

Differential expression of expansin gene family members during growth and ripening of tomato fruit

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Abstract

cDNA clones encoding homologues of expansins, a class of cell wall proteins involved in cell wall modification, were isolated from various stages of growing and ripening fruit of tomato (*Lycopersicon esculentum*). cDNAs derived from five unique expansin genes were obtained, termed tomato *Exp3* to *Exp7*, in addition to the previously described ripening-specific tomato *Exp1* (Rose *et al.* (1997) Proc Natl Acad Sci USA 94: 5955–5960). Deduced amino acid sequences of tomato *Exp1*, *Exp4* and *Exp6* were highly related, whereas *Exp3*, *Exp5* and *Exp7* were more divergent. Each of the five expansin genes showed a different and characteristic pattern of mRNA expression. mRNA of *Exp3* was present throughout fruit growth and ripening, with highest accumulation in green expanding and maturing fruit, and lower, declining levels during ripening. *Exp4* mRNA was present only in green expanding fruit, whereas *Exp5* mRNA was present in expanding fruit but had highest levels in full-size maturing green fruit and declined during the early stages of ripening. mRNAs from each of these genes were also detected in leaves, stems and flowers but not in roots. *Exp6* and *Exp7* mRNAs were present at much lower levels than mRNAs of the other expansin genes, and were detected only in expanding or mature green fruit. The results indicate the presence of a large and complex expansin gene family in tomato, and suggest that while the expression of several expansin genes may contribute to green fruit development, only *Exp1* mRNA is present at high levels during fruit ripening.

Introduction

The growth of expanding plant cells is controlled largely by the pliancy of the primary cell wall, which is composed of rigid cellulose microfibrils coated with, and tethered together by, a matrix of hemicellulosic glycan polysaccharides, all embedded in a coextensive matrix of pectins with smaller amounts of structural glycoproteins [3]. Since the extensibility of the cell wall constrains expansion, cell wall loosening is necessary in order to allow turgor-driven cell enlargement to occur [7, 8]. The walls of expanding vegetative tissues possess numerous enzyme activities that may contribute to a modification of cell wall mechanical

properties, including endo-1,4- β -glucanases [17], xyloglucan endotransglycosylases [12], and expansins [29]. Of these, only expansins exhibit the ability to evoke growth when added to cell walls, as shown by increased cell wall loosening when purified expansin protein was added either to heat-inactivated hypocotyl cell walls *in vitro* or to apical meristems *in vivo* [11, 29]. In addition to vegetative tissues some expansins are also expressed in fruit, since in tomato the mRNA of a divergent expansin gene accumulated specifically during the ripening process [32]. While the activities of endo-1,4- β -glucanases [19, 26] and xyloglucan endotransglycosylases [1, 26] have been found both in growing green and in ripening red tomato fruit, the potential contribution of expansins to the growth of developing green fruit has not yet been examined.

Expansins are a highly conserved group of cell wall-localized proteins that appear to mediate changes in cell wall plasticity during cell expansion or dif-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF059487 (tomato *Exp3*), AF059488 (tomato *Exp4*), AF059489 (tomato *Exp5*), AF059490 (tomato *Exp6*) and AF059491 (tomato *Exp7*).

ferentiation [8]. The precise mechanism of action of expansins is not known, but expansins appear to lack detectable hydrolase or xyloglucan endotransglycosylase activity [28–30], and have been hypothesized to act by disrupting load-bearing non-covalent linkages between cellulose and other cell wall polymers [27, 28]. A possible site of action is the extensive hydrogen bonding between cellulose microfibrils and the backbones of matrix glycans (hemicelluloses), which together form a rigid skeletal network giving the wall its mechanical strength [3]. The matrix glycans are predominantly xyloglucan in dicotyledonous and some monocotyledonous angiosperms, and glucuronoarabinoxylans and mixed linkage 1,3/1,4- β -glucans in grasses, but also include various heteroxylans, heteromannans, heterogalactans and related polysaccharides [3]. Although the identities of the bonds disrupted by expansins have not yet been identified [28], in accordance with these suggestions all expansin proteins described to date possess eight conserved Cys residues in the N-terminal half of the protein with spacing similar to those in the chitin-binding domain of wheat germ agglutinin, and four conserved Trp residues in the C-terminal region of the protein with spacing similar to those in the cellulose-binding domain of microbial cellulases [34]. These conserved amino acid residues presumably play a role in the binding of expansins to cell wall matrix polysaccharides and cellulose [34].

Expansins appear to be ubiquitous in both monocotyledons and dicotyledons, and are present as large multigene families in *Arabidopsis thaliana* and *Oryza sativa* (rice) [6, 9, 34]. The accumulation of expansin proteins or the mRNA for specific expansin gene family members has been correlated with the growth of various plant organs, including cucumber hypocotyls [29], oat coleoptiles [25], tomato leaves [22], maize roots [39] and deepwater rice internodes [4–6]. A further process where expansins may act is in the cell wall disassembly occurring during fruit softening, where the mRNA of tomato *Exp1* has been detected specifically in association with fruit ripening [32]. As part of a larger study to determine the role of the tomato *Exp1* gene product in fruit softening using transgenic tomato plants, we were interested in examining whether particular expansin gene family members are expressed during the expansion of green tomato fruit, and if *Exp1* is the major, or only, expansin expressed during fruit ripening.

Materials and methods

Plant material

Field-grown fruit of tomato (*Lycopersicon esculentum* Mill.) cv. Bonnie Best were staged at 10 mm in diameter, 20 mm in diameter, immature green (IG), mature green (MG) stage 2 or 4, breaker (BR), turning (TU), pink (PK), light red (LR), red ripe (RR) or over ripe (OR), as previously described [15]. The seeds and locules were discarded and pericarp tissue was frozen in liquid N₂ and stored at –80 °C. Greenhouse-grown vegetative tissues of cv. Bonnie Best were collected and frozen similarly.

Reverse transcription-PCR

Total RNA (5 μ g) from tomato fruit at the MG, BR and RR ripening stages was used as template for cDNA synthesis using 0.5 mM dNTPs, 25 ng oligo d(T)₁₅ and 5 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) at 42 °C for 2 h. Products were diluted to 1 ml and 5 μ l was used as template for PCR amplification. Degenerate oligonucleotide primers for PCR were synthesized to two amino acid domains conserved between expansins, MGGACGYG for the sense primer (5'-ATGGGIGGIGCNTG(CT)GGNTA(CT)G-3'), and RNWG(AVQ)NWQ for the antisense primer (5'-(CT)TGCCA(AG)TTN(TGA)(CG)ICCCCA(AG)TTNC-3'), where N = any nucleotide and I = inosine. PCR amplification was carried out using 0.2 mM dNTPs, 2.5 mM MgCl₂, 1 μ M each primer and 2.5 units *Taq* DNA polymerase (Promega) at 94 °C for 5 min, 53 °C for 2 min and 72 °C for 40 min (1 cycle), then 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min (35 cycles). PCR products of the predicted size (ca. 400 to 600 bp in length) were ligated into pBlue-script (Stratagene, La Jolla, CA), transformed into competent *Escherichia coli* strain JM-83, and 20 to 40 clones from each ripening stage analyzed. Thirty-one clones possessing inserts of various sizes were sequenced on both strands using a Li-Cor (Lincoln, NE) model 4000L automatic DNA sequencer, of which 23 clones showed sequence homology with expansins. Of these, 10 clones were identical to tomato *Exp1* [32], none were identical to tomato *Exp2* (C. Catala and A.B. Bennett, personal communication) and the other 13 represented five unique expansin homologue genes, termed tomato *Exp3* to *Exp7*.

cDNA library screening and 5'-RACE

Since the majority of the clones representing the new expansin genes were obtained from green or BR stage fruit cDNA, an aliquot containing 150 000 plaques of a tomato cv. 91347 MG fruit cDNA library constructed in vector Lambda ZAP II (Stratagene) was plated out and screened according to the manufacturer's instructions. The first screen, using as probe an equal mixture of the PCR clones of *Exp3* and *Exp5* which had been end-labeled using [γ - ^{32} P]ATP and T4 polynucleotide kinase, identified 14 positive plaques. The library was then rescreened with a mixture of end-labeled probes of *Exp4*, *Exp6* and *Exp7*, and a further 21 positive plaques were identified. All 35 positive plaques resulting from both screens were purified by a second round of screening. pBluescript phagemids containing the *Eco*RI to *Xho*I-cloned inserts were rescued from all 35 plaques, then sequenced and analyzed. The longest clones of *Exp4* and *Exp5* included the full open reading frame, but the longest clones of *Exp3* were truncated at the 5' end. No clones for *Exp6* or *Exp7* were detected.

To obtain the 5' end of *Exp3*, nested primers for 5'-RACE (Rapid Amplification of cDNA Ends) were synthesized in the antisense direction corresponding to nucleotides 327 to 345 (outside primer) and nucleotides 282 to 300 (inside primer) of *Exp3*. Poly(A)⁺ RNA (50 ng) of MG2 tomato fruit was converted to cDNA using the outside primer and reverse transcriptase as above. Products were tailed with poly(dG) using dGTP and terminal deoxynucleotidyl transferase (Promega) according to the manufacturer's instructions. PCR amplification was carried out as above, except using anchor primer-poly(C) (5'-GGAATTCATCGATGGATCC(C)₁₅) at 94 °C for 2 min, 40 °C for 2 min and 72 °C for 5 min (1 cycle), then the outside primer and anchor primer (lacking the poly(C)₁₅ region) were added and PCR continued at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min (30 cycles). One-fiftieth of the products were amplified in a second round of amplification (40 cycles) using the inside primer and anchor primer, and products cloned into vector pCRII (Invitrogen, Carlsbad, CA). Sequencing of the three longest clones showed that the PCR products contained the expected overlap of 237 bp with the truncated *Exp3* clone, plus 63 bp of new sequence which included an in-frame ATG codon.

Sequence analysis

Predicted amino acid sequences for tomato expansin homologues *Exp3* to *Exp7* were aligned with the predicted amino acid sequence of tomato *Exp1* using ClustalV [18]. Putative signal sequences we identified using the rules of von Heijne [38].

To determine phylogenetic relatedness, the deduced amino acid sequences of full-length mature expansin proteins without signal sequences were aligned as above. Phylogenetic relatedness was determined using PAUP [36], employing bootstrap analysis (100 replications) with random stepwise addition of taxa and tree bisection and reconnection branch swapping. Since the predicted amino acid sequences of tomato *Exp6* and *Exp7* were not full length, the predicted sequences of all the expansin sequences used were truncated at the equivalent points, aligned again as above and phylogenetic analysis repeated (data not shown).

DNA gel blot analysis

Genomic DNA was prepared from young tomato leaves as described [16], and aliquots of 10 μ g digested exhaustively with *Eco*RI, *Hind*III, *Nsi*I or *Xba*I, none of which cut any of the cDNA clones within the regions used as probes. Digested DNA samples were fractionated by electrophoresis on 0.8% agarose gels and then transferred to Duralon-UV membranes (Stratagene).

Six identical blots were hybridized with probes for each of expansin genes *Exp1* and *Exp3* to *Exp7* in 0.25 M Na₂HPO₄ pH 7.4, 1 mM EDTA, 7% (w/v) SDS and 1% (w/v) BSA at 65 °C overnight. For expansin genes for which full-length cDNA sequence information was available, gene-specific probes were amplified by PCR from the 3'-untranslated regions of the genes, the PCR products comprising: for *Exp1*, nt 842 to 1010 (169 bp); for *Exp3*, nt 871 to 1044 (174 bp); for *Exp4*, nt 869 to 1180 (312 bp); and for *Exp5*, nt 780 to 1183 (404 bp). PCR products were gel-purified and used as templates for PCR labeling with [α - ^{32} P]dATP, 3.1 μ M dCTP, dGTP and dTTP, the antisense primer of each primer pair and *Taq* DNA polymerase (10 cycles). Since only one of the primers was present in each labeling reaction, no exponential amplification occurred and only 10 copies of the non-coding strand were synthesized. Probes for *Exp6* and *Exp7* were the 500 bp PCR fragments labeled with [α - ^{32} P]dCTP, random hexamers and Klenow DNA polymerase [33]. DNA gel blots were washed at 65 °C

in 0.5% SDS and either $1\times$ SSC ($1\times$ SSC is 150 mM NaCl, 15 mM sodium citrate) for *Exp1*, *Exp3*, *Exp4* and *Exp5* (15°C below T_m) or $0.15\times$ SSC for *Exp6* and *Exp7* (4°C below T_m). Blots were exposed to X-Omat AR film (Kodak, Rochester, NY) at -80°C with two intensifying screens.

RNA preparation and RNA gel blot analysis

Total RNA was prepared from vegetative and fruit tissues as described [10]. Poly(A)⁺ RNA was selected using Dynabeads (Dyna, Great Neck, NY) according to the manufacturer's instructions. Total RNA (15 μg per lane) or poly(A)⁺ RNA (0.5 μg per lane) was fractionated on gels of 1.2% agarose and 6% formaldehyde [33], then transferred to Duralon-UV membranes. RNA gel blots were hybridized with probes for each of the six tomato expansin genes as described above, and washed at 65°C at the equivalent stringencies to the DNA gel blots above, i.e., 0.5% SDS and $0.75\times$ SSC for *Exp1*, *Exp3*, *Exp4* and *Exp5* (15°C below T_m), or $0.1\times$ SSC for *Exp6* and *Exp7* (4°C below T_m), then exposed to film.

Results

cDNA clones encoding putative full length expansin polypeptides were obtained for the tomato *Exp3*, *Exp4* and *Exp5* genes (Figure 1). The cDNA clone of *Exp3* was 1046 bp in length, and contained a 5'-untranslated region of 53 bp, an open reading frame of 801 bp, and a 3'-untranslated region of 192 bp. The cDNA did not contain an in-frame stop codon upstream of the first deduced Met residue, the presumed translation start. However, primer extension analysis [33] found that the cDNA clone began ca. 40 bp downstream of the transcription start (data not shown), which together with amino acid alignments (Figure 1) suggest that the first deduced Met residue was the initiation point for translation. The cDNA clone of *Exp4* was 1239 bp in length, contained a 5'-untranslated region of 79 bp, an open reading frame of 789 bp and a 3'-untranslated region of 371 bp. This cDNA clone also did not contain an in-frame stop codon upstream of the first deduced Met residue, but primer extension analysis showed that this cDNA clone extended to within 5 bp of the transcription start (data not shown). The *Exp5* cDNA clone was 1225 bp in length, with a 5'-untranslated region of 67 bp, an open reading frame of 717 bp, a 3'-untranslated region of 441 bp, and contained an in-frame stop codon upstream of the first deduced Met

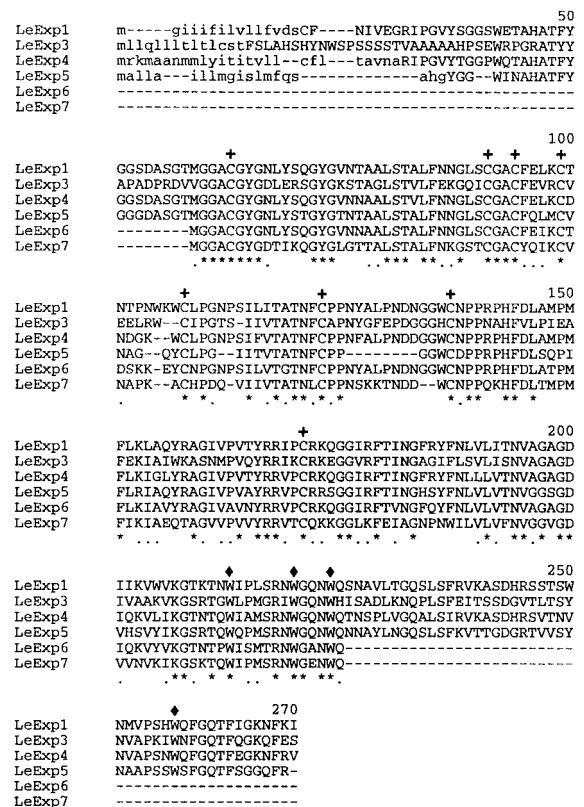


Figure 1. Amino acid sequence alignment. Deduced amino acid sequences of tomato *Exp3*, *Exp4*, *Exp5*, *Exp6* and *Exp7* aligned with tomato *Exp1* [32]. Amino acid identity is indicated by asterisks and conservative changes by dots. Eight Cys residues (crosses) and four Trp residues (diamonds) conserved amongst expansins are indicated. Putative signal sequences are shown in lower case. Note that the tomato *Exp6* and *Exp7* sequences are truncated and lack both N-terminal and C-terminal ends.

residue. The deduced primary translation products encoded by these three cDNAs were 267 amino acids for *Exp3*, 263 amino acids for *Exp4*, and 239 amino acids for *Exp5*. The truncated PCR fragments obtained for *Exp6* and *Exp7* encoded 166 and 162 amino acids, respectively (Figure 1). All five deduced polypeptides possessed the eight conserved Cys residues thought to be characteristic of expansins [34]. Applying the rules of von Heijne [38] to identify signal peptides, signal sequence cleavage sites were predicted in the *Exp3* deduced protein at amino acid 14 (with a second potential cleavage site possessing almost as high a probability at amino acid 18), in the *Exp4* deduced protein at amino acid 26, and in the *Exp5* deduced protein at amino acid 20 (Figure 1). The possession of putative signal sequences by these three deduced proteins predicts that the mature proteins are secreted

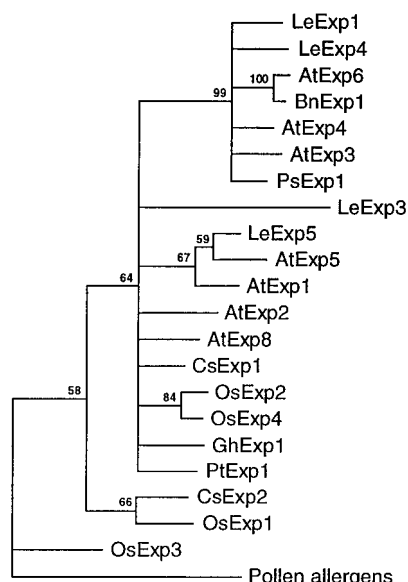


Figure 2. Phylogenetic analysis. Phylogenetic comparison of full-length mature expansin deduced proteins after removal of signal sequences, rooted using a pollen allergen from *Lolium perenne* (GenBank accession number M57474) as outgroup. Numbers on the tree show bootstrap probability *P* values. Sequences and GenBank accession numbers: At (*Arabidopsis thaliana*), Exp1 (U30476), Exp2 (U30481), Exp3 (AC004684), Exp4 (AC003674), Exp5 (U30478), Exp6 (U30480), and Exp8 (AC002336); Bn (*Brassica napus*), Exp1 (AJ000885); Cs (*Cucumis sativus*), Exp1 (U30382), and Exp2 (U30460); Gh (*Gossypium hirsutum*), Exp1 (AF043284); Le (*Lycopersicon esculentum*), Exp1 (U82123); Os (*Oryza sativa*), Exp1 (Y07782), Exp2 (U30477), Exp3 (U30479), and Exp4 (U85246); Ps (*Pisum sativum*), Exp1 (X85187); Pt (*Pinus taeda*), Exp1 (U64892).

to the cell wall. The deduced amino acid sequence of *Exp3* possesses at amino acids 23 to 25 a potential N-glycosylation site (Asn-X-Ser/Thr, where X is any amino acid except Pro), a feature not observed previously in expansins [4, 6, 34] and which are thought not to be glycosylated [9]. However, it is not known if this potential site for N-glycosylation is utilized.

Expansin deduced mature proteins fall into three major phylogenetic branches, one consisting of rice *Exp3* alone, the second of rice *Exp1* and cucumber *Exp2*, and the third of all remaining sequences (Figure 2). This last branch contained a large number of related sequences from both monocotyledons and dicotyledons, within which the ripening-specific expansin tomato *Exp1* [32] was present in a distinct subbranch which also contained three *Arabidopsis* expansins, *Brassica Exp1* and pea *Exp1*. This subbranch also contained tomato *Exp4* (Figure 2) and, when phylogenetic analysis was repeated using truncated amino

acid sequences (data not shown), probably also tomato *Exp6*, both of which are highly similar to tomato *Exp1*. After removal of signal sequences, the *Exp4* deduced protein showed 80.1% amino acid identity and 90.7% similarity to *Exp1*. The partial sequence of *Exp6* was also highly related to *Exp1*, being 82.6% identical over the equivalent region. Both tomato *Exp4* and *Exp6* were more phylogenetically related to tomato *Exp1* than to deduced expansin proteins from other species. The other three expansin genes isolated from tomato fruit were less related to tomato *Exp1*. Tomato *Exp5* was most closely related to *Arabidopsis Exp5*, and together with *Arabidopsis Exp1* formed a subbranch. Tomato *Exp3* was more divergent, and formed a subbranch on its own. When phylogenetic analysis was repeated using truncated amino acid sequences (data not shown), the partial sequence of tomato *Exp7* was most closely related to tomato *Exp3*, with which it showed 50.6% amino acid identity and 71.3% similarity over the equivalent region.

Gene-specific probes were designed from the 3'-untranslated regions of *Exp3*, *Exp4*, *Exp5* and *Exp1* cDNAs, which showed considerable diversity in length and little sequence homology. When used to probe genomic DNA gel blots at moderate stringency, these probes detected restriction fragments of different sizes for each particular gene (Figure 3). This DNA gel blot analysis also suggested that each of these genes is probably present in the genome as a single copy. For *Exp1* and *Exp5* there was an additional very faint band detected in most lanes, which may indicate the presence of distantly related sequences. For *Exp6* and *Exp7*, where 3'-untranslated region sequence information was not available, the original PCR fragments were used as probes of DNA gel blots at high stringency. Each of these probes detected one unique major band, plus one to three minor bands common to both which did not correspond to any of the fruit-expressed tomato expansins described here. Since the DNA sequences of the *Exp6* and *Exp7* probes were only 61% identical and did not cross-hybridize at this stringency, presumably the extra bands seen on the DNA gel blots were due to another expansin gene more similar to both genes than *Exp6* and *Exp7* are to each other. At moderate stringency, the probes for *Exp6* and *Exp7* detected four to eight bands per lane (data not shown), indicating the presence of a highly complex expansin gene family in tomato.

The probes described above were used at the same relative stringencies as was used to probe DNA gel blots to probe RNA gel blots (Figure 4). On gel

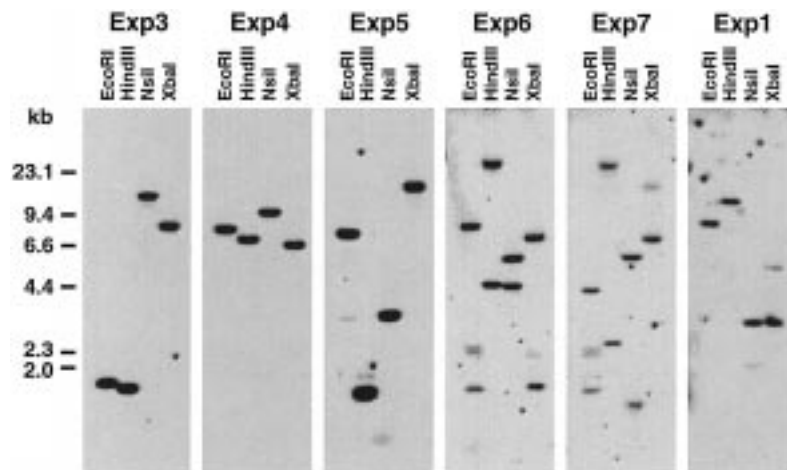


Figure 3. DNA gel blot analysis. Genomic DNA (10 μ g per lane) was digested with the indicated restriction enzyme, fractionated by electrophoresis and gel blots hybridized with radiolabeled probes prepared from the cDNA of each expansin gene. Probes used for *Exp3*, *Exp4*, *Exp5* and *Exp1* were 3'-untranslated regions, and blots were washed at moderate stringency (15 $^{\circ}$ C below T_m). Probes used for *Exp6* and *Exp7* were PCR fragments from coding regions and blots were washed at high stringency (4 $^{\circ}$ C below T_m), as described in Materials and methods.

blots of total RNA, mRNAs for *Exp3*, *Exp4* and *Exp5* were absent in roots and were detected at varying abundances in leaves, stems and flowers (for *Exp3* in all these tissues and for *Exp4* in leaves this required prolonged exposure of the blot to film, data not shown). However, for all three of these genes the highest mRNA abundances occurred in green fruit (Figure 4A). *Exp3* mRNA was present throughout fruit development from the earliest stages to ripe fruit (and was detected in over ripe fruit upon prolonged exposure of the blot to film, data not shown), with high levels during green fruit expansion and maturation and progressively decreasing abundance during fruit ripening. *Exp4* mRNA showed its highest abundance at the earliest stages of fruit expansion when fruit were 10 to 20 mm in diameter and then disappeared in early MG fruit, whereas *Exp5* mRNA peaked in the IG and early MG stages before declining substantially at around the beginning of ripening. In contrast *Exp1* mRNA was not present in green fruit and appeared at high levels at the onset of ripening, as previously described [32].

mRNAs of *Exp6* and *Exp7* were not detected on blots of total RNA (Figure 4A), but were detected on blots of poly(A)⁺ RNA (Figure 4B). The much lower mRNA abundance for these genes presumably explains why they were not detected during cDNA library screening. *Exp6* mRNA accumulated at low levels during rapid fruit expansion when fruit were 10 and 20 mm in diameter and showed highest abundance during the IG and MG2 stages, then declined during the late MG stages. Some very faint bands of

lower molecular weights detected only during the fruit ripening stages, i.e., after BR, may correspond to other expansin homologues or to other proteins. The only other of these expansins which is expressed in ripening but not in green fruit is *Exp1*, but the probe for *Exp6* did not cross-hybridize with the *Exp1* gene on DNA gel blots at this stringency (Figure 3). mRNA for *Exp7* was present at much lower abundance than *Exp6*, and was detected at extremely low levels only when fruit were 10 and 20 mm in diameter.

Thus, during fruit development each of the six expansin genes showed a different and characteristic pattern of expression. mRNAs for all the expansin genes except *Exp1* accumulated during particular stages of developing green fruit, although to varying extents. The only expansins other than *Exp1* whose mRNAs were detected during fruit ripening and softening were *Exp3* and *Exp5*. However, mRNA of *Exp5* was detected at very low levels only during the earliest stages of ripening (BR and TU), and although mRNA of *Exp3* was detected throughout fruit ripening, its mRNA abundances were at least 25- to 50-fold less than *Exp1* and declined as ripening progressed.

Discussion

Reverse transcription-coupled PCR was used to amplify cDNAs of five unique expansin genes from developing tomato fruit, in addition to the previously described tomato *Exp1* [32]. The results show that at

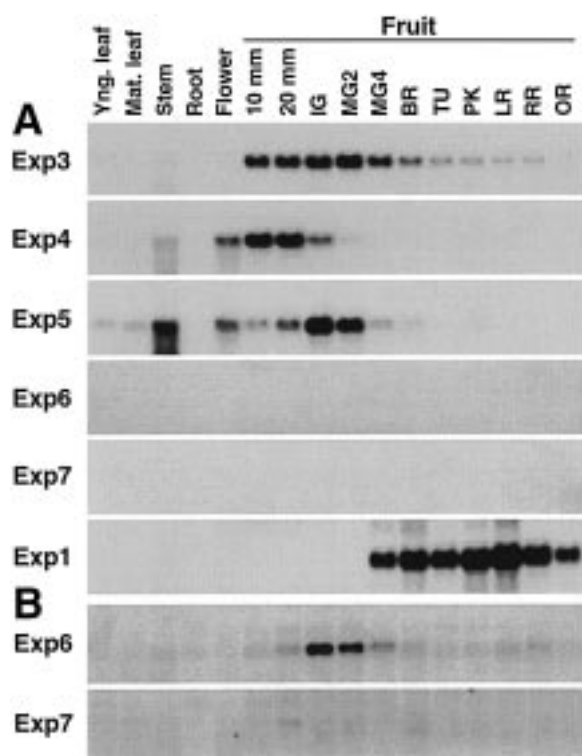


Figure 4. RNA gel blot analysis. A. Relative mRNA abundances of *Exp3*, *Exp4*, *Exp5*, *Exp6* and *Exp7* in various vegetative tissues and during fruit development. mRNA abundance of *Exp1* is shown for comparison. RNA gel blots of 15 µg total RNA per lane were hybridized with radiolabeled probes and washed at stringencies equivalent to Figure 3, as described in Materials and methods. B. mRNA abundances of *Exp6* and *Exp7* on gel blots of 0.5 µg poly(A)⁺ RNA per lane washed at stringencies equivalent to Figure 3.

least six expansin genes are expressed during tomato fruit development, and suggest that since the fruit is only one organ of the growing plant, the expansin gene family of tomato may be as large and complex as that described in other species [6, 9, 34]. Of these six tomato expansin genes, three (*Exp1*, *Exp4* and *Exp6*) possessed a high degree of identity in amino acid sequence and were highly phylogenetically related. The other three genes, tomato *Exp3*, *Exp5* and *Exp7* were less related to tomato *Exp1*, and were more closely related to each other and to pea *Exp1*, an expansin expressed in petals and leaves [31], and to *Arabidopsis* *Exp1* and *Exp5* [34], expansins whose expression characteristics have not yet been studied. Since all six tomato expansin genes are expressed at specific times during fruit development, three of them are also expressed in vegetative tissues and flowers, and little information is presently available on the mRNA

expression of expansins from other species, the correlation if any between phylogenetic similarity and mRNA expression pattern or function remains unclear.

The development of tomato fruit after fruit set begins with a period of rapid cell division, when the fruit are up to approximately 8 mm in diameter, followed by a period of rapid cell expansion during the IG stage, before reaching full size at the MG stage [13]. During the MG stage the seeds and locules develop, then one to two days before any red color is visible on the outside of the fruit, ripening is initiated by a rise in ethylene production which becomes auto-catalytic and triggers other ripening changes [20]. All five of the expansin genes examined in the present study showed the greatest mRNA accumulation early in fruit development: *Exp4* and *Exp7* only during green fruit cell division and expansion, *Exp5* and *Exp6* also during green fruit cell division and expansion but with maximum accumulations slightly later during green fruit expansion and maturation, and *Exp3* with maximal accumulation in green fruit but present throughout fruit development from the 10 mm stage to late in ripening. Presumably the products of these various expansin genes contribute to changes in cell wall properties occurring during green fruit cell division, expansion and maturation. Whether the products of the different genes act on different polysaccharide components or bonds in the wall related to wall expansion or differentiation, or are expressed in different cell types, or are differentially regulated by developmental or hormonal stimuli, remains to be determined. Thus, expansins may play a significant role in the growth and development of expanding green fruit, as they have been shown to do in vegetative tissues [8].

Fruit softening begins at around the BR stage, at the beginning of ripening when the first exterior red color becomes visible on the blossom end of the fruit [26]. Fruit firmness declines progressively during ripening [26], and is thought to be brought about largely by changes occurring in the cell wall. Depolymerization and solubilization of both matrix pectins and matrix glycans occurs during tomato fruit ripening [2, 21, 26, 37], but their contributions to fruit softening have not been elucidated. For example, molecular genetic modification of endopolygalacturonase activity in ripening tomato found that this enzyme was not solely responsible either for polyuronide depolymerization or for fruit softening [2, 14, 23, 35], although it may be involved in textural changes [24]. The role of expansins in fruit development and ripening has not yet been investigated, but accumulation of tomato

Exp1 mRNA was specific for fruit, was ethylene-regulated, and was lacking in the ripening-impaired, non-softening *rin* mutant of tomato [32]. Furthermore, tomato *Exp1* mRNA accumulated at high levels only during ripening, appearing either just before the BR stage in cv. Bonnie Best (Figure 3A) or just after the BR stage in cv. T5 [32]. Accumulation of tomato *Exp1* mRNA continued at high levels throughout ripening, and thus was coincident with fruit softening [32]. Of the five tomato expansin genes investigated in the present study, none showed a pattern of mRNA accumulation similar to *Exp1*, and several showed mRNA accumulation in tissues other than fruit, including leaf, stem and flower (Figure 4). All five genes showed mRNA expression earlier in fruit development than *Exp1* and, of these five genes, only *Exp3* and *Exp5* showed detectable mRNA accumulation during fruit softening. However, mRNA of *Exp5* was present at very low levels only during the early stages of ripening, and although mRNA of *Exp3* was present throughout ripening it was present at substantially lower levels than *Exp1* mRNA, and declined as softening progressed. This suggests that although at least two other expansin genes are expressed at low levels at particular times during ripening, *Exp1* is likely to be the major expansin protein present in the cell walls of softening tomato fruit.

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