



# Expression of six expansin genes in relation to extension activity in developing strawberry fruit

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## Abstract

Expansins are proteins which have been demonstrated to induce cell wall extension *in vitro*. The identification and characterization of six expansin cDNAs from strawberry fruit, termed *FaExp3* to *FaExp7*, as well as the previously identified *FaExp2* is reported here. Analysis of expansin mRNAs during fruit development and in leaves, roots and stolons revealed a unique pattern of expression for each cDNA. *FaExp3* mRNA was present at much lower levels than the other expansin mRNAs and was expressed in small green fruit and in ripe fruit. *FaExp4* mRNA was present throughout fruit development, but was more strongly expressed during ripening. *FaExp5* was the only clone to show fruit specific expression which was up-regulated at the onset of ripening. *FaExp6* and *FaExp7* mRNAs were present at low levels in the fruit with highest expression in stolon tissue. During fruit development *FaExp6* had the highest expression at the white, turning and orange stages whereas expression of *FaExp7* was highest in white fruit. The expression profiles of *FaExp2* and *FaExp5* in developing fruit were similar except that *FaExp2* was induced at an earlier stage. Analysis of expansin protein by Western blotting using an antibody raised against *CsExp1* from cucumber hypocotyls identified two bands of 29 and 31 kDa from developing fruit. Protein extracts from developing fruit were assayed for extension activity. Considerable rates of extension were observed with extracts from ripening fruit, but no extension was observed with protein from unripe green fruit. These results demonstrate the presence of at least six expansin genes in strawberry fruit and that during ripening the fruit acquires the ability to cause extension *in vitro*, characteristic of expansin action.

Key words: Expansins, strawberry, fruit, leaves, cell wall extension.

## Introduction

In order for a plant cell to grow it must be continually able to modify and expand its cell wall, which is composed of cellulose microfibrils tethered together with hemicelluloses and embedded in a matrix of pectins and structural proteins (Carpita and Gibeaut, 1993). Changes in the primary cell wall are not only required for cell expansion but also for developmental events, such as fruit softening. Transient disassembly of the cell wall during growth permits extension without permanently weakening the wall (Shieh and Cosgrove, 1998). In ripening fruits, irreversible changes in wall architecture (Rose and Bennett, 1999) result in progressive loss of firmness. In strawberry fruit, growth occurs throughout development with an increase in cell volume of approximately 1000-fold accompanied by a corresponding 100-fold increase in wall area (Knee *et al.*, 1977). This enormous enlargement relies on the ability of the cell wall to expand or 'loosen'. Growth continues during the ripening phase in which there is a dramatic alteration in fruit texture. The firmness of strawberry fruit declines throughout development, partly as a result of swelling of the cell wall as the hydration status increases. The degradative changes associated with ripening involve multiple structural changes within the cell wall that affect pectins and the cellulose–xyloglucan framework (Redgwell *et al.*, 1997) resulting in increased polyuronide and hemicellulose solubilization (Knee *et al.*, 1977). In strawberry there is a substantial loss of fruit firmness with an 11-fold decrease between unripe and ripe fruit (Redgwell *et al.*, 1997). Of the possible candidate genes involved in cell wall metabolism, cellulase and

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pectate lyase are the only genes isolated from strawberry fruit which show increased expression during ripening (Medina-Escobar *et al.*, 1997; Manning, 1998; Llop-Tous *et al.*, 1999; Trainotti *et al.*, 1999). However, as yet their role in fruit ripening has not been established.

A class of proteins called expansins have been shown to promote cell wall loosening *in vitro* and to catalyse wall extension and stress relaxation in a pH-dependent manner (McQueen-Mason *et al.*, 1992). They do not appear to be conventional enzymes because their loosening effect on the wall is reversible and does not increase with time. Biochemical and biophysical data indicate that expansins bind to the surface of cellulose microfibrils thereby disrupting the hydrogen bonds formed with xyloglucan molecules and so allowing the cell wall to extend. Expansin mRNA has been identified in a range of growing vegetative tissues including cucumber hypocotyls, *Arabidopsis* (Shcherban *et al.*, 1995), rice internodes (Cho and Kende, 1997), tomato meristems (Fleming *et al.*, 1997) and cotton fibres (Shimizu *et al.*, 1997). In *Arabidopsis*, more than 20 expansin open reading frames have been identified (Cosgrove, 1998) and it has become clear that expansins are represented by a substantial multi-gene family (McQueen-Mason and Rochange, 1999). In fruits, expansin cDNAs have been isolated from apricot (GenBank accession number AF038815, unpublished data), sweet cherry (GenBank accession number AAG13983, unpublished data) tomato, and strawberry (Rose *et al.*, 1997; Civello *et al.*, 1999). In tomato, the expression of six expansin genes has been described in unripe and ripening fruit suggesting a role for expansins in fruit development and ripening (Brummell *et al.*, 1999a). The expansin cDNA, *FaExp2*, characterized in strawberry fruit is ripening related and encodes a protein that is most closely related to expansins expressed in apricot fruit (Civello *et al.*, 1999).

Further evidence of the role of expansins in fruit softening is shown by Brummell *et al.* where transgenic tomato plants overexpressing *LeExp1* produced softer fruit than wild-type plants (Brummell *et al.*, 1999b). Overexpression of *LeExp1* altered the depolymerization of structural hemicelluloses whereas polyuronide depolymerization was not directly affected.

The identification of six expansin cDNAs (*FaExp2-7*) with unique expression patterns in developing strawberry fruit is reported here. In addition, it is demonstrated that expansins from ripening strawberry fruit are able to catalyse extension.

## Materials and methods

### Plant materials

Day neutral strawberry plants (*Fragaria ananassa* Duch. cv Brighton) were grown in a glasshouse with a 12/18 °C night/day regime. Fruit were harvested at 4, 7 and 10 d post-anthesis

(DPA) and collectively termed small green fruit, 13 and 16 DPA (large green fruit) and at the white (19 DPA), turning, orange, ripe, and overripe stages. The seeds (achenes) were harvested from small green and ripe fruit. Young, rapidly expanding and mature fully expanded leaf and stolon tissue was harvested from the same plants. Stolon growth was measured daily to determine the expanding and the fully expanded regions. To collect root tissue, strawberry plants were grown hydroponically in Murashige and Skoog nutrient solution with aeration. Expanding root tissue was harvested from the last 2 cm of root tissue including the root tips. Fully expanded root tissue was harvested from the upper section of the roots. All tissue was frozen immediately in liquid nitrogen and stored at -75 °C.

### RNA extraction

Extraction of total RNA from fruits and other tissues was as previously described (Manning, 1991).

### Cloning of expansin cDNAs

First strand cDNA was synthesized from total RNA (5 µg) isolated from small green fruit (7 DPA) and from orange stage fruit using M-MLV reverse transcriptase (SuperScript Pre-amplification System) primed with oligo (dT)<sub>12-18</sub> according to the manufacturer's instructions (Life Technologies). Expansin sequences were amplified by polymerase chain reaction (PCR) using degenerate primers designed to conserved amino acid domains (sense primer, 5' ATGGGIGGIGCNTGYGGNTA 3'; antisense primer, 5' TGCCARTTYTGNCCCCARTT 3'; I = deoxy inosine; N = A, T, C or G, Y, C or T, R, A or G). Amplification was performed with cDNA synthesized from 0.5 µg total RNA for 35 cycles (94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min) and one cycle of 72 °C for 10 min. Reaction products were analysed on a 1.5% (w/v) agarose gel and a single band of ~500 bp was purified by Qiaex II (Qiagen, Crawley UK) and either used directly as a probe or cloned into pPCR-Script (Stratagene). The putative expansin clones were grouped by their *Sau* 3A restriction digest patterns. Analysis of the products on a 2% agarose gel indicated there were six groups of cDNAs. Clones representing each group were sequenced using AmpliTaq DNA polymerase FS (Perkin-Elmer, Warrington UK) and analysed on an Applied Biosystems model 377 sequencer. To check for cross hybridization between the expansin clones, Southern blots were prepared with *Sau* 3A restriction digests of each clone and probed sequentially with the expansin sequences.

### Northern blot analysis

Total RNA (10 µg) from expanding and expanded leaves, roots, stolons and fruit from different stages of development was separated on a 1.4% agarose-formaldehyde gel and capillary blotted onto Hybond N<sup>+</sup> membrane (Roche). The blots were probed with cDNA fragments obtained from day 7 and orange stage fruit radiolabelled with [ $\alpha$ -<sup>32</sup>P]-dCTP (3000 Ci mmol<sup>-1</sup>) by random priming using DNA polymerase (Bioline). Blots were hybridized at 65 °C using 0.25 M sodium phosphate pH 7.4, 7% SDS. The final wash was with 0.2×SSC, 0.1% SDS at 65 °C before exposure to film.

### Analysis of expansin gene expression by RT-PCR

Total RNA (0.1 ng or 100 ng) was used as a template for first strand cDNA synthesis using RT-PCR beads (Amersham-Pharmacia Biotech) with 100 nM oligo d(T)<sub>12-18</sub> primer.

Reactions were incubated at 42 °C for 30 min and denatured at 95 °C for 5 min prior to the addition of expansin gene specific PCR primers (100 nM). Amplification conditions were 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min followed by one cycle of 72 °C for 10 min. Control reactions, to test for contamination by genomic DNA, were prepared by inactivating the reverse transcriptase at 95 °C for 10 min before addition of the gene specific primers. The integrity of the RNA was assessed in control RT-PCR reactions using 0.05 µg RNA and 200 nM 18 S rRNA primers (Clontech). The amplification products were purified using a PCR purification kit (Qiagen) and sequenced as described above. The following expansin gene specific primers were used (5'→3'): *FaExp3* sense TCCCTGGCACCATTGTG, antisense gctctcctgcac tcacc; *FaExp4* sense GGGTTGGGG-TGTGGTTCT, antisense GATGCCTCCCCTTCTTCTG; *FaExp5* sense TCTCGCCCAGCCCGTCTTCCAGCAT, antisense TGGGTTGCCAATTAGTTCTT; *FaExp6* sense TGCGCAAACGACCCGAAC, antisense CACGTCACCTCACAAT; *FaExp7* sense TAACTTCGCGCAGGCCAATGACAAT; *FaExp2* sense CCGGTCTTGCTACGAAATGCGA, antisense AATGAGACGGGGACGATACCAGCG. PCR products were separated on 2% agarose gels and stained with ethidium bromide. To verify that amplification was in the linear range, RT-PCR reactions were run with each template and primer pair. A sample of the reaction was removed every third cycle starting from cycle 20 and blotted onto Hybond N<sup>+</sup> membrane in 0.4 M NaOH. The blots were probed with either an 18S rRNA probe or a mixture of the six expansin cDNAs radiolabeled with [ $\alpha$ -<sup>32</sup>P]-dCTP (3000 Ci mmol<sup>-1</sup>) by random priming using DNA polymerase (Bioline). The blots were then scanned with a PhosphorImager (Molecular Dynamics) and the data analysed using ImageQuant 3.3 software (Molecular Dynamics).

#### Extraction of expansin protein

Attempts to solubilize expansin activity from cell wall preparations of strawberry fruit tissue by extracting with a high salt buffer (McQueen-Mason *et al.*, 1992) were unsuccessful. The reason why this extraction procedure is unsuitable for strawberry fruit is not known but may be related to the mixture of cell wall polysaccharides or the high phenolic content present in the fruit. The method reported here provides a source of active expansin protein. De-achened fruit (5 g) were ground to a powder in a pestle and mortar with liquid nitrogen and macerated further with 25 ml of extraction buffer (mM 3-[*N*-morpholino]propanesulphonic acid (MOPS)/NaOH buffer, pH 7.0, 0.5% (w/v) cetyl trimethylammonium bromide (CTAB), 30% (w/v) glycerol) until the mixture reached room temperature. The extract was then centrifuged at 10000 *g* for 5 min at room temperature in a Sorvall RC5B centrifuge. The supernatant was decanted and clarified by filtration through Miracloth (Calbiochem-Novabiochem). Three volumes of acetone (-20 °C) were added to the supernatant, mixed and incubated on ice for 5 min. The mixture was centrifuged at 5000 *g* for 10 min at 4 °C, the supernatant discarded and the protein pellet washed once with three volumes of acetone (-20 °C) before drying under vacuum. The dried pellets were stored at -20 °C. For comparison on Western blots, total proteins were also extracted by heating in 2×SDS sample buffer (0.125 M Tris-HCl, 4% [w/v] SDS, 20% [v/v] glycerol and 10% [v/v] 2-mercaptoethanol, pH 6.8) according to Laemmli (Laemmli, 1970). SDS-extracted protein was precipitated with 10% trichloroacetic acid (TCA), pelleted by centrifugation and dissolved in 0.1 M NaOH. Protein was assayed by the method of Bradford using a commercial kit

(Pierce, Chester UK) and bovine serum albumin as standard (Bradford, 1976).

#### Assay of expansin activity

Expansin activity was assayed as described earlier (McQueen-Mason *et al.*, 1992) using a custom made extensometer. The protein extracted from 5 g of fruit tissue was resuspended to a concentration of 1.5 mg protein ml<sup>-1</sup> in assay buffer (50 mM sodium acetate, pH 4.5). Aliquots of extract (100 µl) were assayed using 2 mm wide strips of cellulose/xyloglucan composite material derived from *Acetobacter* culture (Whitney *et al.*, 1995, 2000) extended under a constant force of 11 g. Composite material was chosen instead of plant cell wall material as it is a uniform substrate and allows higher rates of extension (data not shown). The basal rate of extension of the composite was determined in assay buffer without protein for 15 min. The buffer was replaced by protein solution and extension monitored for a further 15 min. Expansin activity was expressed as the difference in extension rates before and after the addition of protein. Results are the mean ± SE of three to six measurements for each treatment.

#### SDS-PAGE and Western blotting

Equal amounts of protein (20 µg) extracted by the CTAB and SDS methods were separated on a 12% (w/v) polyacrylamide gel (Laemmli, 1970). Proteins extracted by the CTAB method were denatured in 1×SDS sample buffer prior to SDS-PAGE. Proteins extracted by the SDS method were loaded directly. The separated proteins were blotted onto Hybond-P membrane (Amersham) at 4 °C in 25 mM Tris, 190 mM glycine and 20% (v/v) methanol. The membrane was blocked with 10% (v/v) horse serum, 2 mM sodium azide in phosphate buffered saline-Tween (PBS-T, 80 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, pH 7.5, 0.05% Tween-20) overnight at 4 °C. To detect expansin polypeptides the membrane was incubated for 2 h with cucumber anti-expansin antibodies (Li *et al.*, 1993) diluted 1:1000 in blocking solution. The membrane was washed 3×5 min with PBS-T followed by 3×5 min washes with Tris buffered saline-Tween (TBS-T, 20 mM Tris, pH 7.6, 136 mM NaCl, 0.05% Tween-20). Polypeptides cross-reacting with the expansin antiserum were located with mouse anti-rabbit antibody coupled to alkaline phosphatase (Sigma). The conjugate was diluted 1:1000 with blocking solution in TBS-T and incubated with the membrane for 2 h. After 4×5 min washes in TBS-T the blot was developed with bromochloroindolyl phosphate toluidine salt/nitro blue tetrazolium chloride and the reaction stopped with 10 mM EDTA.

## Results

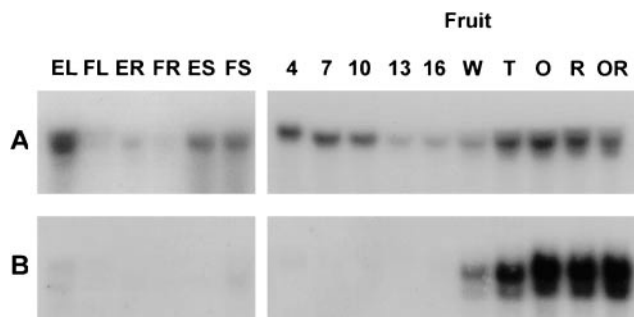
#### Expression of expansin genes in strawberry tissues

To study the role of expansins during fruit development, expansin cDNAs were cloned from rapidly expanding unripe fruit (7 DPA) and from ripening (orange stage) fruit. Using degenerate primers designed to conserved amino acid domains, amplification products of the expected size (~500 bp) were obtained by RT-PCR from day 7 and orange stage fruit. Initially, this mixture of expansin cDNAs from each stage was used as a probe to examine expansin gene expression in developing fruits

and in other strawberry tissues (expanding and expanded leaves, roots and stolons). The probe from the day 7 fruit detected expansin transcripts in all tissues examined and throughout fruit development (Fig. 1A). However, these mRNAs were more abundant in small green fruit (4, 7 and 10 DPA), turning, orange and ripe fruit with lower expression in large green fruit (13 and 16 DPA) and white fruit (19 DPA). The size of the transcript detected by the unripe probe ranged from ~1.6 kb in day 4 fruit to ~1.5 kb at other stages of fruit development. This probe hybridized strongly to RNA from expanding leaves and less strongly to RNA from stolons (both expanding and expanded). There was also some hybridization to RNA from expanding roots, but only low levels of expression were found in the other tissues. In leaves, the size of the transcript was ~1.4–1.5 kb whilst in roots and stolons a transcript of ~1.4 kb was present.

The Northern blot probed with the PCR product amplified from day 7 fruit was reprobed with the PCR product from orange stage fruit. This produced a quite different pattern of expression that indicated very low levels of transcript in young fruit. Transcript levels increased markedly in the fruit from the white to the orange stage and remained constant thereafter. The fruit transcript appeared as a doublet of 1.2 kb and 1.6 kb under high stringency (Fig. 1B). Transcripts of similar sizes were detected in leaf, stolon and root tissue but at much lower levels.

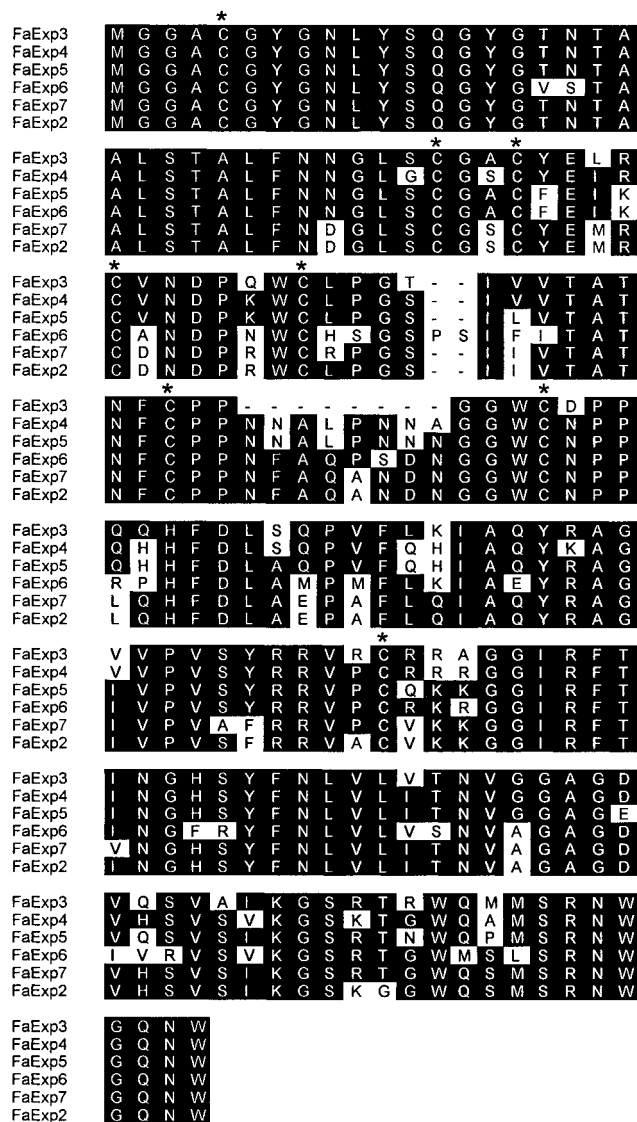
The results from the Northern analysis demonstrate that fruit development is associated with at least two different patterns of expansin gene expression and that expansin mRNA is also present in other strawberry tissues, principally expanding leaves and expanding and fully expanded stolons.



**Fig. 1.** Northern hybridization analysis of expansin gene expression in strawberry leaves, roots and stolons and during fruit development. Total RNA (10 µg) isolated from expanding and fully expanded leaves (EL and FL respectively); expanding and fully expanded roots (ER and FR respectively); expanding and fully expanded stolons (ES and FS respectively); from fruit 4, 7, 10, 13, and 16 d post-anthesis and from white (W), turning (T), orange (O), ripe (R), and overripe (OR) fruit was separated by gel electrophoresis, transferred to Hybond<sup>+</sup> membrane and hybridized with <sup>32</sup>P-labelled cDNA probes. (A) Day 7 unripe expansin probe; (B) orange expansin probe.

*Molecular characterization of strawberry expansins*

Restriction digests of cloned PCR products indicated that several expansin genes are expressed in strawberry fruit. In total, six different expansin PCR products were identified (Fig. 2), ranging in length from 464–494 bp. Two of the clones were isolated from day 7 unripe fruit (*FaExp3* and *FaExp4*) and four from orange stage fruit (*FaExp5–FaExp7* and *FaExp2*). Clone *FaExp2* has been previously identified in ripe fruit (Civello *et al.*, 1999). All six clones showed sequences typical for alpha-expansins (Shcherban *et al.*, 1995).



**Fig. 2.** Alignment of the strawberry expansin partial amino acid sequences deduced from cDNA sequences. Gaps are shown as dashes; letters on black backgrounds indicate identical amino acids. Proteins were aligned using the clustal method of DNASTAR Megalign (DNASTAR inc., Madison, WI). The eight conserved cysteines (\*) typical of expansins are indicated.

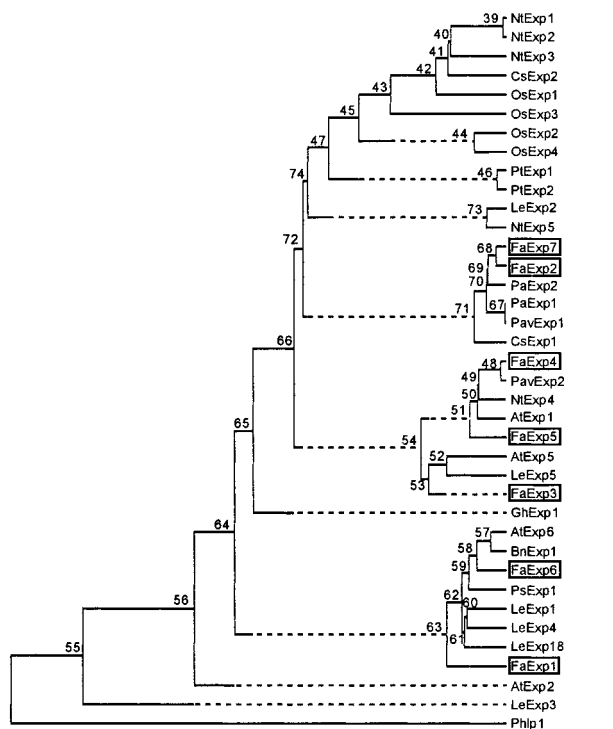
The expansins from unripe fruit (FaExp3 and FaExp4) share 84% amino acid identity and are closely related to expansins isolated from fruit of other species over the equivalent region (Fig. 3). FaExp3 shares 85% similarity with the tomato fruit expansin, LeExp5, (Brummell *et al.*, 1999a) whilst FaExp4 is highly related (98%) to PavExp2 from sweet cherry (*Prunus avium*, Genbank AAG13983, unpublished data). *LeExp5* mRNA is maximally expressed in unripe tomato during green fruit expansion and maturation (Brummell *et al.*, 1999a, b) whereas *FaExp3* is expressed slightly earlier in fruit development during cell division and expansion.

Interestingly, the ripening related expansins, FaExp2 and FaExp5, have a higher amino acid similarity to FaExp7 (96%) and AtExp1 (88%) from *Arabidopsis*

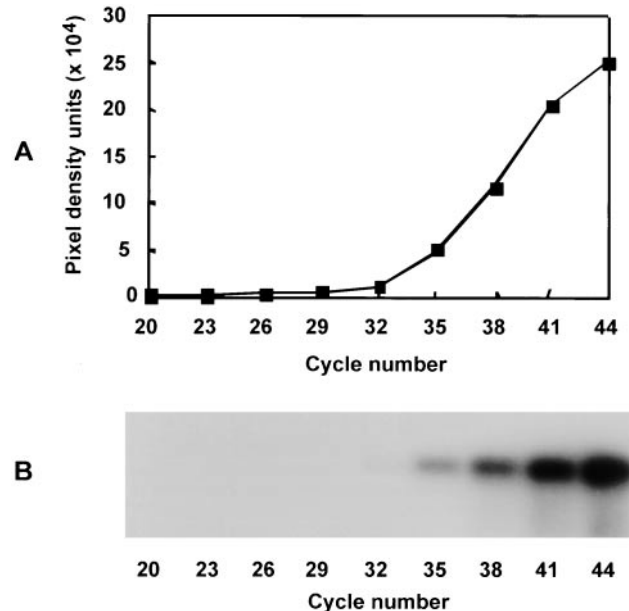
*thaliana* (Shcherban *et al.*, 1995) respectively, than to each other. In addition, FaExp2 and FaExp7 are highly related to the apricot fruit expansins PaExp2 (Genbank AF038815, unpublished data) and PaExp1 (Genbank U93167, unpublished data) respectively, being 94% and 95% similar over the equivalent region. The truncated amino acid sequences of the fruit expansins PaExp1, PaExp2, PavExp1 and the strawberry expansins FaExp2 and FaExp7 represent a separate cluster which is phylogenetically distinct from the tomato expansins. Strawberry, apricot and sweet cherry belong to the family Rosaceae, but at present no expression data are available for the apricot and sweet cherry expansins. FaExp1 and FaExp6 have least homology with the other strawberry expansins, with FaExp6 being most closely related (91%) to AtExp6 (Shcherban *et al.*, 1995).

#### RT-PCR analysis of expansin transcript accumulation

Southern analysis of the expansin clones revealed cross hybridization between different expansins and therefore an RT-PCR approach was used to characterize the expression of specific expansin genes. The procedure (Halford *et al.*, 1999), enabled DNA fragments of specific size to be amplified from the six different expansin genes using gene specific primers and either 0.1 ng or 100 ng of total RNA template. All amplifications were in the linear range



**Fig. 3.** Phylogenetic comparison of deduced amino acid sequences from truncated expansins. The tree was constructed using the Megalign program, Clustal method, with a PAM250 residue weight table and rooted with PhP1 pollen allergen as an outgroup. Numbers on the tree show bootstrap probability (*P*) values. Sequences are GenBank accession numbers: At (*Arabidopsis thaliana*), Exp1 (U30476), Exp2 (U30481), Exp5 (U30478), and Exp6 (U30480); Bn (*Brassica napus*), Exp1 (AJ000885); Cs (*Cucumis sativus*), Exp1 (U30382) and Exp2 (U30460); Fa (*Fragaria × ananassa*), Exp1 (AF163812) and Exp2 (AF159563); Gh (*Gossypium hirsutum*), Exp1 (AF043284); Le (*Lycopersicon esculentum*), Exp1 (U82123), Exp2 (AF096776), Exp3 (AF059487), Exp4 (AF059488), Exp5 (AF059489), and Exp18 (AJ004997); Nt (*Nicotiana tobaccum*), Exp1 (AF049350), Exp2 (AF049351), Exp3 (AF049352), Exp4 (AF049353), and Exp5 (AF049354); Os (*Oryza sativa*), Exp1 (Y07782), Exp2 (U30477), Exp3 (U30479), and Exp4 (U85246); Pa (*Prunus armeniaca*), Exp1 (U93167) and Exp2 (AF038815); Pav (*Prunus avium*) Exp1 (AAG13982) and Exp2 (AAG13983); Phlp1 (*Phleum pratense*) pollen allergen (X78813); Ps (*Pisum sativum*), Exp1 (X85187); Pt (*Pinus taeda*), Exp1 (U64892) and Exp2 (U64890).

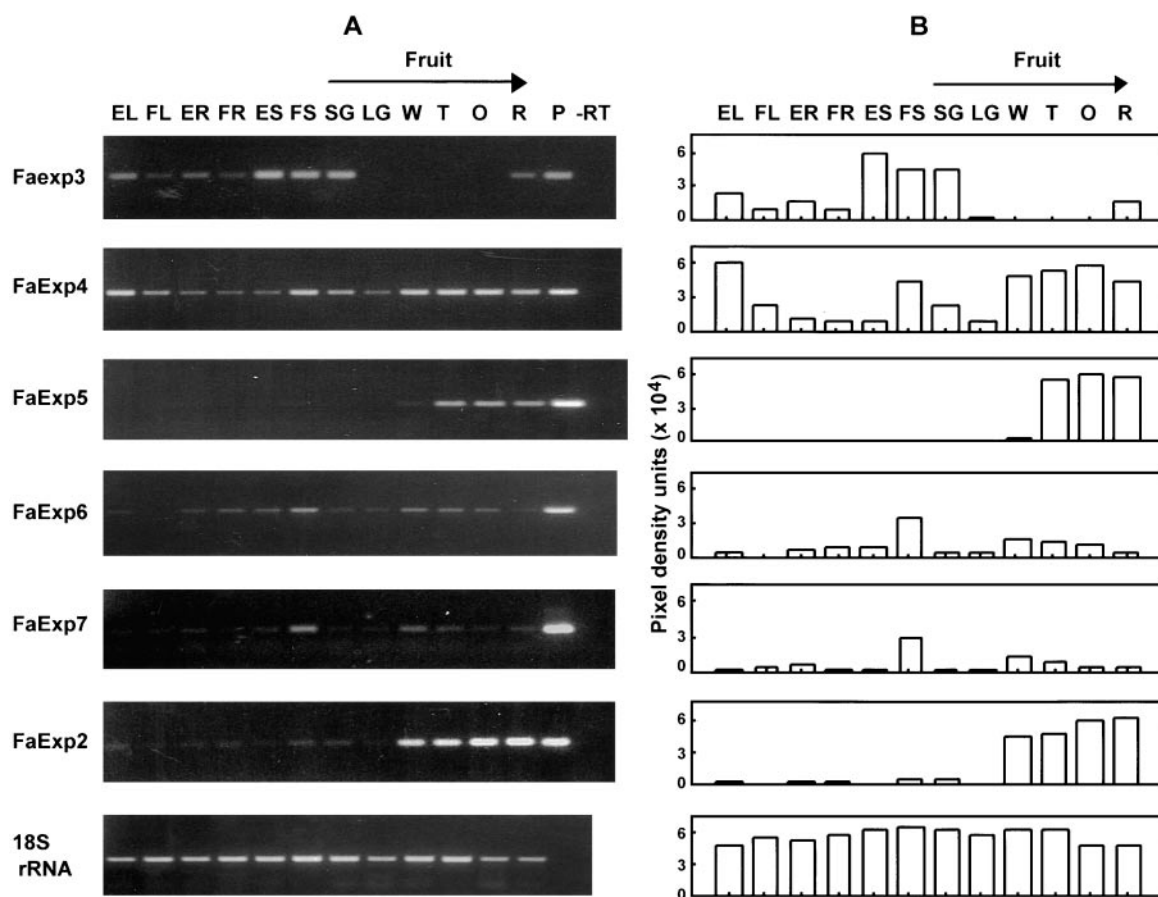


**Fig. 4.** PCR amplification profile of an expansin gene. Gene specific primers for clone *FaExp4* were used in a PCR reaction to amplify a cDNA fragment of the corresponding gene after reverse transcription of 0.1 ng of total RNA from expanding leaves. An aliquot was removed every third cycle starting at cycle 20, separated on a 2% agarose gel and then blotted onto Hybond N<sup>+</sup> membrane. The blot was hybridized with a <sup>32</sup>P-labelled unripe probe and the signal was quantified using a PhosphorImager and Imagequant 3.3 software (A). The same blot was then exposed to autoradiography film (B).

between 35 and 41 cycles (Fig. 4) and 35 cycles were used as standard. Control RT-PCR reactions using 18S rRNA primers were also linear between 35 and 41 cycles and produced a single band of similar intensity with all samples confirming that equivalent amounts of template were present (Fig. 5). The conditions for all RT-PCR reactions were identical except for clone *FaExp3* in which 1000-fold more template was necessary in order to visualize the products. The RT-PCR products were sequenced and all show identity with the corresponding cDNAs.

Figure 5 shows the expression profiles of all six genes analysed on 2% (w/v) agarose gels and stained with ethidium bromide. Each gene had a unique expression profile during fruit development and differed in its expression in other tissues. The spatial expression of *FaExp3* and *FaExp4* was similar in leaf, root and stolon tissues but could be distinguished quantitatively by the vast difference in the amount of template required for PCR amplification. Amplification from a plasmid DNA template under identical conditions produced PCR

fragments of similar intensity for each cDNA. This suggests that the higher quantity (1000-fold) of total RNA template required to amplify *FaExp3* is due to the low abundance of the corresponding message. Clones *FaExp3* and *FaExp4* had distinct patterns of expression in the fruit, *FaExp3* being most abundant in small green fruit then declining sharply to undetectable levels in the white, turning and orange stages before being expressed again in ripe fruit. The expression of *FaExp4* was very different, its transcript being present in high levels at all stages of fruit development, but with a small reduction in large green fruit. Genes encoding *FaExp6*, *FaExp7* and *FaExp2* identified from orange stage fruit were expressed at lower levels in leaf, root and stolon tissues than *FaExp3* and *FaExp4* from unripe fruit. The only fruit specific expansin identified, *FaExp5*, was expressed in the later stages of fruit development from turning to fully ripe. In contrast, *FaExp6* and *FaExp7* were most highly expressed in fully expanded stolon tissue. During fruit development, *FaExp6* was more abundant at the white, turning



**Fig. 5.** Tissue specific expression profile of strawberry expansin genes. Total RNA (0.1 ng for *FaExp2*, *FaExp4*–7; 100 ng for *FaExp3*; 0.05 pg for 18S rRNA) from expanding and expanded leaves, roots and stolons and from small green (SG), large green (LG) and ripening fruit (see legend for Fig. 1) was used as a template for RT-PCR analysis using gene specific primers, plasmid (P) and minus reverse transcriptase (-RT) controls. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide (A). The products were blotted onto Hybond N<sup>+</sup> and hybridized with either a <sup>32</sup>P-labelled unripe/ripe expansin probe or a <sup>32</sup>P-labelled 18S rRNA probe. The signal was quantified using a PhosphorImager and Imagequant 3.3 software (B).

and orange stages whilst *FaExp7* was mostly expressed in white and turning fruit. Clone *FaExp2* exhibited weak expression in leaves, stolons, roots, and green fruit but was strongly up-regulated in fruit from the white stage onwards. Collectively, the RT-PCR data support the Northern analysis data (Fig. 1) in that expansins are mostly expressed during the later stages of development when anthocyanin production begins and the fruit start to ripen.

#### Analysis of expansin activity and protein levels in developing fruit

In order to correlate expansin action with the expression of expansin genes, acid-induced extensibility was measured in protein extracts from all stages of fruit development using a cellulose/xyloglucan cell wall composite (Whitney *et al.*, 1995, 2000). When protein extracts were prepared from strawberry fruits using the method described earlier (McQueen-Mason *et al.*, 1992) it was not possible to obtain measurable extension activity. The CTAB method described in this paper dramatically increased extension activity extracted from the fruit. Extension was greatest with extracts from orange, ripe and overripe fruit with some extension observed in white and turning fruit (Fig. 6A). Little extension was present in fruit before the turning stage or in achenes. However, the results from Northern and RT-PCR analysis indicate that expansins are also expressed in small green fruit. To test the possibility that inhibitors of extension may have been present in unripe fruit, tissue from ripe and small green unripe fruits was mixed in equal proportions before extraction and assay. There was no significant reduction in extension activity of ripe fruit when co-extracted with small green unripe fruit (Fig. 6B). Therefore, it seems unlikely that inhibitors are affecting the assay of unripe fruit leading to a loss of extension. It may be that expansins expressed in unripe tissues do not effectively disrupt the cellulose/xyloglucan bonds, but instead act upon a different cell wall component. As yet a suitable substrate has not been identified.

The protein extracted by the CTAB method for the extension assays was also analysed by Western blotting. The cucumber expansin (CsExp1) antibody bound to protein bands of ~29 and 31 kDa that differed in their relative abundance during fruit development (Fig. 7A). The 31 kDa band increased in extracts from fruit 7 DPA to 16 DPA and then declined at the white and turning stages. This band was not present in fruit 4 DPA, orange, ripe or overripe fruit. The smaller band (29 kDa) first appeared in white fruit and then increased until the ripe stage with similar amounts in overripe fruit. Both expansin protein bands were present in white and turning fruit. In order to assess the efficiency of the CTAB extraction method, total protein from all stages of fruit

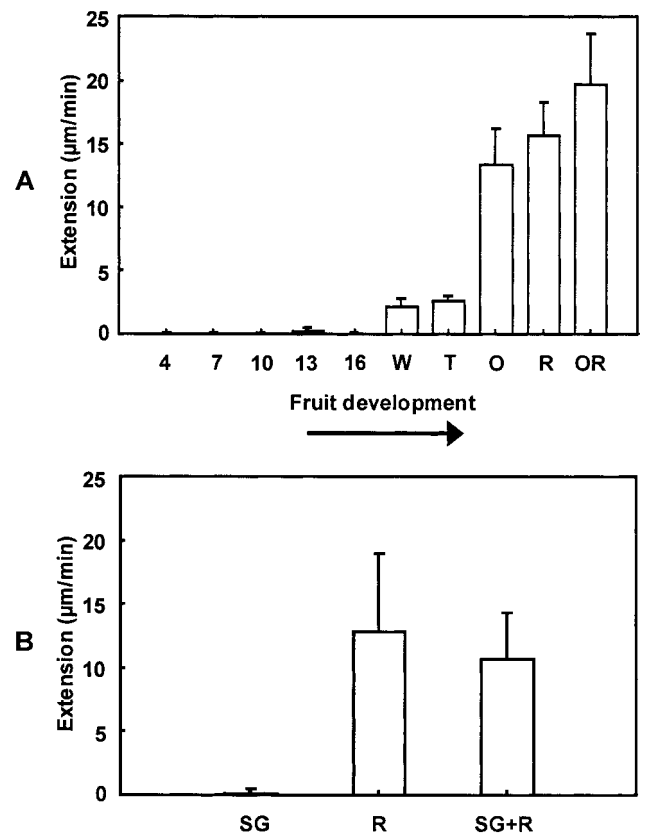


Fig. 6. Acid-induced extension of cell wall composites by strawberry fruit cell wall proteins. Protein (150 µg) was resuspended in assay buffer (50 mM sodium acetate, pH 4.5) and assayed using 2 mm wide strips of cellulose/xyloglucan composite material extended under a constant force of 11 g. Expansin activity is expressed as the difference in extension rates before and after the addition of protein. Extension rates are shown in extracts from developing fruits (A) and in extracts obtained after mixing small green and ripe fruit in equal proportions (B). Results are the mean  $\pm$  SE of 3–6 measurements for each treatment. For abbreviations see legend for Fig. 1 and Fig. 5.

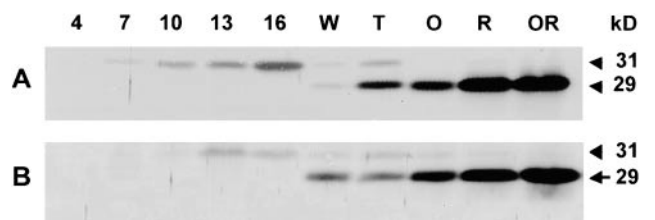


Fig. 7. Immunoblot analysis of protein extracts from strawberry fruit. Protein (20 µg) from CTAB extracts (A) and SDS extracts (B) was separated on a 12% SDS polyacrylamide gel, blotted onto Hybond-P and probed with antiserum against the cucumber expansin (CsExp1). Two bands were identified corresponding to 29 and 31 kDa. For abbreviations see legend for Fig. 1.

development was also prepared using a standard SDS method (Laemmli, 1970). Analysis of the SDS extracts by Western blotting showed a similar pattern of immunostaining to that of the CTAB extracts (Fig. 7B). However, the CTAB method appears to be more effective than

SDS at extracting immunoreactive material from unripe fruit whereas the reverse was true for fruit at the white stage. These data confirm the validity of using the CTAB method for extracting active expansin protein.

The presence of two immunoreactive bands with different sizes and distinct patterns of distribution during fruit development correlates with the expression data from RT-PCR and Northern analysis. The initial increase in the 29 kDa expansin protein at the onset of ripening parallels the extension data suggesting this is likely to be the ripening related expansin encoded by *FaExp2*. The subsequent expression of *FaExp5* may contribute to the increasing intensity of the 29 kDa band as the fruit ripens.

## Discussion

Six expansin cDNAs from strawberry fruit have been identified and characterized, two from rapidly expanding unripe fruit (7 DPA) and four from ripening (orange stage) fruit. Each expansin has a unique expression profile during fruit development and some are also expressed in other tissues such as leaves, roots and stolons. The patterns of expression during fruit development fall into three main groups: (a) those that show an up-down-up profile (*FaExp3* and *FaExp4*); (b) those that show a down-up-down profile (*FaExp6* and *FaExp7*) and (c) those which increase during development (*FaExp5* and *FaExp2*). Civello *et al.* also observed a ripening-related expression profile for *FaExp2* (Civello *et al.*, 1999). The up-down-up pattern of expression has been observed with other genes expressed during strawberry fruit development (Manning, 1998). Interestingly, the clones which share the greatest homology do not necessarily have similar expression profiles. For example, *FaExp7* and *FaExp2* have 96% amino acid identity and yet are expressed at very different times during fruit development.

The deduced amino acids sequences indicate that all the strawberry clones are conventional alpha-expansins with the characteristic eight conserved cysteine residues. Expansins represent a highly conserved gene family but the functions of numerous isoforms expressed in fruit remain to be determined. It is interesting that the same number of expansins have been identified in strawberry and tomato fruit with the likelihood, that other, more lowly expressed genes remain to be discovered. In strawberry, growth of the receptacle after petal fall is initially due to a combination of cell division and cell expansion. Cell division ceases after about 7 d, the subsequent growth of the fruit being accounted for by a massive increase in cell expansion (Knee *et al.*, 1977). Fruit growth and the ripening associated softening are two overlapping processes in strawberry that may require different modifications of the plant primary cell wall. Growth itself is likely to involve a series of integrated cell wall transitions.

Expansins may have a similar role in the enlargement of strawberry fruit to those expressed in vegetative tissues (Cosgrove, 1997). The two clones expressed in unripe fruit (*FaExp3* and *FaExp4*) are also expressed in expanding and expanded leaf, root and stolon tissues. However, these two clones are not just expressed in small green fruit. Clone *FaExp4* is highly expressed throughout development whilst *FaExp3*, although silent for much of the fruit's development, is also expressed in ripe fruit. Since *FaExp4* appears to be expressed in all tissues and throughout fruit development it may have a general role in cell wall modification during plant growth. *FaExp6* and *FaExp7* expression is enhanced in white and turning fruits and these developmental stages represent a transition to ripening that is marked by an increase in anthocyanin production. This phase is also characterized by changes in the abundance of numerous mRNAs (Manning, 1998). Auxin is essential for strawberry fruit growth but also represses ripening and the expression of many ripening-enhanced genes (Manning, 1994). The effects of auxin on expansin gene expression have not been addressed in this study, but the expression of *FaExp2* (Civello *et al.*, 1999) was reported to be insensitive to auxin. Expression profiles of the ripening-related clones *FaExp2* and *FaExp5* indicate that these expansins might be responsible for the increased extension activity observed from ripening fruit. They may have a key role in the terminal phase of cell wall modification.

Similarities between patterns of expression are evident for some of the expansins from strawberry and tomato (Brummell *et al.*, 1999a) during equivalent stages of fruit development. However, none of the tomato expansins so far described have expression profiles resembling those of *FaExp3* and *FaExp4* in strawberry (Brummell *et al.*, 1999a). This is not surprising considering that tomato and strawberry fruits differ fundamentally in their growth characteristics and cell wall composition. In tomato, growth ceases prior to ripening and softening is accompanied by substantial pectin depolymerization (Huber and O'Donoghue, 1993) whereas in strawberry, growth continues and there is little change in the molecular size distribution of pectins during ripening (Huber, 1984). In both fruits, growth and ripening are tightly regulated events whose co-ordination may depend on the timing and degree of expression of individual expansin genes. Although the temporal regulation of expansins in strawberry has been described, little is known about their spatial distribution in fruits, another factor that could explain the complexity of this family of proteins.

The roles of different fruit expansins remain uncertain. Suppression of the abundance of the ripening-specific expansin *Exp1* in transgenic tomatoes to low levels (3% of wild-type levels) resulted in an increase in fruit firmness at all stages of ripening (Brummell *et al.*, 1999b). However, although firmer than the controls, the transgenic fruit

still showed the characteristic increase in softening during the ripening stages. Therefore, some of the components necessary for progressive cell wall breakdown appear to act independently of this expansin. The accessibility of depolymerases to cell wall polymers may be regulated indirectly by expansins. In strawberry, ripening-enhanced enzymes such as cellulase (Llop-Tous *et al.*, 1999) and pectate lyase (Medina-Escobar *et al.*, 1997) may act synergistically with expansins to effect complete cell wall disassembly.

One of the key questions that remains unanswered is how do expansins differ in their functional properties? It was not possible to measure extension activity in unripe green fruit although transcripts of several expansins were present at this stage. Failure to detect extension could have been due to (a) low activity of the protein, (b) poor extraction efficiency or (c) inappropriate substrate used in the assay. Western analysis suggests that there is less expansin protein in unripe green fruit compared to ripening fruit and this alone could account for the apparent lack of activity observed with the extension assay. It has been suggested that the likely substrate of the cucumber expansin (CsExp1) in cell walls is the cellulose xyloglucan matrix, but that other (1–4)  $\beta$ -glucan to (1–4)  $\beta$ -glucan hydrogen bonded contacts can also serve as substrates (Whitney *et al.*, 2000). Expansin action was also shown to be greater on composites containing long chain xyloglucans than on those containing shorter chains (Whitney *et al.*, 2000). Expression of the corresponding recombinant proteins will enable a better understanding of their properties.

Evidence here and elsewhere is accumulating for the increasingly complex nature of the proteins involved in cell wall modification. This is exemplified by the expansins and unravelling the roles of these proteins in fruit development will be a major challenge for the future.

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