Detection of Expansin Proteins and Activity during Tomato Fruit Ontogeny[1]

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Expansins are plant proteins that have the capacity to induce extension in isolated cell walls and are thought to mediate pH-dependent cell expansion. J.K.C. Rose, H.H. Lee, and A.B. Bennett ([1997] Proc Natl Acad Sci USA 94: 5955–5960) reported the identification of an expansin gene (*LeExp1*) that is specifically expressed in ripening tomato (*Lycopersicon esculentum*) fruit where cell wall disassembly, but not cell expansion, is prominent. Expansin expression during fruit ontogeny was examined using antibodies raised to recombinant LeExp1 or a cell elongation-related expansin from cucumber (CsExp1). The LeExp1 antiserum detected expansins in extracts from ripe, but not preripe tomato fruit, in agreement with the pattern of *LeExp1* mRNA accumulation. In contrast, antibodies to CsExp1 cross-reacted with expansins in early fruit development and the onset of ripening, but not at a later ripening stage. These data suggest that ripening-related and expansion-related expansin proteins have distinct antigenic epitopes despite overall high sequence identity. Expansin proteins were detected in a range of fruit species and showed considerable variation in abundance; however, appreciable levels of expansin were not present in fruit of the *rin* or *Nr* tomato mutants that exhibit delayed and reduced softening. LeExp1 protein accumulation was ethylene-regulated and matched the previously described expression of mRNA, suggesting that expression is not regulated at the level of translation. We report the first detection of expansin activity in several stages of fruit development and while characteristic creep activity was detected in young and developing tomato fruit and in ripe pear, avocado, and pepper, creep activity in ripe tomato showed qualitative differences, suggesting both hydrolytic and expansin activities.

Synthesis, assembly, and modification of the primary cell wall are features common to both cell expansion and cell differentiation and represent fundamental determinants of plant architecture. An excellent model system in which to study cell wall dynamics, including wall assembly, restructuring, and disassembly, are the contiguous stages of fruit development. In most plant species fruit ontogeny may be divided into several distinct developmental phases (Gillaspy et al., 1993). These comprise early fruit growth accompanied by cell division, a period of cell expansion that generates more substantial and prolonged growth until maximal fruit size is reached, and finally ripening, involving numerous metabolic pathways including those leading to textural changes and softening. Similar changes in the structure and composition of the cell wall and in the complement of associated enzymes have been observed in expanding cells and ripening fruit (Rose and Bennett, 1999).

In growing tissues the cell wall acts to restrict increases in cell volume, and so controlled turgor-driven cell expansion relies on the coordination of cell wall loosening with wall biosynthesis and assembly to maintain structural integrity and strength. Under acidic conditions in vitro, plant tissues grow more rapidly, giving rise to the "acid-growth" theory of cell expansion (Rayle and Cleland, 1992). Similarly, when cell walls are clamped in a constant-load extensometer and placed in low pH buffers, they exhibit prolonged elongation (Cosgrove, 1989), consistent with a pH-dependent mechanism of wall extension or wall creep. Heat-inactivated walls can be induced to elongate in a similar fashion by applying a protein extract from growing walls and purification of the growth-promoting activities from cucumber hypocotyls and oat coleoptiles identified a class of proteins that were termed expansins (McQueen-Mason et al., 1992; Li et al., 1993). A number of expansin genes have now been cloned and divided into two broad classes based on sequence homology (Cosgrove, 1998). One class, termed α-expansins, include the original cucumber expansin gene (*CsExp1*) and homologs from numerous species, many of which have been associated with rapidly growing vegetative tissues. Another class, β-expansins, share approximately 25% amino acid identity with α-expansins and include genes encoding a class of grass pollen allergens and closely related sequences

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that are expressed in vegetative tissues (Cosgrove et al., 1997). The mechanism of action of expansins is still obscure; however, several pieces of evidence have suggested specific cell wall components that may be targeted (Cosgrove, 1998; Rose and Bennett, 1999).

A recent, and perhaps surprising, finding was that an α-expansin gene from tomato (*LeExp1*) is specifically and abundantly expressed at the level of mRNA accumulation in ripening fruit where cell expansion does not occur (Rose et al., 1997). Phylogenetic analysis suggested that *LeExp1* sequence aligned within an evolutionarily divergent clade of the super-family of expansin genes. Expansins were proposed to contribute to cell wall disassembly in ripening fruit, although this hypothesis was based solely on the expression patterns of LeExp1 mRNA. The mRNA expression patterns of other tomato expansins suggest roles for divergent expansins at different stages of fruit ontogeny (Brummell et al., 1999).

In this paper we describe the expression of expansin proteins during fruit development in wild-type and ripening-impaired mutant tomato fruit, using antibodies raised to either a recombinant LeExp1 protein or to an expansin purified from cucumber hypocotyls (CsExp1). The results support the idea that distinct expansin isoforms are expressed in elongating cells and in ripening fruit. We also report the first detection of expansin-like activity in protein extracts from developing and ripening fruit and contrast the activities in ripening tomato fruit with those from several other species.

RESULTS

Identification of Expansin Subgroups

Figure 1 shows a dendrogram generated from deduced amino acid sequences corresponding to α-expansins from several species, together with the sequence of a related pollen allergen (*Phlp1*) that is more similar to β-expansins (Cosgrove, 1998). The sequences of the deduced polypeptides, without the predicted signal sequences, were aligned using CLUSTAL IV. Values corresponding to matching percentages of branching orders are indicated at each branch point. The sequences align within four principal groups (labeled A–D, after Link and Cosgrove, 1998) in addition to two more distantly related sequences from Arabidopsis (*AtExp5*) and tomato (*LeExp3*). The sequences of six full-length tomato expansins that are currently in the databases align within three of the groups and exhibit 50% to 90% amino acid identity to each other. Two partial length sequences, *LeExp6* (accession no. AF059490) and *LeExp7* (accession no. AF059491), which are most similar to *LeExp1* and *LeExp3*, respectively (Brummell et al., 1999), are not shown. Subgroup A includes *LeExp1*, the fruit ripening-related expansin (Rose et al., 1997) *LeExp4*, which is expressed in young developing fruit and flowers (Brummell et al., 1999), and *LeExp18*, which is expressed in cells exhibiting high meristematic activity (Reinhardt et al., 1998). Subgroup C includes *LeExp5*, which is expressed in late fruit growth and mature stems (Brummell et al., 1999). Subgroup B contains several sequences from genes whose expression has been associated with rapid cell expansion, including *CsExp1* from cucumber (McQueen-Mason et al., 1992), *GhExp1* expressed during cotton fiber elongation (Orford and Timmis, 1998), and a rice expansin *OsExp4* (Cho and Kende, 1997a). In addition two newly submitted sequences from apricot (*PaExp1* and *PaExp2*) are described in their database entries as being identified from ripening fruit.

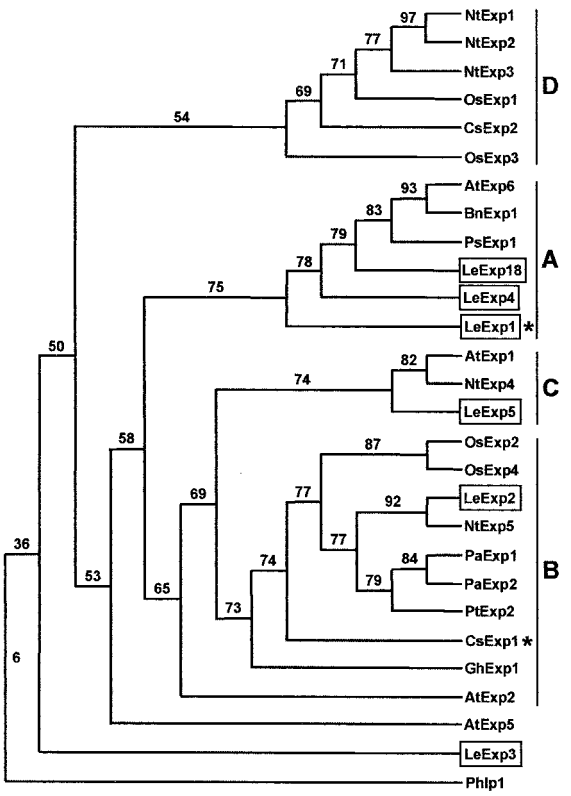

Figure 1. Phylogenetic analysis of expansin genes. The dendrogram was generated based on an alignment of the deduced amino acid sequences of 27 expansin genes together with a pollen allergen (*Phlp1*), using CLUSTAL. Tomato expansin genes are boxed and the sequences corresponding to the polypeptides used to generate the antisera described in this paper (CsExp1 and LeExp1) are indicated with asterisks. Subgroups A through D are highlighted with vertical lines, and values corresponding to matching percentages of branching orders are indicated at each branch point. Additional details and accession numbers are listed in "Materials and Methods."

The polypeptides used to generate expansin antibodies for the western-blot analyses described in this paper correspond to genes that align within subgroup A (*LeExp1*) and subgroup B (*CsExp1*) and are indicated in Figure 1 with an asterisk. LeExp1 and CsExp1 share 69% amino acid identity, following removal of the predicted signal sequences.

Heterologous Expression of LeExp1 Protein

The coding region of the mature LeExp1 polypeptide was expressed in *Escherichia coli* as a fusion protein with a 6-His residue tag at the N terminus to facilitate subsequent purification. Following induction of transcription of the *LeExp1* gene with isopropylthio-β-galactoside (IPTG), a polypeptide with an apparent molecular mass of approximately 25 to 27 kD, the predicted molecular mass of the His-tagged LeExp1 protein, was observed to accumulate to relatively high levels in total protein extracts (Fig. 2). The polypeptide was not detected in uninduced cultures. Analysis of subcellular fractions localized the induced polypeptide to insoluble inclusion bodies with no apparent accumulation of soluble protein in the incubation medium, cytosol, or periplasm despite the use of a range of induction and incubation conditions. However, following application and binding of urea-solubilized, IPTG-induced culture extracts to a nickel column, a polypeptide with a molecular mass of approximately 27 kD eluted in a urea gradient and was isolated as a relatively pure protein fraction (Fig. 2). Protein was not detected in equivalent gradient fractions following application of proteins from uninduced cultures (Fig. 2).

LeExp1 and CsExp1 Antibodies Show Low Cross-Reactivity

Antibodies raised to recombinant tomato expansin LeExp1 or partially purified native cucumber expansin CsExp1 (Li et al., 1993) were used in immunoblot analyses of LeExp1 recombinant protein, partially purified cucumber expansin (CsExp1), and a crude protein extract from red ripe tomato fruit (Fig. 3). The LeExp1 antibody detected a 25-kD protein in the recombinant LeExp1 protein fraction and in the ripe fruit extract (Fig. 3A), but showed no cross-reactivity to the cucumber protein, even following over-exposure of the blot to film (Fig. 3A, right panel). The preimmune serum corresponding to the LeExp1 antiserum showed no cross-reactivity to any of the protein extracts (data not shown). The CsExp1 antibody detected a major protein of approximately 25 kD in the cucumber expansin sample in addition to a band of approximately 14 kD (Fig. 3B). However, the CsExp1 antibody showed extremely low cross-reactivity with the ripe fruit protein extract, and hybridization with recombinant LeExp1 was only detectable following prolonged exposure of the blot to film (Fig. 3B, right panel). Both the LeExp1 and CsExp1 antisera recognized an approximately 55-kD polypeptide, which we speculate may represent a homo- or heterodimeric expansin complex.

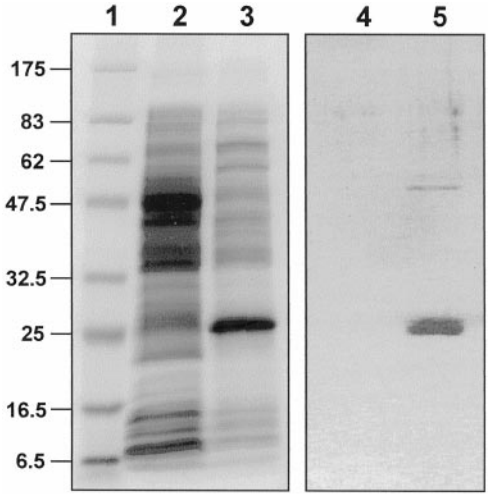

Figure 2. SDS-PAGE gel of LeExp1 expressed in *E. coli*. Samples comprised total protein extracts of an uninduced *E. coli* culture (lane 2) or a culture induced with 2 mM IPTG (lane 3), and eluant fractions isolated following binding of the total cell protein extracts to nickel resin: eluted protein from noninduced cultures (lane 4) and protein eluted from cultures induced with IPTG for 5 h (lane 5). Protein samples were separated by SDS-PAGE and stained with Coomassie Blue. M_r markers (lane 1) are indicated.

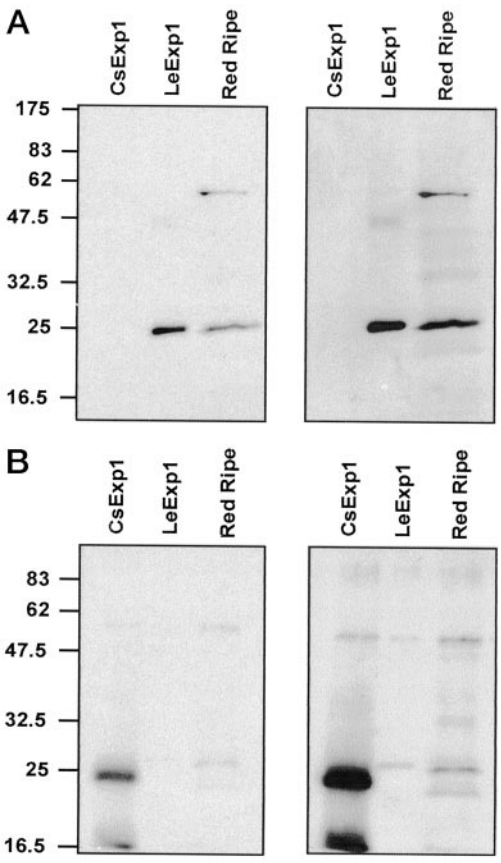

Figure 3. Cross-reactivity of expansin antibodies. Immunoblot analysis of partially purified native cucumber expansin (CsExp1), recombinant tomato expansin (LeExp1), and a protein extract from red ripe tomato fruit with antisera raised to either (A) recombinant LeExp1 or (B) purified CsExp1. M_r markers are indicated. Right panels are the same as the left panels except they are over-exposed to reveal faint bands.

Immunodetection of Expansin Proteins in Wild-Type and Mutant Tomato Fruit

Figure 4A shows an immunoblot analysis of proteins extracted with high-salt buffer from tomato fruit pericarp cell walls at stage II (small green rapidly expanding fruit), mature green (fully expanded), breaker, turning, and red ripe using antibodies raised to recombinant LeExp1 or native CsExp1. The LeExp1 antibody (Fig. 4, left panel) cross-reacted strongly with a 25-kD polypeptide at the breaker developmental stage, which marks the onset of ripening and autocatalytic ethylene production, as well as turning and red ripe (fully ripe) stages. A faint cross-reactive 25-kD band was detected in the stage II and mature green protein extracts following overexposure of the blot to film in addition to several less cross-reactive bands of various molecular weights.

Immunoblot analysis of the same protein samples using the CsExp1 antibody revealed a different pattern of immunoreactive proteins (Fig. 4A, right panel). Cross-reactive 25-kD polypeptides were detected in fruits at stage II, mature green, breaker, and turning with a peak of expression at the breaker stage, whereas virtually no cross-reactivity was detected in extracts from red ripe fruit.

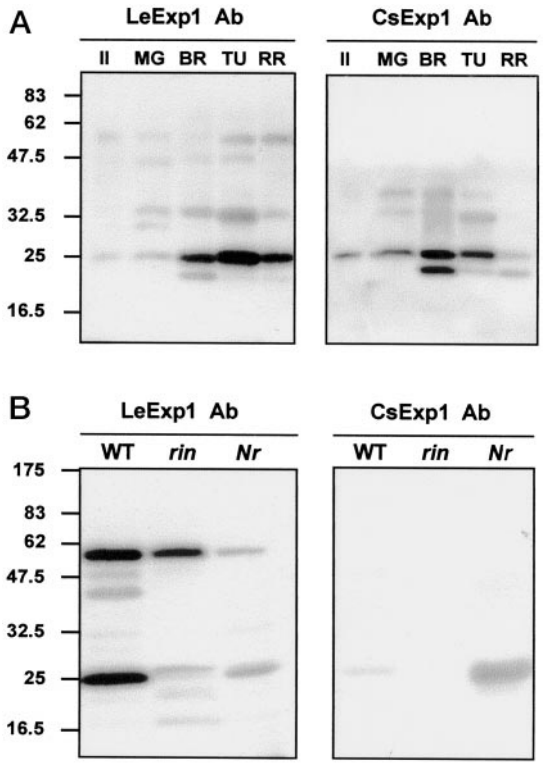

Figure 4. Detection of expansin proteins during fruit ontogeny. Immunoblot analysis of expansin protein expression (A) during tomato fruit development (cv T5), comprising stage II (small, rapidly expanding green fruit), MG (mature green), BR (breaker), TU (turning), and RR (red ripe; B) tomato fruit (cv Ailsa Craig) of the wild-type (Ac) and the ripening mutants *rin* (ripening inhibitor) and *Nr* (never ripe) that were harvested at a preripe stage and treated with 100 μL L^{-1} ethylene. Membranes were cross-reacted with antibodies to LeExp1 (left panels) or CsExp1 (right panels) expansins. M_r markers are indicated.

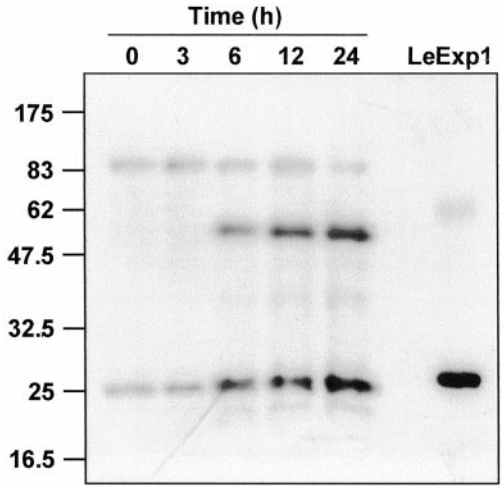

Figure 5. Ethylene regulation of expansin expression. Immunoblot analysis of expansin protein expression in protein extracts of ACC synthase antisense tomato fruit treated with ethylene over a time course of up to 24 h, using antibodies to LeExp1. Recombinant LeExp1 is included as a positive control. M_r markers are indicated.

Expansin protein expression was further examined in the pleiotropic tomato mutants *rin* (ripening inhibitor) and *Nr* (never ripe), each of which exhibits a range of phenotypes including reduced fruit softening. Figure 4B shows immunoblot analyses of proteins from red ripe cv Ailsa Craig wild-type tomato fruit and equivalent-age ethylene-treated *rin* and *Nr* mutant fruit, using the LeExp1 and CsExp1 antibodies. The LeExp1 antibody detected high levels of a 25-kD protein in wild-type fruit extracts, but only faint cross-reactive bands were observed in extracts from the *rin* and *Nr* mutants following prolonged exposure of the immunoblot to film (Fig. 4B, left panel). A 55-kD protein was also detected in wild-type and *rin* fruit. The CsExp1 antibody showed no substantial labeling of proteins, although very faint bands were detected in the wild-type and *Nr* protein extracts following over-exposure of the immunoblot to film (Fig. 4, right panel).

Induction of LeExp1 Protein in 1-Aminocyclopropane-1-Carboxylic Acid (ACC) Synthase Antisense Fruit

LeExp1 protein accumulation was examined in transgenic tomato fruit expressing an antisense gene for ACC synthase. These fruit synthesize less than 1% of the wild-type levels of ethylene and fail to ripen unless exogenous ethylene is applied (Oeller et al., 1991). Figure 5 shows a western-blot analysis using the LeExp1 antibody of protein extracts from transgenic fruit treated for up to 24 h with 10 μL L^{-1} ethylene. A faint 25-kD band was detected at the 0- and 3-h time points, however expression levels were substantially higher after 6 h and continued to in-

crease throughout the time course. Similarly, a hybridizing band of approximately 55 kD was detected at the 6-h time point and accumulated further until 24 h. An additional faint band at approximately 78 kD was also detected and showed equal abundance throughout the time course. The recombinant protein positive control migrated as a slightly higher M_r polypeptide, possibly due to the presence of additional His that were included in the recombinant protein.

Detection of Expansin Proteins in a Range of Fruit Species

Proteins were extracted from the cell wall fractions of nine species of fruit with a buffer containing 1.5 M sodium chloride, a salt concentration likely to be sufficient to disrupt ionic interactions between cell wall proteins and wall polymers. Extracts were size-fractionated on SDS-PAGE gels and were either stained with Coomassie Blue (Fig. 6A) or transferred to nitrocellulose membrane for subsequent immunoblot analysis with the LeExp1 antibody (Fig. 6B). Based on one-dimensional gel electrophoresis, the protein populations showed substantial variation between species with each extract containing a distinct profile. In certain samples, such as those from persimmon, kiwi, and strawberry, one or two polypeptides appeared to comprise more than 80% of the total protein. Immunoblot analysis indicated that expansin protein also showed substantial variation in expression levels between extracts. In particular, a 25-kD cross-reactive band was extremely abundant in pear fruit, showing more than 10-fold greater levels than in tomato extracts and more than 50-fold greater levels than in extracts from kiwifruit and strawberry. Expression was only detectable in pepper following prolonged exposure of the blot to film. A cross-reactive band of approximately 55 kD was also present at varying intensities, although the relative abundance, compared with the 25-kD polypeptide, was not constant.

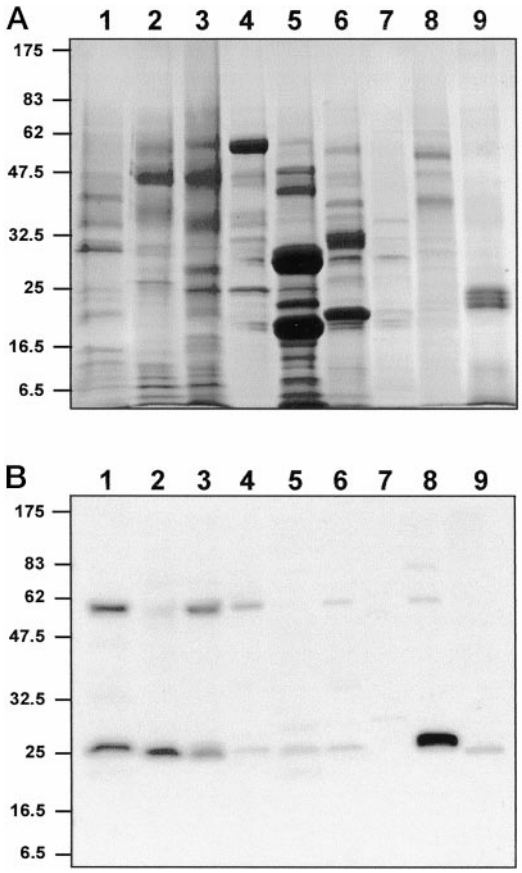

Figure 6. Expansin expression in a variety of fruit species. A, SDS-PAGE gel of proteins extracted with 1.5 M NaCl from the cell walls of a variety of ripening fruit, stained with Coomassie Blue. B, Immunoblot analysis of the same fruit extracts shown in A, using the LeExp1 antibody. Lane 1, Tomato; 2, melon; 3, avocado; 4, persimmon; 5, kiwifruit; 6, strawberry; 7, pepper; 8, pear; and 9, pineapple. M_r markers are indicated.

Detection of Expansin Activity during Fruit Ontogeny

Expansin or "creep" activity was assayed as described by Cosgrove (1989). Briefly, heat-inactivated cucumber hypocotyls were clamped in a constant-load extensometer and allowed to equilibrate in pH 4.5 buffer where they exhibited slow initial linear extension (Fig. 7). After 20 to 30 min, an aliquot of the buffer was removed and replaced with an equal volume of protein extract. At least eight replicate experiments were conducted per sample, and typical traces are shown. Creep activity was present although at fairly low levels in extracts from stage II and mature green tomato fruit and induced either transient or prolonged extension, whereas the extracts from breaker fruit had negligible activity levels (Fig. 7A). Replicate assays of red ripe tomato fruit extracts yielded variable results however, unlike the other protein samples, in most cases red ripe extracts promoted sudden breakage of the hypocotyl after a 150 to 300 μm extension (3%–6%). In some replicates, this was preceded by low creep activity, similar in magnitude and kinetics to the stage-II sample, whereas in other cases the hypocotyl broke without first exhibiting creep. Two profiles are shown to illustrate the variable response (Fig. 7A).

Protein extracts from ripening avocado, pepper, and pear fruit also exhibited creep activity (Fig. 7B), although with different profiles. Avocado and pepper extracts induced moderate, prolonged, or transient responses, whereas the pear sample showed relatively high-creep activity and promoted continuous extension over the 2-h time course of the experiment, at which point the extension rate showed no evidence of decreasing. An overall increase in hypocotyl segment length of 500 μm or 10% of the clamped hypocotyl length occurred without breakage, characteristic of a moderate expansin response.

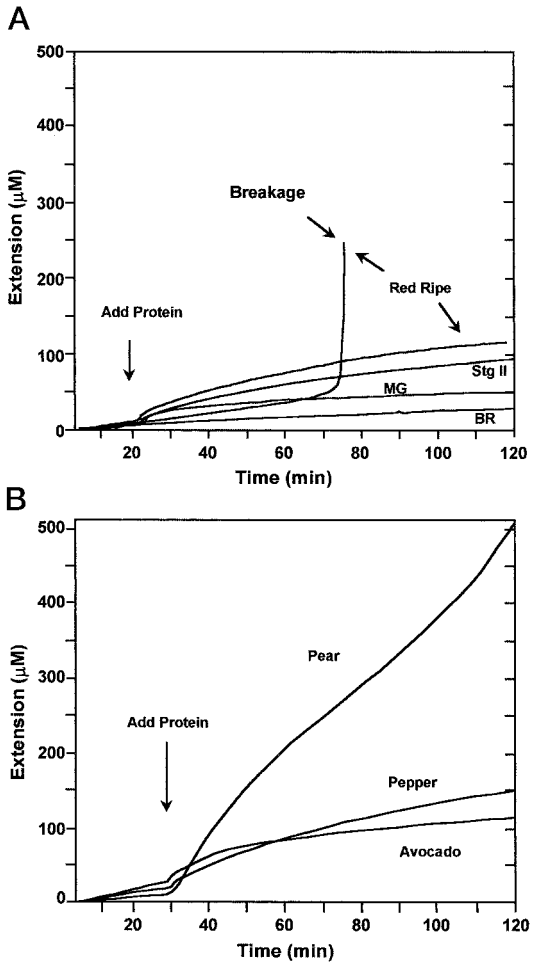

Figure 7. Wall extension induced by protein extracts from developing and ripening fruit. Heat-inactivated cucumber hypocotyls were clamped in an extensometer and incubated in 50 mM sodium acetate buffer, pH 4.5, for 20 to 30 min. At the indicated time (arrows), an aliquot of buffer was removed from the incubation solution and replaced with the same volume of buffer containing a protein extract from the cell walls of (A) tomato fruit at the stage II (Stg II), mature green (MG), breaker (BR), or red ripe developmental stages (B) ripe avocado, pepper, and pear fruit. The curves shown are representative of the extension responses seen in three to seven replicate experiments.

DISCUSSION

Families of expansin genes have been identified in several plant species, and these may be divided into two broad phylogenetic classes, α-expansins and β-expansins, which have differing properties such as solubility in addition to phylogenetic divergence (Cosgrove, 1998). The functional significance of the phylogenetic grouping is not known; however, members of each subgroup appear to contain characteristic sequence subdomains (Link and Cosgrove, 1998). This suggests that the phylogenetically divergent subgroups may reflect expansin isoforms with differing biochemical properties such as substrate affinities or pH optima and may fulfill unique and diverse functions in plant development.

The Tomato Expansin Gene Family

Limited data are available with respect to the expression patterns of divergent expansins from a single species; however, in this regard tomato represents the best characterized species to date. At least 10 distinct tomato expansin genes have been identified (for further information, see http://www/bio.psu.edu/expansins), eight of which have been assigned database accession numbers and six of which correspond to full-length coding sequences. *LeExp1* was the first reported tomato expansin gene and was described as being expressed at high levels, at the level of mRNA accumulation, in ripening fruit, but not in growing vegetative tissues (Rose et al., 1997). Phylogenetic analysis revealed that the deduced amino acid sequence of *LeExp1* aligned with those of ripening-related expansins from strawberry and melon. Subsequent descriptions of the expression patterns of other tomato expansins indicate that the phylogenetic grouping of the tomato expansin gene family (Fig. 1) may be more complex than simply reflecting "expansion-related" and "ripening-related" classes. For example, *LeExp4* is expressed in growing fruit (Brummell et al., 1999) but is more closely related to *LeExp1* in subgroup A than *LeExp2*, which aligns within subgroup B (Catalá et al., 2000).

The detection of many expansins during tomato fruit ontogeny may reflect not only divergent functional roles and differences in wall structure and composition at specific developmental stages but also the multiplicity of cell and tissue types in which expansins may be specifically expressed. These include the columella, locules, placental tissue, seeds, the pericarp (which can be further subdivided into endocarp, mesocarp, and exocarp), epidermis, and distinct layers of collenchymatous cells (Gillaspy et al., 1993). In addition, the vascular system with its heterogeneous associated cell types extends throughout the fruit. Given the wide range of cell types with associated differences in wall composition and the variation in cell shape and size within a fruit, it is perhaps not surprising that multiple expansin genes have been detected with apparently overlapping patterns of expression in crude preparations of fruit tissue.

Immunological Discrimination between Expansin Isoforms

Expansin proteins and activity have been detected in growing vegetative tissues of several species (Cosgrove and Li, 1993; Keller and Cosgrove, 1995; Wu et al., 1996; Cho and Kende, 1997b), however, to date the expression of expansins in fruit has been restricted to the detection of mRNA (Rose et al., 1997; Brummell et al., 1999). We observed that antisera raised to either LeExp1 or CsExp1 proteins showed extremely low cross-reactivity (Fig. 3A). Given that *LeExp1* and *CsExp1* align within divergent expansin

subgroups, this suggested that the LeExp1 and CsExp1 antisera held the potential to examine the expression of structurally divergent expansin proteins.

Using antibodies raised to recombinant LeExp1 protein, ripening-specific expression of LeExp1 was confirmed at the level of protein accumulation (Fig. 4A). The pattern of expression of this protein correlated with the accumulation of LeExp1 mRNA (Rose et al., 1997). Weak cross-reactivity was detected in the stage II and mature green fruit extracts, despite the previously reported absence of LeExp1 mRNA in these stages (Rose et al., 1997). This may reflect the polyclonal nature of the antiserum and result from a low degree of cross-reactivity to other divergent tomato expansins in developing fruit. In contrast, the CsExp1 antiserum recognized expansins in the expanding stage-II fruit and showed increasing expression through development, peaking at the breaker stage before declining to barely detectable levels in the red ripe fruit extract. This expression pattern does not match the pattern of mRNA accumulation of any expansin gene described to date, suggesting that the CsExp1 antibody is either recognizing an unreported expansin protein or a combination of expansins that cumulatively give rise to the observed expression pattern.

LeExp1 Is Not Expressed in Ripening-Impaired Mutant Fruit

A number of genetic mutants have been identified in tomato that exhibit impaired ripening phenotypes and these represent useful tools to examine ripening-regulated signal transduction pathways. Rose et al. (1997) described the absence of LeExp1 mRNA accumulation in the *rin* mutant, whereas detection of wild-type levels in the *Nr* mutant was ascribed to the leakiness of the mutation in the cv Ailsa Craig genetic background. Figure 4B shows that, in common with the pattern of mRNA expression, expansin protein was barely detectable in the *rin* fruit extract, however antibody cross-reactivity was also low in the *Nr* fruit extract. This contrasts with the relatively high levels of LeExp1 mRNA in *Nr* in the cv Ailsa Craig genetic background (Rose et al., 1997) and may reflect differential post-transcriptional regulation of LeExp1 expression between wild-type and *Nr* fruit, or differences in experimental treatments since different sets of fruit were used to isolate protein or mRNA.

LeExp1 Accumulation Is Ethylene Regulated

The existence of two signal transduction pathways in ripening tomato fruit has been suggested: one that is ethylene-dependent and another that is ethylene-independent, but developmentally regulated (Theologis et al., 1993; Lelièvre et al., 1997). Through the use of antisense technology it has been possible to discriminate between developmental or ethylene regulation of specific ripening-related genes by examining expression in transgenic plants expressing antisense genes for either of two genes required for ethylene biosynthesis; ACC synthase or ACC oxidase (Lelièvre et al., 1997). Rose et al. (1997) reported that *LeExp1* expression was regulated by ethylene at the level of mRNA accumulation in ACC synthase antisense tomato fruit. We examined the expression of LeExp1 protein in ethylene-treated ACC synthase antisense fruit (Fig. 5), and the close correlation with the timing of induction of LeExp1 mRNA in the same fruit series (Rose et al., 1997) indicates that there is no substantial temporal delay between transcript and protein accumulation. This result supports the model that expansin-mediated wall modification is an early event in fruit ripening.

Detection of Expansins in Divergent Ripening Fruit Species

Fruit softening is typically accompanied by depolymerization and solubilization of several classes of cell wall polysaccharides and by elevated levels of genes, proteins, and enzymatic activities associated with wall degradation. Research into the mechanisms of fruit softening to date has generally highlighted the role of cell wall hydrolases, such as polygalacturonase and endo-1,4-β-glucanases (or "cellulases"). The identification of an abundant ripening-specific expansin (Rose et al., 1997) raises the novel possibility that since to date expansins have not been identified as hydrolases (Cosgrove, 1998) processes other than polysaccharide hydrolysis may also make significant contributions to fruit softening. We examined the expression of expansin protein in a range of fruit, corresponding to evolutionarily divergent species that have previously been characterized to some degree with respect to their ripening-related wall changes (Fig. 6). Based on immunoblot analyses, expansin protein abundance varied considerably among different species of fruit with by far the highest levels detected in ripening pear, moderate levels in tomato, melon, and avocado, low levels in persimmon, kiwifruit, strawberry, and pineapple, and an almost undetectable signal in pepper. The significance of high levels of expansin in pear is not clear. In relation to the other fruit species examined, pear did not exhibit the most rapid or extensive softening, and qualitative or quantitative cell wall changes or wall-related enzyme activities have not been reported in pear that are not also common to other fruit. In a similar manner correlations between rapid softening, unique wall properties, and expansin protein levels were not apparent in other fruit. We conclude that expansin-mediated wall modification is a common event in fruit ripening, although the relative expression levels in different species do not suggest obvious biochemical modes of action or conserved mechanisms of regulation.

Detection of Expansin Activity during Fruit Ontogeny

Expansin activity is typically detected in a reconstitution assay by applying a protein extract to isolated heat-killed hypocotyl segments clamped under tension in an acidic buffer (Cosgrove, 1998). To date, acid-extension activity has been reported in a variety of elongating vegetative tissues (Cosgrove, 1998), suspension-cultured cells (Link and Cosgrove, 1998), and in pollen extracts (Cosgrove et al., 1997). Figure 7 represents the first report of expansin-like activity in crude protein extracts from a variety of fruit developmental stages. Activity was determined using heat-killed cucumber hypocotyl tissue since this is experimentally more robust than tomato hypocotyls, exhibits a high degree of reproducibility, and has been extensively characterized. Furthermore, Keller and Cosgrove (1995) reported that the acid extension activities of tomato and cucumber walls were similar and that tomato expansin extracts were equally or more active on cucumber hypocotyls as on tomato leaf tissue.

Overall activity levels were somewhat low compared with activity induced by extracts from growing vegetative tissues (Li et al., 1993; Keller and Cosgrove, 1995; Cho and Kende, 1997b). It is interesting that creep activity was almost undetectable in the breaker fruit sample despite the fact that this sample contained relatively high levels of immunoreactive expansins as detected by both the LeExp1 and the CsExp1 antisera (Fig. 4A). Conversely, less expansin was detected in the stage-II fruit, but the creep activity was greater. This suggests that either creep activity may be a consequence of an expansin that was not detected by either of the antibodies or that expansin activity is regulated/limited by some additional factor. The sudden breakage of the hypocotyl segments, observed following the application of red ripe fruit protein (Fig. 7A), is a typical consequence of the action of hydrolytic enzymes (Cosgrove and Durachko, 1994). A variety of divergent ripening-related cell wall hydrolases have been identified in ripening tomato fruit (Fischer and Bennett, 1992; Rose and Bennett, 1999). We suggest that the red ripe fruit extension profiles may reflect the combined activities of both ripening-related expansins and hydrolases, perhaps acting synergistically on load-bearing linkages within the cell wall. Furthermore, the differences between the extension profiles induced by the ripe pear or tomato extracts (Fig. 7, A and B) suggest quantitative, or more likely qualitative, differences in critical cell wall structures/linkages that are targeted by the complement of proteins in the respective extracts.

The detection of phylogenetically related expansin proteins and expansin-like activities in both expanding and ripening fruit suggests that the basic mechanism of action of ripening-related and expansion-related expansins is likely to be similar. However, immunological differences between expansion-related and ripening-related expansin isoforms hint at functional and biochemical variability. This cannot be confirmed until the biochemical basis of expansin activity is determined, which remains the major goal in understanding the role of expansins in plant growth and development.

MATERIALS AND METHODS

Plant Materials

Developing and ripening fruit, used as the source of material for Figures 3, 4A, and 7, were harvested from field-grown (Davis, CA) tomatoes (*Lycopersicon esculentum* cv T5) and assigned a developmental stage as described in Rose et al. (1997). Fruit used as a source of protein for Figure 4B (*L. esculentum* cv Ailsa Craig) were from greenhouse-grown tomatoes; either wild type or nearly isogenic for the *rin* and *Nr* mutations. Transgenic tomatoes expressing an ACC synthase gene (Oeller et al., 1991), used as the source of material for Figure 5, were greenhouse grown.

The tomato (cv T5) and melon (*Cucumis melo* cv Reticulatus F_1-α) fruit used as a source of protein for Figure 6 were field grown (Davis, CA), whereas fruit from other species (cultivars listed in parentheses when known) were obtained from a local farmers' market: avocado (cv Hass), persimmon (cv Fuyu), kiwifruit, strawberry (cv Red Sweet), red bell pepper, pear (cv Bosc), and pineapple. All fruit were determined to be ripening based on flesh firmness, production of ethylene (described in Rose et al., 1998), or external color depending on the species. In all cases fruit tissue was harvested at the indicated stages, immediately frozen in liquid nitrogen, and stored at −80°C.

Ethylene Treatments

Wild-type, *rin*, and *Nr* tomato fruit used as a source of protein for Figure 4B were harvested 35 DPA and treated with 10 μL L^{-1} ethylene for 14 d, as described in Gonzalez-Bosch et al. (1996). ACC synthase antisense transgenic fruit (Fig. 5) was treated with 10 μL L^{-1} ethylene for up to 24 h as described in Rose et al. (1997).

Protein Extraction

Frozen fruit tissue from each developmental stage or species was powdered in liquid nitrogen and thawed in 1,500 mL of ice-cold buffer A (50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.0, 5 mM dithiothreitol, 3 mM sodium metabisulfite, 2 mM EDTA, 0.1% [w/v] polyvinylpyrrolidone [M_r of 40,000], and 0.1% [v/v] Triton X-100) in the ratio of 1 g of tissue/3 mL of buffer, and homogenized for 1 min at 1°C. Samples were centrifuged at 10,000*g* for 30 min and the pellets washed three times by resuspending in ice-cold distilled water, followed by centrifugation. The pellets were each resuspended in 500 mL of buffer B (50 mM HEPES, pH 7.0, 5 mM dithiothreitol, 3 mM sodium metabisulfite, 2 mM EDTA, and 1.5 M sodium chloride) and stirred at 1°C for 12 h. Samples were centrifuged as before, the supernatants

removed, the pellets re-extracted in 100 mL of buffer 2 for 2 h at 1°C, centrifuged, and the supernatants combined. Ammonium sulfate was added to a concentration of 0.4 g mL^{-1} and stirred at 1°C overnight. The suspensions were centrifuged at 10,000*g* for 30 min, the supernatants decanted, and the pellets resuspended in a minimum volume of buffer C (as for buffer B but without sodium chloride). Protein concentrations of the extracts were determined using a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Phylogenetic Alignments

The deduced amino acid sequences used to generate the phylogenetic tree in Figure 1 were aligned using the default parameters of DNASIS for WINDOWS, (gap penalty values 10 and all other parameters as the default) software version 2.5, based on the CLUSTAL IV algorithm (Hitachi Software, San Bruno, CA) as outlined in Higgins and Sharp (1998). The GenBank accession numbers are: Arabidopsis AtExp1, U30476; *AtExp2*, U30481; *AtExp5*, U30478; *AtExp6*, U30480; rape *BnExp1,* AJ000885; cotton *GhExp1*, AF043284; cucumber *CsExp1*, U30482; *CsExp2*, U30460; pea *PsExp1*, X85187; *Phleum* pollen allergen *Phlp1*, X78813; pine *PtExp2*, U64890; rice *OsExp1*, Y07782; *OsExp2*, U30477; *OsExp3*, U30479; *OsExp4*, U85246; tobacco *NtExp1*, AF049350; *NtExp2*, AF049351; *NtExp3*, AF049352; *NtExp4*, AF049353; *NtExp5*, AF049354; tomato *LeExp1*, U82123; *LeExp2*, AF096776; *LeExp3,* AF059487; *LeExp4*, AF059488; *LeExp5*, AF059489; *LeExp18*, AJ004997; and apricot *PaExp1*, U93167, *PaExp2*, AF038815.

Recombinant Protein Expression

The *Sph*I and *Hin*dIII restriction sites were introduced by PCR into the 5′ and 3′ ends, respectively, of the *LeExp1* coding sequence minus the N-terminal signal sequence. The 5′ primer (GTGTTTACGCATGCGGTTCATGG) corresponded to nucleotides 104 to 126 of *LeExp1* and the 3′ primer (TCCTAAGCTTAACAACACTCTGAAATATC) to nucleotides 857 to 885. PCR was carried out in 50 μL of final volumes using 1 unit of AmpliTaq (Perkin-Elmer, Norwalk, CT), 10 mM Tris (tris[hydroxymethyl]aminomethane)-HCl, pH 8.3, 50 mM KCl, 200 μM dNTPs, 3 mM $MgCl_2$, and 1 μM of the above primers with 0.5 μg of LeExp1 cDNA (described in Rose et al., 1997) for 35 cycles (94°C for 1 min, 44°C for 1.5 min, and 72°C for 1.5 min). The resulting 788-bp DNA fragment was gel-purified and cloned into the pQE30 QIAexpress vector (Qiagen, Santa Clarita, CA). DNA sequence was subsequently confirmed with universal and specific internal primers (Genset Corporation, La Jolla, CA), using an ABI 377 (Perkin-Elmer) using dye terminator chemistry with AmpliTaq DNA polymerase, FS (*Taq*; FS;Perkin-Elmer/Applied Biosystems Division, Foster City, CA).

Expression of recombinant LeExp1 using the QIAexpress system (Qiagen) followed the procedures outlined in the QIAexpressionist handbook, 2nd edition, 1992 (Qiagen). Briefly, the pQE30 vector harboring LeExp1 was transformed into *Escherichia coli* M15 cells, containing the pREP4 repression plasmid. The vector allowed the addition of a 6× His tag to the N terminus of the recombinant protein, promoting binding to nickel-nitrilotriacetic acid agarose resin, supplied with the QIAexpress kit (Qiagen). Cell cultures were grown according to the manufacturer's instructions in Luria-Bertani medium then induced with 2 mM isopropyl β-D-thiogalactoside for up to 5 h. Pelleted cells were lysed and following centrifugation, the supernatant applied to a column of nickel-nitrilotriacetic acid agarose resin under denaturing conditions, and eluted from the column in a descending pH gradient according to the protocol 7 of the QIAexpressionist handbook. Fractions from the column were analyzed by SDS-PAGE (12.5% [v/v] acrylamide) and Coomassie Blue staining.

Antibody Production and Immunoblot Analysis

Strips of SDS-polyacrylamide gel containing approximately 2.0 mg of recombinant LeExp1 protein were freeze-dried, powdered, and emulsified in 2 mL of Freund's incomplete adjuvant and 2 mL of phosphate-buffered saline (PBS). The antigen was injected into two rabbits, booster injections were given at 14, 21, 35, and 49 d, each containing approximately 300 μg of recombinant protein, and serum was collected at 75 d.

Protein extracts (10 μg per lane) were separated by SDS-PAGE on 4% to 20% polyacrylamide gels (Novex, San Diego). Gels were subsequently stained with Coomassie Blue R-250 or transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Life Science, Cleveland). Immunoblot analysis used the ECL western-blotting kit (Amersham Life Science). Membranes were blocked in 3% (w/v) bovine serum albumin and 0.02% (w/v) sodium azide in sterile PBS-Tween and incubated sequentially with LeExp1 antiserum (diluted 1:1,500 in PBS, 0.1% [v/v] Tween), or CsExp1 antiserum (described in Li et al., 1993) (diluted 1:1,000 in PBS, 0.1% [v/v] Tween), followed by a 1:20,000 dilution of the horesradish peroxidase-conjugated secondary antibody, and chemiluminescent reagents before exposure to Hyperfilm ECL (Amersham Life Science). These and intermediate washing steps were according to the manufacturer's instructions. The same protein extracts were used for immunoblot and activity assays.

Expansin Activity Assays

Fruit protein extracts (protein concentrations used in the assays normalized to correspond to starting fresh weight: tomato stage II, 5.5 $\mu g\ \mu L^{-1}$; mature green, 1.2 $\mu g\ \mu L^{-1}$; breaker, 0.7 $\mu g\ \mu L^{-1}$; red ripe, 0.8 $\mu g\ \mu L^{-1}$; pear, 2.5 $\mu g\ \mu L^{-1}$; pepper, 4.3 $\mu g\ \mu L^{-1}$; and avocado, 3.0 $\mu g\ \mu L^{-1}$) were diluted 1:1 with 50 mM sodium acetate, pH 4.5, and the pH adjusted to 4.5. Samples were assayed for expansin activity using heat-inactivated cucumber hypocotyl walls clamped in a constant-load extensometer, as described in Cosgrove (1989). The initial lengths of the segments were approximately 5 mm. After equilibrating the walls for 15

min in buffer, 100 μL of buffer was removed and replaced with 100 μL of the diluted protein extract.

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