have been examined. A high-quality cDNA library prepared from ripe strawberry fruit was differentially screened for ripening-related clones using cDNA from ripe and white fruits. From 112 up-regulated clones obtained in the primary screen, 66 differentially expressed clones were isolated from the secondary screen. The partial sequences of these cDNAs were compared with database sequences and 26 families of non-redundant clones were identified. Northern analysis confirmed that all of these cDNAs were ripening-enhanced. The expression of many of their corresponding genes was negatively regulated in auxin-treated fruit. These sequences, several of which are novel to fruits, encode proteins involved in key metabolic events including anthocyanin biosynthesis, cell wall degradation, sucrose and lipid metabolism, protein synthesis and degradation, and respiration. These findings are discussed in relation to the role of these genes in determining fruit quality characteristics.

Key words: Auxin – *Fragaria* – Fruit quality – Fruit ripening – Gene expression – Metabolic pathways

Introduction

Strawberries are widely grown throughout the temperate regions of the world and are universally popular as a fresh fruit crop (Manning 1993). The biochemical events that occur during the ripening of strawberry result in important changes affecting fruit quality. These include the accumulation of red anthocyanin pigments, the loss of cell wall structure causing softening, an increase in the content of sucrose and hexoses, and the production of volatile flavour compounds. The biochemical characterization of these changes has been difficult because of the presence of high levels of phenolic compounds and viscous polysaccharides that interfere with enzyme isolation. To overcome these problems, molecular approaches have been used, firstly, to examine the extent of gene expression occurring during strawberry fruit development and, secondly, to identify the specific genes involved in ripening.

Some of the qualitative changes in gene expression that occur in developing strawberry fruit and their hormonal control have been described in a preliminary study (Manning 1994). In that study total RNA isolated from receptacle tissues was translated in vitro and the products analysed by two-dimensional polyacrylamide gel electrophoresis. Changes in the abundance of more than 50 mRNAs were observed between the immature and overripe stages, most of these occurring at or just before the appearance of anthocyanin pigmentation, used as a marker of ripening. As the fruit matured the expression of one group of genes increased, whereas another group showed a marked decrease before the onset of ripening. However, the expression of another groups of genes was prominent in immature green and ripe fruit but much reduced or undetectable just before the turning stage. Previous studies demonstrated that strawberry fruit ripening is independent of ethylene and therefore can be categorised as non-climacteric (Given et al. 1988a). Auxin, produced by the achenes, is known to be essential for fruit growth but also acts as a repressor of ripening in this fruit. Evidence from in-vitro translation data indicates that expression of the ripening-enhanced genes in strawberry is co-ordinated by the hormone auxin (Manning 1994).

The in-vitro translation data predict that in strawberry the highest number of genes related to ripening

Abbreviations: ACP = acyl carrier protein; AR = auxin regulated; CHR = chalcone reductase; CHS = chalcone synthase; EF-2 = elongation factor 2; F3H = flavonoid-3-hydroxylase; NAA = 1-naphthaleneacetic acid; OMT = *O*-methyltransferase; PCR = Polymerase chain reaction; PDC = pyruvate decarboxylase; POA = phenoxyacetic acid; UDPG = UDP-glucose; UFGT = UDPglucose:flavonol 3-*O*-glucosyltransferase

will be obtained by comparing fruit at the white and red stages. Wilkinson et al. (1995) used polymerase chain reaction (PCR) differential display to compare differences in gene expression between white and red strawberry fruit. Five mRNAs were identified as being ripening-enhanced, with three having homology to known proteins, including chalcone synthase. In another study, a cDNA subtractive library was used to compare genes expressed in red and green fruit (Medina-Escobar et al. 1997b). A cDNA encoding pectate lyase, an enzyme that may have a role in cell wall pectin degradation in strawberry (Medina-Escobar et al. 1997a), and eight other genes were isolated.

This study describes the isolation of 26 ripening-related clones from strawberry using a standard differential screening method. The possible functions of these genes in relation to fruit metabolism and to quality traits in strawberry are discussed.

Materials and methods

Plant material. Day-neutral strawberry (*Fragaria ananassa* Duch. cv. Brighton) plants were grown as described by Manning (1994). Freshly picked fruits (40 g) were collected at the white (16 d post-anthesis, with green achenes) and ripe (fully red) stages, frozen in liquid N_2 and stored at −75 °C. Stages of fruit and treatment of de-achened fruit with 1-naphthaleneacetic acid (NAA) and phenoxyacetic acid (POA) were as previously described (Manning 1994).

Isolation of RNA. Achenes were removed from the frozen fruits with a scalpel and the de-achened receptacles were ground to a fine powder in liquid N_2. Total nucleic acids were extracted from the powdered tissue and total RNA isolated by LiCl precipitation as previously described (Manning 1991).

Isolation of poly(A)$^+$mRNA and construction of a cDNA library from ripe fruit. Messenger RNA was isolated from total RNA by oligo(dT)-cellulose column chromatography (Bantle et al. 1976). Double-stranded (ds) cDNAs were prepared from oligo (dT)-primed poly(A)$^+$mRNA from white and ripe fruit using the System Plus Kit (Amersham Life Sciences, Amersham, Bucks, UK) according to the manufacturer's instructions. The ds cDNAs from ripe fruit were cloned into the λgt10 vector using *Eco*RI linkers (BRL, Paisley, Renfrewshire, UK) to produce a primary cDNA library of 2.95×10^4 clones. Insert sizes ranged from 0.25 to 4.0 kb in a random sample of clones.

Differential screening. The unamplified library from ripe strawberry fruit was differentially screened using cDNA from white and ripe fruit tissue. In the primary screen, 1343 plaques were plated onto four 10-cm square plates and duplicate lifts made from each onto Hybond-N nylon membranes (Amersham). Probes were prepared by labelling cDNA from white and ripe fruit with digoxigenin (DIG) using random primed labelling (Boehringer-Mannheim, Lewes, Sussex, UK). One of each pair of membranes was hybridized to DIG-labelled ds cDNA from ripe fruit, and the other duplicate membrane was hybridized to DIG-ds cDNA prepared from white fruit. Hybridizations were at 65 °C using 8 ng · ml^{-1} probe in Hybsol hybridization buffer (Yang et al. 1993). Post-hybridization washes were at room temperature for 2 × 5 min with 2 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) in 0.1% SDS, followed by two 15-min washes with 0.1 × SSC in 0.1% SDS at 68 °C. Labelled probe bound to the membranes was detected colorimetrically using an anti-DIG alkaline phosphatase conjugate (Boehringer). Plaques preferentially binding to DIG-labelled cDNA from ripe fruit were picked into phage dilution buffer and replated at low density in 10-cm square plates with 25 compartments. For the secondary screen, duplicate plaque lifts were made onto 2.0-cm square membranes and reprobed as before with DIG-labelled ripe and unripe cDNA. Isolated plaques preferentially hybridizing to ripe cDNA were picked for further characterization.

Characterization of ripening-related clones. Lambda DNA was prepared from the ripening-related clones, restricted with *Eco*RI and analysed by agarose gel electrophoresis. Fragments from clones with multiple inserts were isolated by Qiaex resin (Qiagen, Dunmow, Essex, UK) and duplicate quantities of cDNA dot-blotted onto Hybond-N membranes. Inserts from ripening-enhanced cDNAs were identified by probing with ripe and white cDNA as described above and sub-cloned into an *Eco*RI-digested pBK-CMV vector (Stratagene, Cambridge) for sequencing.

Sequencing. Differential screening produced 66 putative ripening-related clones. Lambda clones having single inserts were sequenced with reverse and forward λgt10 primers (Sigma, Southampton, UK) using a dye terminator cycle-sequencing ready reaction kit (Prism; Applied Biosystems, Warrington, Lancs., UK) with either AmpliTaq or AmpliTaq FS DNA polymerase (Applied Biosystems). Lambda clones not giving good sequence were sub-cloned into the pBK-CMV vector and these and the sub-cloned multiple inserts were sequenced as above, but with M13 reverse and forward primers. The partial sequences obtained from each strawberry clone were edited to remove vector sequences and compared with sequences in the GenEMBL database using the FASTA, TFASTA and BLASTX programs of the Software Package (version 8) from the Genetics Computer Group (University of Wisconsin, Madison, Wis., USA). One of the cDNAs (FAN R3) was fully sequenced using internal primers.

Northern analysis. The expression of the putative ripening-enhanced clones was assessed in a range of strawberry tissues and in fruit treated with auxin. Total RNA, sufficient for several northern blots, was isolated from the receptacle tissue of fruits at 4, 7, 10, 13, 16, 19, 22 (turning) days after anthesis, and from the orange, ripe and overripe stages. RNA was also prepared from auxin treated de-achened fruit (Manning 1994), from mature leaves and petioles and from roots of plants grown in solution culture in Murashige and Skoog nutrient mixture (Sigma). The RNA was dissolved at 1 μg · μl^{-1} in loading buffer (2.2 M formaldehyde, 50% formamide, 5% (w/v) glycerol, 50 μg · ml^{-1} ethidium bromide, 20 mM Mops, 5 mM sodium acetate, 1 mM EDTA, 0.01% bromophenol blue) and stored at −75 °C. Samples of RNA (10 μg) were denatured for 15 min at 65 °C and separated on 1.4% formaldehyde-agarose gels with an electrode buffer containing 0.22 M formaldehyde (Tsang et al. 1993). The RNA was transferred to positively charged membranes (Boehringer) by capillary blotting and fixed by UV light for 5 min. Transcripts were detected either with DIG-labelled probes as described above, or with ^{32}P-labelled probes. For the latter, membranes were prehybridized and hybridized in 5 × Denhardt's solution [1 × Denhardt's = 20 mg % Ficoll 400 (Pharmacia, St. Albans, Herts, UK), 20 mg % polyvinylpyrrolidone, 20 mg % bovine serum albumin (Fraction V; Sigma)], 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 1% SDS and 100 μg · ml^{-1} denatured salmon sperm DNA at 65 °C, using selected cDNA clones labelled with [^{32}P]dCTP used as probes. The membranes were washed twice in 3 × SSPE in 0.1% SDS for 20 min at 65 °C followed by 0.3 × SSPE in 0.1% SDS for 20 min at 65 °C before exposure to X-ray film at −75 °C.

Results

Screening of a ripe cDNA library. From the initial screen of 1343 plaques, 112 ripening-enhanced clones were selected. Following the secondary screen and

Table 1. Characterization of ripening-related cDNA clones from strawberry receptacles

Clone	Number of clones	Transcript size	Database accession number for strawberry clone	Putative identity	Related sequence and accession number	% homology	Overlap
FAN R1	1	1.2	AF041384	*O*-methyltransferase	Almond X83217	81	320
FAN R2	1	1.6	AF041385	Flavonoid-3-hydroxylase (1)	Grape X75965	65	167
FAN R3[a]	3	0.9	AF041386	Acyl carrier protein	Swamp oakY10994	68	408
FAN R5	1	1.4		Chalcone synthase (1)	Apple X68977	72	337
FAN R10	1	1.6	AF041387	No significant homology			
FAN R13	1	2.6	AF041388	Expressed sequence tag	*Arabidopsis* T45068	62	382
FAN R16b	1	2.5	AF041389	Pyruvate decarboxylase	*Arabidopsis* U71122	72	697
FAN R26	1	1.5	AF041390	Meristem pattern gene	*Petunia* X92205	63	273
FAN R31c	6	1.2	AF041391	Chalcone reductase	Soybean X55730	67	652
FAN R33a	2	3.8	AF041392	Elongation factor 2	Sugarbeet Z97178	75	583
FAN R33b	2	1.2	AF041393	Auxin-regulated 1	Tobacco Y56266	68	287
FAN R40	1	2.6	AF041394	No significant homology			
FAN R48	5	1.9	AF041395	No significant homology			
FAN R55a	1	2.0	AF041396	Flavonoid-3-hydroxylase (2)	Apple X71360	80	549
FAN R56	1	1.6	AF041397	Expressed sequence tag	*Arabidopsis* N97151	61	321
FAN R60	3	1.6	AF041398	Chalcone synthase (2)	*Vigna* X74821	77	472
FAN R61c	19	1.4	AF041399	Auxin-regulated 2	Mungbean U20808	69	707
FAN R62	2	2.4	AF041400	No significant homology			
FAN R74	1	1.9	AF041401	UDPglucose glucosyltransferase	Grape AF000371	64	358
FAN R84	4	1.5	AF041402	Flavonoid-3-hydroxylase (3)	Apple X69664	76	452
FAN R85	1	1.9	AF041403	Flavonoid-3-hydroxylase (4)	Apple X71360	69	348
FAN R93c	1	1.5	AF041404	Cysteine proteinase	*Phaseolus* Z99953	70	339
FAN R97	3	2.0	AF041405	Cellulase	*Arabidopsis* X98543	72	300
FAN R106c	1	2.3	AF041406	Malonyl-CoA decarboxylase	Goose L21171	54	534
FAN R109	2	2.0	AF041407	UDPglucose IAA-glucosyltransferase	*Arabidopsis* Z97339	64	451
FAN R110b	1	2.8	AF041408	Sucrose transporter	Spinach X67125	56	599

[a]This clone was fully sequenced

sub-cloning of the multiple inserts, 66 putative ripening-enhanced cDNAs were obtained and partially sequenced. Clone FAN R3 was later fully sequenced using internal primers.

Northern analysis. All the clones described in Table 1 were shown by northern blot analysis to be up-regulated between day 16 and the orange stage of development (Fig. 1). Several clones appear to be fruit-specific since their transcripts were undetectable in leaf, petiole and root tissues. The levels of expression in the segments of de-achened fruit treated with water (control) were generally lower than those in normal ripe fruit and equated with fruit at the turning stage (e.g. FAN R48 and FAN R62, in Fig. 1). Although removal of the achenes accelerated anthocyanin accumulation in the control segments, they did not become fully ripe by the time the fruit were sampled.

Identification of ripening-enhanced clones. The sequence of the cDNA clone FAN R1 is closely related to *O*-methyltransferases (OMTs) from other species (Table 1), the predicted amino acid sequence from a 497-bp region being 84% identical to a homologue from alfalfa (P28002) (Gowri et al. 1991).

The differential screen isolated seven ripening-enhanced strawberry clones belonging to a family of cDNAs encoding a putative flavonoid-3-hydroxylase (F3H). Comparisons of the strawberry sequences and analysis of their database alignments reveals that clones FAN R2, FAN R55a, FAN R84 and FAN R85 represent four different homologues of F3H (F3H-1 to F3H-4, respectively). Of these, FAN R55a had the highest degree of identity to sequences in the databases, clone X71360 from apple being the most closely related. Expression patterns of the putative F3H genes were similar during fruit development and in auxin-treated fruit (Fig. 1). Transcripts were present in immature fruit and declined to a low level before increasing again just prior to ripening. However, the expression of the transcript hybridizing to FAN R55a was relatively low at the turning stage, showing that this gene is activated about 3 d after the other homologues.

Three similar and nearly full-length cDNAs from ripe strawberry encode the same acyl carrier protein (ACP). A representative clone, FAN R3, which was fully sequenced, differs from the homologue Aj001446 (database, unpublished) from the fruit of *Fragaria vesca*, the wild strawberry, by 19 bp over a 674-bp overlap, the translated product differing by nine amino acids. An ACP from *Cuphea lanceolata*, Swamp Oak, (Y10994) has significant similarity to the strawberry clone. The coding region of FAN R3 predicts a protein with a highly conserved domain around the phosphopantotheine-binding site at serine 39, typical of plant ACPs (Slabas et al. 1987).

Transcripts with high similarity to two chalcone synthases (CHS) were isolated from strawberry as

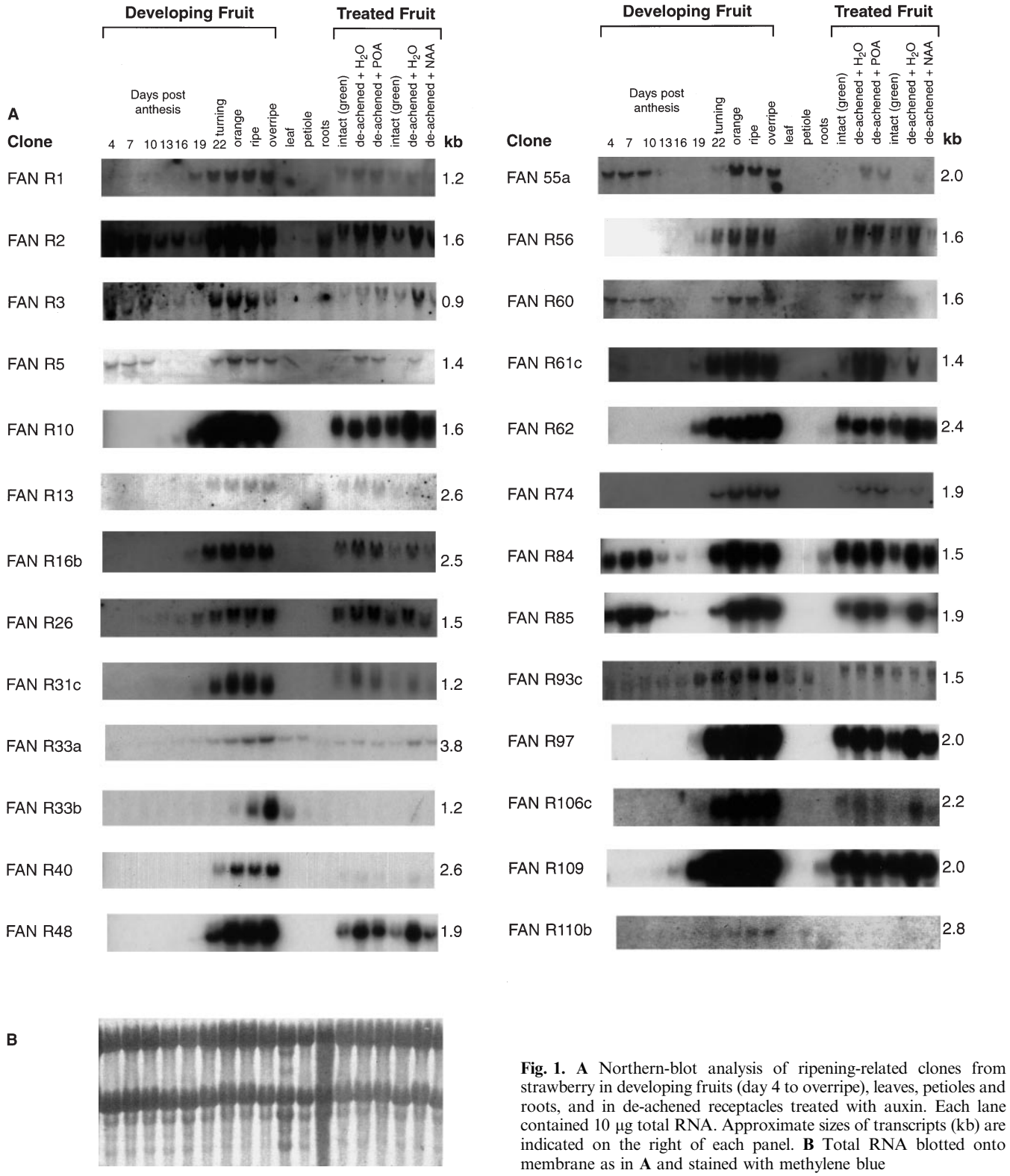

Fig. 1. **A** Northern-blot analysis of ripening-related clones from strawberry in developing fruits (day 4 to overripe), leaves, petioles and roots, and in de-achened receptacles treated with auxin. Each lane contained 10 μg total RNA. Approximate sizes of transcripts (kb) are indicated on the right of each panel. **B** Total RNA blotted onto membrane as in **A** and stained with methylene blue

ripening-enhanced cDNAs. The partial sequence of one of these (FAN R5) encodes CHS-1 which appears to be identical to the CHS (clone RJ5; U19942) isolated by Wilkinson et al. (1995) using the PCR differential display technique. However, the expression of CHS-1 found in immature fruit (10 d post-anthesis) is much higher than that reported by Wilkinson et al. (1995) for RJ5 in fruit at an equivalent stage of development. Of the five cDNAs reported by these authors, RJ5 is the only clone with identity to our strawberry clones. Three cDNAs, represented by FAN R60, encode a different chalcone synthase (CHS-2) which at the amino acid level has highest homology to D26594, a CHS from camellia (Takeuchi et al. 1994). CHS-1 is more similar to the

D26595 homologue from the same species. The pattern of expression of CHS-2 (FAN R60) in developing fruit is similar to that of CHS-1 (FAN R5).

A non-redundant clone from strawberry (FAN R16b) has high homology to pyruvate decarboxylase (PDC), being most like the sequence from *Arabidopsis* (U71122, unpublished). The deduced protein shares significant similarity with PDCs from a number of crop plants including pea, maize and rice. It is one of the more abundant transcripts in the fruit, its expression rising sharply at day 19 to reach a maximum value at the orange stage. It does not appear to be expressed in the leaves, petioles and roots.

Clone FAN R26 shares homology with members of the *no apical meristem* (*nam*) gene family encoding proteins with unknown function (Souer et al. 1996). The strawberry sequence is also similar to the *Arabidopsis* clones ATAF1 and ATAF2 (GenBank accession numbers X74756 and X74755, respectively). The deduced sequences of all these clones are remarkably similar at the N-terminal region, the peptide fragment GFRFHPTDEEL being identical.

Six of the clones induced in ripening strawberry are apparently encoded by the same gene, a putative chalcone reductase (CHR). Clone FAN R31c, a representative member of this group, is most similar to a cDNA isolated from suspension-cultured cells of soybean (X55730; Welle et al. 1991).

One ripening clone from strawberry (FAN R33a) shows an unexpected similarity to elongation factor 2 (EF-2), an essential component of eukaryotic protein synthesis. The highest similarity to this clone was found in a recent but unpublished cDNA sequence (Z97178) from sugar beet, the only other homologues sequenced in higher plants being those from tobacco (Y10991) and rice (D21277). Strawberry EF-2 also has significant sequence homology to EF-2s from *Chlorella* (M68064; Schnelbogl and Tanner 1991), yeast (D89151), nematode (Z81068) and *Giardia* (D29835). The transcript hybridising to FAN R33a was present at a low level in day-4 fruit but differed from the other transcripts in that it continuously increased during development, reaching a maximum level in overripe fruit. It was also expressed in leaves and petioles. EF-2 catalyses the translocation of peptidyl tRNA from the aminoacyl site to the peptidyl site on the ribosome in a GTP-dependent reaction. It is interesting to note that another elongation factor (EF-Tu) was used by Wilkinson et al. (1995) as a probe to monitor the loading of strawberry RNA on northern blots since its expression was approximately constant throughout fruit development.

Two strawberry cDNAs (FAN R33b and FAN R33c), apparently *Eco*RI fragments of the same clone, auxin-regulated 1 (AR-1), share homology with groups of genes whose precise functions within the cell are unknown. Amongst these homologues are genes whose expression is induced by auxin (see Table 1) and genes that encode glutathione transferases found in a wide range of plant species, including leaf mustard (Y13898), which has the highest identity to the corresponding strawberry cDNA. Another group of homologues belongs to the *par* genes (Takahashi and Nagata 1992) which are expressed during the transition from the G_o to the S phase of the cell cycle. The amino acid sequence predicted from the partial sequence of FAN R33b is 59% identical to each of the tobacco clones parA (P25317), parB (M29274) and parC (X64398) over regions of 90, 90 and 91 amino acids, respectively. Expression of FAN R33b is distinguished from the other ripening-related clones in that it does not increase until the fruit is at the orange stage and then continues to increase considerably in overripe fruit.

One of the strawberry clones, auxin-regulated 2 (AR-2), was particularly abundant, accounting for 29% and 1.4% of the ripening-related and total clones, respectively. A representative of these cDNAs, FAN R61c is almost identical to a clone from *Fragaria vesca* (Aj001445, unpublished database data), postulated to be a metallothionein protein, although FASTA and BLAST searches did not confirm such a function. However, FAN R61c is similar to an auxin up-regulated gene from mungbean (Table 1; Chen et al. 1996), but shows no similarity to any other sequences. FAN R61c was not expressed in developing fruit until the white (day 19) stage, when there was a dramatic increase in expression, attaining the highest levels in fully ripe fruit. This gene was repressed in de-achened fruit treated with NAA but not with POA, and was not expressed in leaves, petioles and roots.

Two types of clones putatively requiring the cofactor UDP-glucose (UDPG) are expressed in ripening strawberry fruit. The partial sequence of one of these (FAN R74), a non-redundant clone, encodes a polypeptide with similarity to the *Bronze-1* genes of maize (60% identity and 72% similarity over 32 amino acids; Furtek et al. 1988) and barley (40% identity and 72% similarity over 32 amino acids; Wise et al. 1990). The *Bronze-1* alleles encode UDPglucose:flavonol 3-*O*-glucosyltransferase (UFGT), one of the essential and terminal steps in anthocyanin biosynthesis involving the 3-*O*-glycosylation of flavonoids. FASTA and BLAST searches of the databases reveal significant base homology of the strawberry clone to genes encoding other UDPG-binding proteins in grape (see Table 1), and other plant species.

The other group of UDPG-dependent clones from strawberry comprises two similar cDNAs represented by FAN R109. This clone has highest homology to three coding regions of a genomic clone (Z97339, unpublished data) from *Arabidopsis* encoding a putative indole-3-acetate β-glucosyltransferase. Importantly, FAN R109 is also similar to a cDNA from maize (L34847) that was expressed in *Escherichia coli* as a recombinant protein which catalysed the formation of 1-*O*-β-D-indol-3-ylacetyl-glucose from UDPG and IAA (Szerszen et al. 1994). Other homologues of the strawberry clone include UFGTs, typified by *Bronze-1* (P16166), and UDP-glucuronosyltransferases from a number of species including tomato (ERT1b; X72729; Picton et al. 1993), rat (P19488; Mackenzie 1990) and human (P36509; Ritter et al. 1992). Differences between the expression patterns of FAN R74 and FAN R109 indicate that they

might have separate functions. The transcript of FAN R74 was absent in the non-fruit tissues of the plant and was repressed to a greater degree by NAA than was the transcript of FAN R109. FAN R74 was appreciably more highly expressed in young fruit than was FAN R109 (Fig. 1).

A cDNA (FAN R93c) encoding a putative cysteine proteinase was isolated from strawberry. The partial sequence is most similar to a cDNA from kidney bean (Z99953) and has significant homology to cysteine proteinases from *Vicia sativa* (Z30338) and tomato (Z14028). Other homologues include stress-related cysteine proteinases induced by glucose starvation in maize (Q10716), by dehydration in pea (X54358) and *Arabidopsis* (P43296), and by wounding in tobacco (Z13959). These homologues and the deduced polypeptide from strawberry appear to belong to a group of cysteine proteinases with the modified ERFNIN concensus sequence (Karrer et al. 1993) of ERFNAQ. A vacuolar-like targeting signal of IRQV (Chrispeels and Raikhel 1992) is also present in the predicted N-terminal propeptide. Strawberry cysteine proteinase does not appear to be closely related to a ripening-related protease from citrus (Alonso and Granell 1995), a non-climacteric fruit, which is ethylene-inducible. FAN R93c is unrelated to the SENU3 cysteine proteinase expressed in senescing leaves of tomato (Drake et al. 1996). The expression of FAN R93c differs significantly from the majority of the strawberry clones in that it is not repressed by auxin (NAA) in de-achened fruit.

Transcripts encoding cell-wall-degrading enzymes are likely to be isolated in soft fruits such as the strawberry, whose firmness decreases rapidly during ripening. Three similar cDNAs encoding a putative endo-1,4-β-D-glucanase (cellulase) were isolated. The clone FAN R97 is representative of these and most closely resembles the cellulase sequences from *Arabidopsis* (Shani et al. 1997). It also shares homology with cellulases from a number of fruits including avocado (Tucker et al. 1987), peach (Trainotti et al. 1997), pepper (Ferrarese et al. 1995) and Cel2 from tomato (Lashbrook et al. 1994). Cellulases in plants generally have a low identity to fungal cellulases. However, the translation product deduced from the partial sequence of the strawberry clone, does have significant homology to a fragment of the cellulase clone from *Thermomonospora fusca* (Lao et al. 1991), with 50% identity and 63% similarity over 30 amino acids.

One of the strawberry cDNAs, FAN R106c, has homology to malonyl-CoA decarboxylase from goose, the translated product having 36% identity and 52% similarity to the corresponding amino acid sequence (P12617) over a 68 residue overlap.

Soluble sugars accumulate to about 8% of the fresh weight in ripe strawberries. The differentially expressed clone FAN R110b from strawberry has homology with sucrose transporters from a range of species including spinach (X67125, see Table 1; Riesmeier et al. 1992), *Arabidopsis*, tobacco and potato.

The remaining ripening-enhanced clones either showed homology to expressed sequence tags (FAN R13 and FAN R56) of unknown function or were unrelated to any database sequences (FAN R10, -40, -48 and -62).

Discussion

The isolation, identification and expression pattern of 26 ripening-related cDNAs described here supports earlier suggestions from translation studies (Manning 1994) that numerous changes in gene expression are involved in the biochemical events associated with ripening of strawberry fruit. Putative functions have been assigned to many of these genes based on their convincing homology to database sequences. Single genes and families of genes with two or more homologues have been isolated, which are likely to be involved in key metabolic pathways related to colour, texture, respiration, carbohydrate composition and flavour. In agreement with earlier analysis of translation products (Manning 1994), strawberry ripening-related genes are negatively regulated by auxin, except for cysteine proteinase and a few late up-regulated clones with weak expression in de-achened fruit. Auxin was previously shown to delay ripening in strawberry (Given et al. 1988a) and has recently been shown to delay ripening in grape and to alter the expression of genes developmentally regulated in the fruit (Davies et al. 1997). Auxin inhibited the expression of the up-regulated genes, and retarded the silencing of down-regulated genes in ripening grapes.

One of the most dramatic visual changes to occur as strawberry fruits ripen is the rapid increase in anthocyanin content. Six of the gene families isolated appear to encode enzymes involved in phenylpropanoid metabolism and the formation of the coloured anthocyanin products. These co-ordinately up-regulated genes are CHS, CHR, F3H, OMT and two UDP-dependent glycosyl transferases. Of these, CHS and F3H had a different pattern of expression, their transcripts being present in immature and ripe fruit, but much reduced at the white stage. This up-down-up pattern of expression was observed previously in the in-vitro translation products from strawberry (Manning 1994), and more recently for similar genes from grape, another non-climacteric fruit (Boss et al. 1996). Transcripts of CHS, F3H, phenylalanine ammonia-lyase, chalcone isomerase, dihydroflavonol 4-reductase and leucoanthocyanin dioxygenase increased in the second phase (veraison) of fruit development, coinciding with anthocyanin accumulation in the skin of the grape berry. As in strawberry, UFGT (CHR was not investigated) was not expressed in grape in the early stages of fruit development. The similarity between grape and strawberry in expression of these structural genes suggests there are common points in the control of anthocyanin biosynthesis in fruits that may be different from those found in flowers.

It is likely that the two cDNA clones encoding UDP-requiring enzymes are involved in the biosynthesis of different products in the fruit. One of these (FAN R74) probably encodes a UFGT, which regulates the terminal

step of anthocyanin formation. An increase in the activity of a UFGT has been demonstrated in ripening strawberry fruit (Given et al. 1988b). In addition to anthocyanins, a range of flavonol-3-glucosides and flavonol-3-glucuronides are found in strawberry (Ryan 1971). Whether the formation of these compounds results from the action of several enzymes with different specificity or from a single enzyme with a broad specificity is unknown. The other UFGT-like homologue may catalyse the addition of different sugar moieties to the phenylpropanoids in strawberry. An alternative and intriguing possibility is that one of the postulated UDP-dependent strawberry enzymes conjugates IAA in the fruit. Ester-conjugated IAA has been detected in strawberry receptacles (Archbold and Dennis 1984), and glycosylation could be a mechanism for regulating the concentration of free (or unconjugated) IAA in the fruit.

Softening in strawberry fruit coincides with a marked increase in the solubilization of polyuronides from the cell wall (Knee et al. 1977). Of several candidate cell-wall-degrading enzymes examined in strawberry, cellulase activity shows the closest correlation with fruit softening and increases in strawberry fruit during the later stages of ripening (Abeles and Tanaka 1990). Cellulase is the only clone involved in cell wall hydrolysis to be isolated from our ripening-enhanced cDNAs, although a pectate lyase clone, which also appears to be fruit specific, has recently been characterized from strawberry (Medina-Escobar et al. 1997a). Cellulase activity has been detected in a number of ripe fruits, both climacteric and non-climacteric, in elongating zones of flowering stems (Shani et al. 1997) and in petiole, fruit and flower abscission zones. The role of cell wall hydrolases has been most intensively studied in tomato, which has relatively high polygalacturonase activity but low cellulase activity, approximately 1% of that in strawberry. However, down-regulation of polygalacturonase does not abolish softening in tomato, indicating that no single enzyme plays a dominant role in the changes in texture associated with ripening fruits.

The involvement of ACP, an essential component of fatty acid synthetase, in the ripening of strawberry is unexpected since the lipid composition of the fruit changes little during development (Couture et al. 1988). However, numerous volatile flavour compounds are produced by the fruit (Zabetakis and Holden 1997). These include several alcohols, aldehydes and esters that may be secondary lipid metabolites derived from linolenic and linoleic acids via the action of lipoxygenase and hydroperoxide lyase (Gardner 1991). The products of these enzymes are volatile aldehydes of chain lengths C_6 and C_9. The formation of alcohols and esters from such aldehydes is believed to be catalysed by alcohol dehydrogenase and alcohol acyltransferase (Perez et al. 1996). Acyl carrier protein has previously been studied in relation to lipid accumulation in tissues such as seeds (Slabas et al. 1987), but in strawberry fruit ACP may be involved in flavour biogenesis. We hypothesize that de novo synthesis of ACP is required to maintain the production of volatile flavour compounds that are lost from the fruit.

Another ripening-related gene from strawberry that may have a role in fatty acid biosynthesis is malonyl-CoA decarboxylase. The activity of malonyl-CoA decarboxylase has been detected in a number of plants (Hatch and Stumpf 1962) but the only eukaryotic organism in which the nucleotide sequence encoding this enzyme has been described is the goose (Jang et al. 1989). A specialized organ in the goose, the uropygial gland, accumulates branched-chain fatty acids with multiple methyl side chains. Malonyl-CoA decarboxylase regulates fatty acid biosynthesis in this tissue by removing malonyl-CoA ensuring that methylmalonyl-CoA is the only chain-elongating substrate for fatty acid synthase (Kolattukudy et al. 1987). Alternatively, this enzyme may prevent the harmful accumulation of malonyl-CoA in the mitochondrion (Courchesne-Smith et al. 1992).

Relatively few regulatory genes have been isolated from fruits. In climacteric fruits, it is likely that ripening is regulated by genes involved in the pathway of ethylene signal transduction (Bleecker and Schaller 1996). Two types of ripening-enhanced, but auxin-repressed, genes from strawberry (AR-1 and AR-2) are related to genes whose expression is induced by auxin in other species. The deduced product from some of the AR-1 homologues is glutathione transferase, while the function of AR-2 and its homologue expressed in mungbean hypocotyls is unknown. However, AR-2 accounts for a high proportion of the strawberry clones, indicating this gene may have an important role in the ripening of this fruit.

Cysteine proteinases have a role in protein degradation, either for the remobilization of amino acids, as occurs in seeds and senescing tissues such as leaves, or for the selective removal of damaged proteins. An increase in cysteine proteinase expression occurs in strawberry fruit when there is little change in protein content (Manning 1994). This implies that the enzyme is not involved in general protein breakdown. Analysis of the changes in gene expression in strawberry fruit indicates that ripening involves de-novo synthesis of one set of proteins in the fruit while another set declines. Cysteine proteinases such as that encoded by FAN R93c may regulate the rate of protein turnover by removing proteins that are no longer required for the development of the fruit.

To our knowledge the putative sucrose transporter from strawberry is the first to be described in a fruit. The long-distance transport of photoassimilates from source organs (leaves) via the phloem to sink organs such as seeds, roots and fruits involves loading and unloading processes requiring the activity of various transporters. Sucrose is the principal carbohydrate translocated in strawberry and its corresponding transporters are likely to regulate the import of this sugar into the fruit. More than 70% of the total sugars in the ripening fruit accumulate in the vacuole (John and Yamaki 1994). Glucose and fructose increase in the vacuole in parallel with sucrose, indicating that some of the sucrose imported may be hydrolysed by invertase. Sugars in the

fruit are important components of flavour and balance fruit acidity. They also have an osmotic potential which influences turgor pressure, the driving force for cell and fruit enlargement. Furthermore, sugars may affect developmental processes by modulating the expression of other genes, for example CHS (Tsukaya et al. 1991).

Before sensitive gas-chromatographic methods were developed for the determination of ethylene, the distinction between climacteric and non-climacteric fruits was based on respiration (Kidd and West 1930). While attention has focused on ethylene in recent years, respiration in fruits has been neglected. There have been few studies on the enzyme PDC and as far as we are aware, the putative strawberry clone is the first to be described from a fruit. Pyruvate decarboxylase, which catalyses the formation of acetaldehyde from pyruvate, acts at a branch point in the glycolytic pathway between aerobic and anaerobic metabolism. Strawberry fruit exhibit a low rate of respiration during ripening and PDC may be important in regulating the supply of pyruvate to the aerobic pathway in this fruit. Alternatively, PDC may supply precursors for the biosynthesis of volatile flavour compounds as suggested by Hong and Harlander (1989) who demonstrated the formation of esters from strawberry tissue cultures.

The developmental regulation of gene expression observed in ripening strawberry results in major changes in the biochemistry and composition of the fruit. For the first time a gene has been identified in a fruit encoding a putative translation factor (EF-2) induced during ripening. This finding suggests that mechanisms operate in strawberry to enhance the de-novo synthesis of specific proteins in addition to transcriptional regulation. The role of elongation factors in plants has not been established. The activity of a partially purified EF-2 from wheat germ has been examined (Smailov et al. 1993). However, another family of elongation factors of the 1-α type, homologous to the bacterial factor EF-Tu, has been implicated in the control of plant development through the regulation of protein synthesis (Ursin et al. 1991). EF-1α is thought to affect plant growth and differentiation by interacting with microtubules (Durso and Cyr 1994).

The finding that a *nam*-like transcript is induced in ripening strawberry fruit is difficult to reconcile with a role in apical development. However, the proposal by Souer et al. (1996) that *nam* may influence the rate of cell expansion may be relevant in a fruit that continues to enlarge during the later phases of development.

Standard differential screening has proved to be highly effective in isolating ripening clones in several fruits including tomato (Slater et al. 1985; Picton et al. 1993) and banana (Medina-Suarez et al. 1997). In strawberry, this strategy appears to be more efficient than either PCR-differential display (Wilkinson et al. 1995) or a novel MAST-PCR-SBDS method (Medina-Escobar et al. 1997). In the latter, only 0.2% of the clones in a subtracted library, expected to be significantly enriched for ripening cDNAs, were isolated as ripening-enhanced. The cDNAs described here indicate some of the key biochemical pathways likely to be involved in strawberry fruit ripening. We are currently evaluating the role of a number of these clones in the biochemical and compositional changes affecting the fruit using transgenic plants.

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