Two Divergent Endo-β-1,4-glucanase Genes Exhibit Overlapping Expression in Ripening Fruit and Abscising Flowers

Coralie C. Lashbrook, Carmen Gonzalez-Bosch,[1] and Alan B. Bennett[2]

Mann Laboratory, Department of Vegetable Crops, University of California, Davis, California, 95616

Two structurally divergent endo-β-1,4-glucanase (EGase) cDNAs were cloned from tomato. Although both cDNAs (Cel1 and Cel2) encode potentially glycosylated, basic proteins of 51 to 53 kD and possess multiple amino acid domains conserved in both plant and microbial EGases, Cel1 and Cel2 exhibit only 50% amino acid identity at the overall sequence level. Amino acid sequence comparisons to other plant EGases indicate that tomato Cel1 is most similar to bean abscission zone EGase (68%), whereas Cel2 exhibits greatest sequence identity to avocado fruit EGase (57%). Sequence comparisons suggest the presence of at least two structurally divergent EGase families in plants. Unlike ripening avocado fruit and bean abscission zones in which a single EGase mRNA predominates, EGase expression in tomato reflects the overlapping accumulation of both Cel1 and Cel2 transcripts in ripening fruit and in plant organs undergoing cell separation. Cel1 mRNA contributes significantly to total EGase mRNA accumulation within plant organs undergoing cell separation (abscission zones and mature anthers), whereas Cel2 mRNA is most abundant in ripening fruit. The overlapping expression of divergent EGase genes within a single species may suggest that multiple activities are required for the cooperative disassembly of cell wall components during fruit ripening, floral abscission, and anther dehiscence.

INTRODUCTION

Cell wall disassembly is a significant component of many physiological and developmental processes, including vegetative growth, fruit ripening, and the abscission of plant organs. The dissolution of cell wall polysaccharides during these events requires the activity of multiple cell wall hydrolases, each potentially responding to a unique subset of developmental, hormonal, and environmental cues. The mobilization of cell wall hydrolases for degradative processes is regulated in part via changes in gene expression (Fischer and Bennett, 1991) and permits the modulation of architectural constraints required for transitions in plant growth and development. In some systems, cell wall metabolism has the capacity to generate biologically active pectic (Tong et al., 1986; Brecht and Huber, 1988; Campbell and Labavitch, 1991) and hemicellulosic (York et al., 1984; Farkas and Maclachlan, 1988; McDougall and Fry, 1988) cell wall fragments capable of further regulatory activity. Thus, cell wall disassembly has both a structural and informational role in plant development.

Tomato fruit ripening has proven to be an excellent model system for studying the contributions of individual hydrolases to plant cell wall modifications. During ripening, both pectic and hemicellulosic components of tomato cell walls are extensively modified (Gross and Wallner, 1979; Huber, 1983). The solubilization of pectins by the ripening-induced enzyme polygalacturonase (PG) has been most extensively studied. However, PG-mediated pectin degradation alone cannot account for the significant changes in tomato fruit texture that accompany ripening. In transgenic tomato plants, reduction of PG mRNA levels to 0.1% of wild-type levels significantly alters the degree of pectin polymerization observed in ripening fruit cell walls (Smith et al., 1990), which affects a variety of fruit quality parameters (Kramer et al., 1990; Schuch et al., 1991) but does not prevent fruit softening (Smith et al., 1990). Conversely, expression of a chimeric PG transgene in the tomato ripening mutant rin resulted in pectin solubilization and depolymerization at near wild-type levels but not fruit softening (Giovannoni et al., 1989; DellaPenna et al., 1990). The maintenance of normal rheological properties of ripe tomato fruit in the absence of PG-mediated pectin degradation indicates that textural changes associated with ripening are dependent upon cell wall dissolution processes catalyzed by other enzymes. Analysis of cell walls isolated from ripening tomato fruit has not detected the release of oligosaccharide fragments derived from crystalline cellulose (Gross and Wallner, 1979; Ahmed and Labavitch, 1980), whereas solubilization of cell wall components believed to be derived from the hemicellulosic matrix is evident (Huber, 1983). The involvement of endo-β-1,4-glucanases (EGases; β [1:4] 4-glucan hydrolase; EC

---

[1] Present address: Departamento de Bioquimica y Biologia Molecular, Facultad de Biologicas, Burjassot, Valencia 46100, Spain.
[2] To whom correspondence should be addressed.

3.2.1.4) in hemicellulose degradation during vegetative growth and fruit ripening has been implicated (Hayashi et al., 1984; Hatfield and Nevins, 1986). Although the term "cellulase" has been widely used to describe these endoglucanases, the term is misleading in view of the current lack of evidence for EGase-catalyzed cellulose degradation.

The presence of EGase activity in ripening tomato fruit (Hall, 1963, 1964; Dickinson and McCollum, 1964) and in abscising tomato flowers (Roberts et al., 1984; Tucker et al., 1984) has been established, and a role for EGases in cell wall dissolution processes within bean abscission zones (Tucker et al., 1988; Tucker and Milligan, 1991) and in avocado fruit (Tucker et al., 1987; Cass et al., 1990) is suggested by studies of EGase gene expression. Currently, the nature of the cell wall substrate(s) modified by these enzymes and thus their physiological function are unknown. To better assess the physiological function of EGases in a system amenable to molecular genetic manipulation, we have cloned EGase cDNAs from tomato and have determined that they belong to a gene family with at least four divergent members. Unlike ripening avocado fruit and bean abscission zones, in which a single EGase mRNA predominates, EGase expression in tomato reflects the overlapping expression of structurally divergent EGase gene family members. The structure and differential expression of two members of the tomato EGase gene family, EGase1 (Cel1) and EGase2 (Cel2), are described in this report.

RESULTS

Two Tomato Endoglucanases Are Structurally Divergent

cDNA clones encoding two tomato EGase genes were isolated using oligonucleotide probes corresponding to amino acid sequence domains conserved in bean and avocado EGases (Tucker et al., 1987; Tucker and Milligan, 1991). In Figure 1A, comparison of the deduced amino acid sequences of tomato Cel1 and Cel2 revealed several common features. Cel1 and Cel2 cDNAs have a hydrophobic N-terminal signal sequence characteristic of proteins targeted via the endomembrane pathway to locations including the cell surface and contain within their coding regions N-glycosylation consensus motifs (Asn-X-Ser/Thr). Cel1 and Cel2 cDNAs encode mature proteins ranging in predicted size from 51 to 53 kD, with basic predicted pIs of 7.3 and 8.2, respectively. Cel1 and Cel2 cDNAs also contain a number of short, highly conserved domains located in equivalent positions of all plant EGases sequenced thus far. Nevertheless, an overall sequence comparison of Cel1 and Cel2 reveals only 50% amino acid sequence identity. This is the lowest identity yet observed between two plant EGases and is consistent with our observation that Cel1 and Cel2 cDNAs do not cross-hybridize, even under conditions of relaxed stringency.
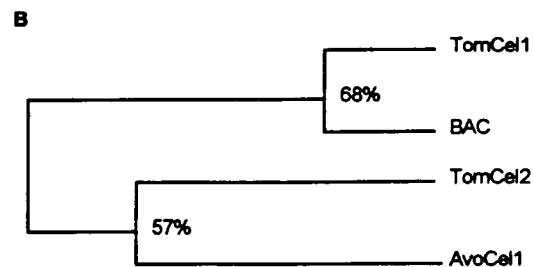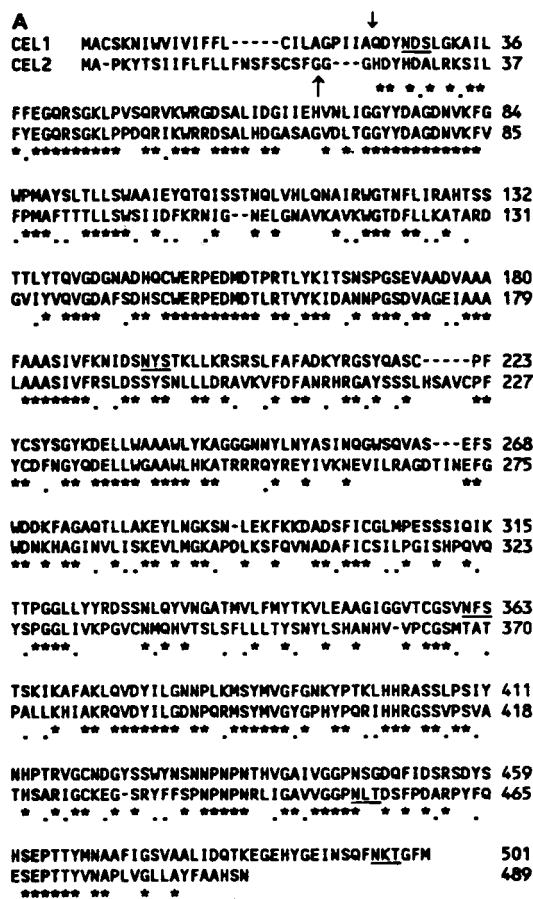
Figure 1. Structural and Evolutionary Relationships between Cel1 and Cel2.

(A) Comparison of the deduced amino acid sequences of tomato Cel1' and Cel2. The putative cleavage sites of N-terminal signal sequences are designated by arrows. N-glycosylation consensus motifs are underlined. Identical amino acids are designated by asterisks; similar amino acids are represented by dots. Dashes were introduced to optimize alignment. The nucleotide sequence of Cel1 has been assigned GenBank accession number U13054. The GenBank accession number for Cel2 is U13055.

(B) Possible evolutionary relationships between cloned plant EGases. The dendrogram is based on comparisons of deduced amino acid sequences corresponding to mature protein domains. Tom Cel1 and Cel2, tomato EGase1 and EGase2; BAC, bean abscission zone cellulase (Tucker and Milligan, 1991); AvoCel1, avocado fruit cellulase (Tucker and Milligan, 1991).

Figure 1B illustrates the sequence comparisons between tomato Cel1 and Cel2, bean abscission zone EGase, and avocado fruit EGase. This dendrogram suggests that the structural divergence of EGases into the two families shown occurred prior to the evolutionary divergence of major dicotyledonous families. The comparison also suggests that both avocado and bean could possess members of both families. Genomic DNA gel blot analysis of both species suggests that this is the case (Tucker and Milligan, 1991).

Genomic DNA gel blot analysis shown in Figure 2 indicates that Cel1 and Cel2 are transcribed from distinct genes of low copy number. At both moderate (20°C below the melting temperature; T_m −20°C) and high (T_m −5°C; data not shown) stringencies, Cel2 hybridizes to a small set of large genomic fragments, whereas Cel1 hybridizes to a different array of smaller fragments. Restriction fragment length polymorphism analysis has assigned *Cel1* to tomato chromosome 8 and *Cel2* to chromosome 9 (C.C. Lashbrook, K.B. Alpert, and J.W. deVerna; unpublished data). Neither *Cel1* nor *Cel2* maps to a previously defined locus.

Cel1 and Cel2 mRNAs Accumulate in Fruit in Response to Developmental and Hormonal Signals

RNase protection assays were used to assay the accumulation of mRNAs corresponding to Cel1 and Cel2 over the course
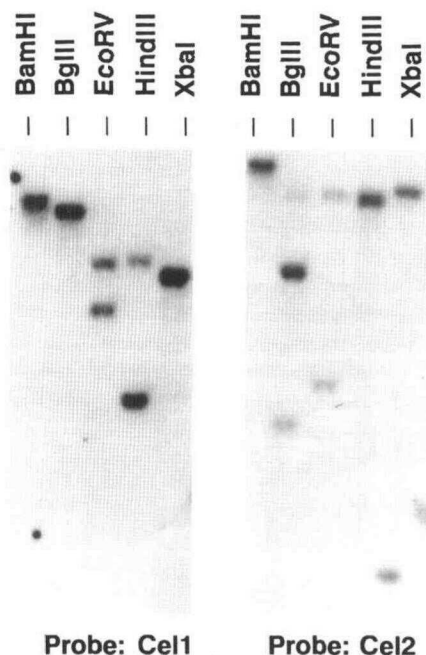
Figure 2. Genomic DNA Gel Blot Analysis of Cel1 and Cel2.

Ten micrograms of genomic DNA cut with the indicated restriction enzymes was hybridized at moderate stringency to random primed Cel1 or Cel2 cDNA probes. Single sites for HindIII and BglII are present within the cDNAs of Cel1 and Cel2, respectively.
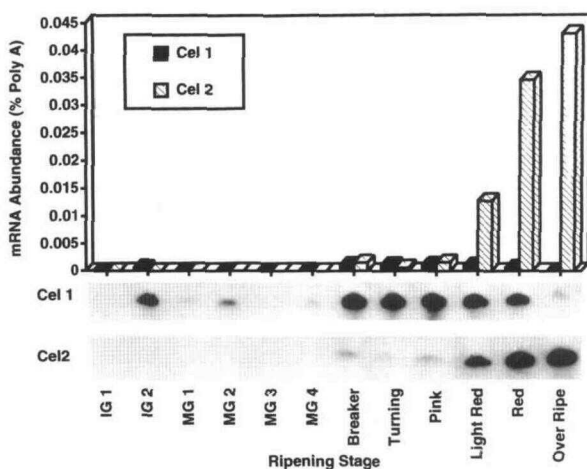
Figure 3. Cel1 and Cel2 mRNA Expression over the Course of Tomato Fruit Development and Ripening.

Specific transcript levels, expressed as the percentage of poly(A)+ mRNA present in tomato fruit, were quantified by densitometric analysis of autoradiographs of protected fragments from RNase protection assays relative to transcript-specific standard curves. A direct comparison of image intensities was not possible because of different exposure requirements for the Cel1 and Cel2 signals.

of tomato fruit development. Figure 3 indicates that Cel1 mRNA transiently accumulates in immature green (IG) fruit, suggesting a possible role for Cel1 in fruit cell expansion, the liquefaction of locules, or other fruit maturation events. It is likely that Cel1 expression contributes to the elevated EGase enzyme activity previously observed in early stages of tomato fruit development (Hall, 1964; Hobson, 1968; Babbitt et al., 1973). At the onset of ripening, Cel1 mRNA accumulation is evident again, reaching its maximum level at the pink stage of ripening in air-treated fruit and declining as the fruit becomes overripe. This pattern of mRNA accumulation during ripening is similar to that observed for PG (Speirs et al., 1989). However, the maximum level of Cel1 mRNA accumulated during ripening is up to 1000-fold less than that reported for PG (Sato et al., 1985; DellaPenna et al., 1987). Assuming that poly(A)+ mRNA constitutes ~3% of total cellular RNA, the maximum Cel1 mRNA abundance attained during ripening is 0.002% of the poly(A)+ population of tomato pericarp, while PG mRNA levels of up to 2% of the poly(A)+ RNA in the same tissue have been reported (DellaPenna et al., 1987). Figure 3 also illustrates that Cel2 mRNA, first detectable in breaker fruit, remains at low levels until late in ripening when significant levels of Cel2 mRNA accumulate in ripe fruit. Ultimately, Cel2 mRNA represents almost 0.05% of pericarp mRNA: levels ~20-fold greater than those of Cel1 mRNA.

Because fruit selected for RNA analysis was developmentally staged on the basis of ethylene evolution (see Methods), it was possible to evaluate the accumulation of Cel1 and Cel2 mRNA in relation to the induction of autocatalytic ethylene biosynthesis. Both EGase mRNAs are nondetectable in late stages of mature green (MG) fruit, when ethylene synthesis has

commenced (Figure 3), their presence subsequently becoming evident at the breaker stage of ripening. Thus, the timing of both Cel1 and Cel2 mRNA accumulation follows the initiation of ethylene synthesis. The relationship between Cel1 mRNA induction and ethylene synthesis is complicated by the transient expression of Cel1 mRNA during the late phases of fruit maturation, prior to ethylene production. The low levels of Cel1 mRNA detected in the MG1 and MG2 stages may represent low levels of transiently expressed Cel1 mRNA from the IG2 stage persisting into the earliest stages of fruit ripening. Thus, Cel1 appears to be developmentally as well as hormonally regulated.

To further assess the potential regulation of EGase expression by ethylene, ripening tomato fruit were treated with the ethylene action inhibitor 2,4-norbornadiene (NBD), a competitive inhibitor of the ethylene receptor (Sisler and Yang, 1984). Figure 4 shows that the accumulation of mRNAs from both Cel1 and Cel2 was severely inhibited in NBD-treated fruit tested at two ripening stages, suggesting that the accumulation of Cel1 and Cel2 mRNAs is regulated by ethylene. The inhibitory effect of 2000 μL L^{-1} NBD on Cel1 and Cel2 mRNA accumulation was reversed by treatment with 1000 μL L^{-1} ethylene (data not shown).

### Cel1 and Cel2 mRNAs Are Differentially Expressed in Fruit and Flowers

Both Cel1 and Cel2 mRNA transcripts are present in a wide variety of tomato plant tissues, although at quite different levels
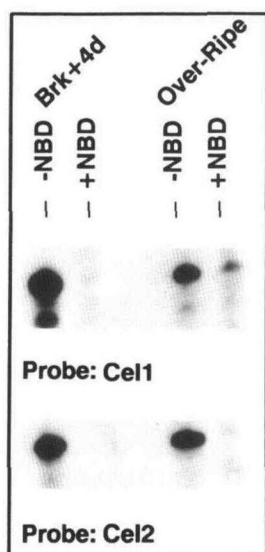
Figure 4. Effect of NBD on EGase Expression during Fruit Ripening.

MG fruit of equivalent internal ethylene content were treated with 2000 μL L^{-1} NBD (+) until nontreated controls (−) reached the ripening stages Brk + 4d (4 days after breaker stage) and overripe (4 days after full red stage). Total RNA isolated from fruit was subjected to RNase protection analysis.
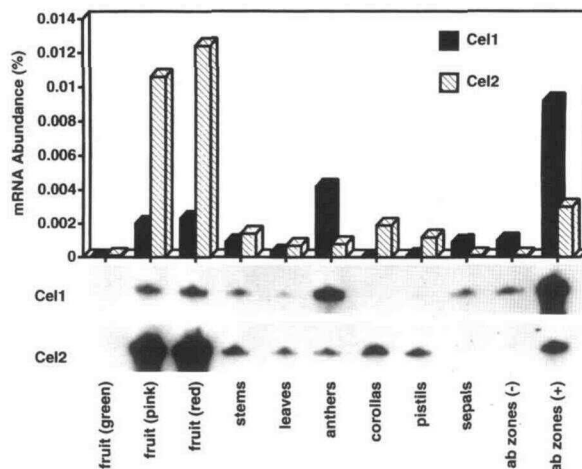
Figure 5. Tissue Distribution of EGase mRNAs.

Cel1 and Cel2 mRNA levels in various tomato tissues were quantified by RNase protection analysis. Fruit RNA was isolated from the pericarp at three stages of ripening. Vegetative tissues were harvested from young field-grown tomato plants. Floral tissues were isolated from field-grown tomato flowers on the day of anthesis. Abscission zones were isolated from floral explants that abscised (+ ab) or failed to abscise (− ab) after being held 4 days in water-filled tubes. mRNA abundance is expressed as the percentage of poly(A)$^+$ mRNA present in each tissue.

of abundance. As shown in Figure 5, ripening tomato fruit have a high level of total EGase mRNA, which is contributed mainly by Cel2. In contrast, abscission zones and anthers also have significant levels of total EGase mRNA, comprised mainly of Cel1 mRNA. Floral abscission and anther dehiscence are functionally similar events, involving the separation of cell layers, and it is possible that Cel1-catalyzed cell wall degradation is an integral part of both processes. Alternatively, Cel1 may participate in cell wall degradative processes associated with earlier stages of anther development. In sweet pea, two EGase isoforms purified from developing anthers appeared to be identical to isoforms purified from abscising flower buds (Sexton et al., 1990). Because sweet pea EGase accumulation preceded pollen grain maturation, a potential role for the enzyme in the breakdown of walls including those surrounding the tapetum was suggested.

Ethylene Accelerates EGase mRNA Accumulation and Flower Abscission

Cel1 and Cel2 mRNA levels were assayed over the course of floral abscission to determine the temporal relationship existing between the abscission process and the expression of specific EGases. Maximum abscission of tomato flowers held in the presence or absence of 10 μL L^{-1} ethylene occurred after 4 days in air or after 2 days in ethylene as shown in Figure

6. This is consistent with numerous observations in other species that ethylene accelerates the abscission process (reviewed by Sexton et al., 1985). Figure 6 indicates that Cel1 mRNA was completely absent at anthesis but accumulated within the flower abscission zones held in air as cell separation progressed. As complete abscission was approached, Cel1 mRNA levels declined. Cel2 mRNA, which was detectable at anthesis, accumulated further at the onset of abscission and, like that of Cel1, declined in abundance as complete abscission was approached. Ethylene accelerated the abscission of tomato flowers and accelerated the rate of accumulation of both Cel1 and Cel2 mRNAs to their maximum levels. Although ethylene treatment of flowers accelerated the timing of EGase mRNA accumulation, it did not increase the maximum level of Cel1 or Cel2 mRNA ultimately accumulated.

Table 1 provides additional evidence for the dependence of Cel1 and Cel2 EGase expression on ethylene in abscission zones. Tomato flower abscission was significantly reduced, and the accumulation of both Cel1 and Cel2 mRNA was virtually abolished in abscission zones of flowers treated with 2000 μL L^{-1} NBD. Both abscission and EGase mRNA abundance in the presence of NBD were subsequently restored to control levels by the addition of 15 μL L^{-1} ethylene, demonstrating that NBD effects were completely reversible by ethylene. Taken together, the data in Figure 6 and Table 1 indicate that both Cel1 and Cel2 mRNA levels are regulated by ethylene and are temporally correlated with the abscission process.
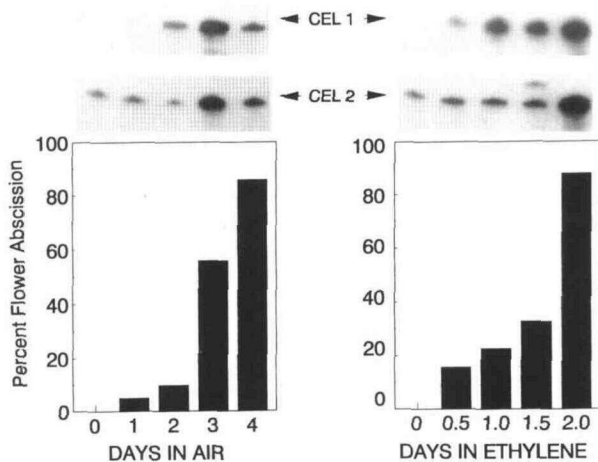
Figure 6. EGase mRNA Accumulation during Tomato Flower Abscission.

Floral explants harvested within a day of anthesis were held in water-filled tubes for 4 days in air or 2 days in ethylene. At designated times, the percentage of abscission of 100 flowers was determined by inflection of floral pedicels at their abscission zones. mRNA prepared from these populations, containing both abscised and nonabscised flowers, was subjected to RNase protection analysis. A direct comparison of signal intensities was not possible because of different exposure requirements for the Cel1 and Cel2 signals.

Table 1. Effect of NBD on EGase mRNA Accumulation in Tomato Flower Abscission Zones

Treatment	Abscission Rate (% Abscised)	EGase mRNA Abundance (% Control)	
		Cel1	Cel2
Air (control)	66	100	100
Air and NBD, 2000 μl L^{-1}	9	1	0
C$_2$H$_4$, 15 μL L^{-1}, and NBD, 2000 μL L^{-1}	71	107	100

Flowers were subjected to designated gas treatments in sealed jars for 4 days and scored for abscission by inflection of pedicels at their abscission zones. Specific EGase transcript levels in mRNA prepared from at least 100 abscission zones were quantitated by RNase protection analysis.

Tomato EGase mRNA Levels Are Induced by Cell Separation and Differentially Localized in Abscission Zones and Flanking Pedicel Tissue

In the abscission-related experiments described above, we examined abscission zone EGase mRNA levels isolated from populations of flowers at defined time points, with each population containing differing proportions of both abscised and nonabscised members. To determine the temporal relationships between tomato Cel1 and Cel2 mRNA accumulation and the actual separation of the flower abscission zone cell layers, specific EGase transcript levels within abscised and nonabscised abscission zones selected from a flower population exhibiting ~50% abscission were compared in Figure 7. In addition, to determine if tomato Cel1 and Cel2 mRNA accumulation extended beyond the region of cells actually undergoing separation, mRNA levels were quantitated in noncontiguous pedicel tissue flanking the abscission zone. Cel1 and Cel2 mRNAs were present at basal levels in nonabscised tissue but increased ~20- and 30-fold, respectively, in the abscission zones of abscised flowers. Cel2 mRNA accumulation was primarily limited to abscission zones, where it constituted up to 0.003% of the mRNA. Cel2 mRNA was also detectable in proximal pedicel tissue, although at levels ~10-fold less than in the abscission zone. In contrast, Cel1 mRNA in abscised abscission zones constituted over 0.01% of the mRNA present. Notably, comparably high levels of Cel1 mRNA were observed in abscised abscission zones and in the distal pedicel tissue that was not undergoing cell separation. The abscission of tomato flowers has been shown to be initiated in parenchymatous cells distal to cells that later form the separation layer in both ethylene-treated (Roberts et al., 1984) and non-ethylene–treated tomato flowers (Jensen and Valdovinos, 1967). In tomato flowers, significant EGase enzyme activity has been measured in extracts of abscission zones and distal tissues but not in preparations from proximal tissue (Tucker et al., 1984). The association of Cel1, but not Cel2, mRNA with
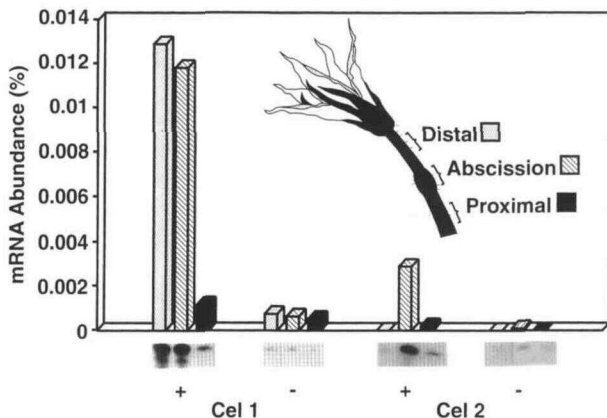
Figure 7. Expression of Cel1 and Cel2 in Abscised and Nonabscised Tomato Flowers.

Several hundred tomato flowers were held in water-filled tubes until ~50% had abscised as measured by inflection of each floral pedicel at its abscission zone. RNA was prepared from abscission zones and flanking pedicel tissue of the abscised (+) and nonabscised (−) members of the population, and EGase transcript levels were quantified using RNase protection assays.

distal tissue sections suggests that the enzyme activity previously reported may correspond to the Cel1 protein product.

DISCUSSION

We have described the primary structure and pattern of mRNA accumulation of Cel1 and Cel2, two members of a multigene EGase family in tomato. Both Cel1 and Cel2 mRNAs accumulate in ripening fruit and in flower abscission zones as well as at lower levels in other vegetative and floral organs. Although their pattern of mRNA accumulation is overlapping, Cel1 mRNA predominates in abscission zones and anthers, whereas Cel2 mRNA predominates in ripening fruit. Cel1 and Cel2 exhibit low sequence identity and fail to cross-hybridize at moderate to low stringency. The structural divergence of these two tomato EGases may reflect the evolution of functional differences. The presence of elevated levels of Cel1 mRNA in physiological contexts involving cell separation may be consistent with the observation of significant amino acid sequence identity (68%) between tomato Cel1 and the bean abscission zone EGase. Cel2, in contrast, is most abundant in ripening fruit and exhibits a greater amino acid sequence identity to avocado fruit EGase (57%).

The existence of multiple, divergent EGase genes in tomato invokes the question of whether similar gene families may exist in other plants. Analysis of the EGase genomic organization in avocado and bean suggests that EGase gene families in those species are quite small (Tucker et al., 1987; Tucker and Milligan, 1991). Gene cloning strategies based upon the detection of conserved plant EGase domains have not been used in bean and avocado; thus, it is not known whether other divergent EGase mRNAs also accumulate in these species, although genomic DNA gel blot analysis is consistent with the presence of two divergent gene families in avocado (Tucker and Milligan, 1991). Given the failure of tomato Cel1 and Cel2 to cross-hybridize with each other, genomic DNA gel blot analysis using either probe might greatly underestimate the complexity of the tomato EGase gene family. On the basis of substrate specificity and chromatographic separation behavior, five distinct tomato EGase activities have been described in ripening tomato fruit pericarp and locules (Maclachlan and Brady, 1992). At least four divergent EGase genes are present in tomato (reviewed in Brummell et al., 1994), with Cel1 and Cel2 representing major genes expressed in abscission zones and fruit, respectively.

The high sensitivity of the RNase protection assay used in this study allowed the detection of both Cel1 and Cel2 mRNAs, both present at levels below the limit of detection by RNA gel blot hybridization. It is notable that in tomato the combined amount of Cel1 and Cel2 mRNAs rarely exceeds 0.05% of the mRNA population. In contrast, both ripening avocado fruit and bean abscission zones exhibit much higher levels of EGase mRNA (Christoffersen et al., 1984; Tucker et al., 1988). Tomato EGase enzyme activity is also considerably lower than that observed in avocado fruit (Lewis et al., 1974), which is consistent with the low mRNA levels that we detected.

The precise physiological role of EGases in plant development is not known, although their involvement in cell wall disassembly occurring during fruit ripening and abscission has been frequently proposed. The detection of Cel1 and Cel2 in a variety of tissues throughout the tomato plant suggests that EGases may be associated with additional developmental processes that involve rearrangements in cell wall architecture. The overlapping accumulation of Cel1 and Cel2 mRNA in ripening fruit and abscission zones suggests the possibility that the protein product of each mRNA has distinct biochemical functions required to cooperatively bring about required changes in cell wall architecture. Alternatively, each EGase may have identical properties but differ primarily in spatial and temporal expression, sharing some overlap perhaps as a built-in redundancy of function. The latter interpretation is not supported by the differential EGase substrate specificities that have been observed in distinct tomato fruit EGase activities separated chromatographically (Maclachlan and Brady, 1992) or by the significant differences in primary structure between Cel1 and Cel2.

On the basis of primary sequence comparisons, tomato Cel1 and Cel2 can be assigned to the E family of cellulases (Henrissat et al., 1989; Beguin, 1990), a group of bacterial and plant EGases whose structural similarities are believed to underlie commonalities in catalytic function. It has been suggested that plant and microbial members of this EGase family

evolved from a common ancestor (Navarro et al., 1991), yet important distinctions have evolved between prokaryotic and eukaryotic cellulases of the E family. Perhaps most significant is that plant EGase sequences identified to date lack the cellulose binding domains and cellulosome anchoring motifs of their microbial counterparts. Thus, there is at present no structural evidence that plant endoglucanases are capable of participating in degradation of crystalline cellulose. It has been difficult to clearly define the biochemical function of purified plant EGases, possibly because of the unrecognized existence of multiple EGases in each tissue. There is evidence that EGases from different sources act on different hemicellulosic substrates (Hayashi et al., 1984; Hatfield and Nevins, 1986; Durbin and Lewis, 1988). Having several EGase genes cloned from a single source now provides a unique opportunity to assess their specific biochemical function by expression of single genes in heterologous systems. In addition, experiments to assess the physiological function of each tomato EGase in transgenic plants with altered expression of each gene are underway.

METHODS

Tomato Endo-β-1,4-glucanase Cloning

Degenerate oligonucleotide hybridization probes corresponding to two conserved domains of avocado and bean endo-β-1,4-glucanases (EGases), GGYYDAGDN and CWERPEDMD (Tucker et al., 1987; Tucker and Milligan, 1991), were synthesized with the sequences 5'-TTA-(G)TCICCIGCA(G)TCA(G)TAA(G)TAICCICC-3' and 5'-TCCATA(G)T-CT(C)TCIGGICGT(C)TCCCAA(G)CA-3', respectively, where I is deoxyinosine. Oligonucleotides were ^{32}P-radiolabeled at their 5' termini with T4 polynucleotide kinase (Bethesda Research Laboratories) to a specific activity exceeding 5×10^8 dpm μg^{-1} and were used to screen a ripe tomato fruit cDNA library in the plasmid vector pArc (DellaPenna et al., 1986) using screening methods described in the Bethesda Research Laboratories newsletter Focus (1984; volume 6, pages 1 to 3) and elsewhere (Hanahan and Meselson, 1980). Hybridizing colonies were purified, and the cloned inserts partially sequenced. Two of the cDNAs with significant sequence identity to EGases of bean and avocado were designated tomato Cel1 and Cel2. The Cel1 clone isolated from the primary library screen contained a complete coding sequence. The initial clone of Cel2 was only partial length and was used to rescreen the library, yielding a full-length clone. Full-length Cel1 and Cel2 cloned inserts released from the pArc vector by Smal digestion were subcloned into pBluescript (SK+ and KS+, respectively) vectors for use in subsequent experiments.

Each strand of Cel1 and Cel2 was sequenced by the dideoxy chain termination method (Sanger et al., 1977) using sequence-specific primers complementary to domains of the cDNA or using vector sequence primers for sequencing into cDNA deletions generated by exonuclease III. Sequence data was recorded using the Microgenie software program (Beckman Instruments, Palo Alto, CA) and analyzed with the PC Gene system (IntelliGenetics, Inc., Mountain View, CA). Oligonucleotides were synthesized by Operon Technologies (Alameda, CA) or the University of California–Davis Protein Structure Laboratory.

Plant Materials and Tissue Preparation

Fruit and flowers were harvested from field-grown tomatoes (*Lycopersicon esculentum* cv Castlemart). Fruit were harvested at the mature green (MG) stage and allowed to ripen in humidified 20-L bottles subjected to continuous flow (20 L hr^{-1}) of air or 10 μL L^{-1} ethylene at 20°C. Air tanks contained open vials of mercuric perchlorate (250 mM in perchloric acid) to absorb ethylene. Fruit were harvested at various stages of ripening and scored by color using U.S. Department of Agriculture standards (U.S. Department of Agriculture, 1975) as well as by ethylene production rate using a gas chromatograph fitted with a flame ionization detector. Fruit selected for expression analysis exhibited the following ethylene production rates: 0.02 to 0.1 (mature green 1; MG1), 0.2 to 0.3 (MG2), 0.4 to 0.5 (MG3), 0.6 to 0.7 (MG4), 1 to 3 (breaker), 3 to 5 (turning), 5 to 8 (pink), 6 to 9 (light red), and 2 to 6 nL per g fresh weight of tissue per hr (red). MG fruit within these defined ethylene evolution ranges also exhibited internal anatomical features corresponding to stages MG1 to MG4 (Su et al., 1984). Immature green 1 (IG1) fruit was defined as fruit with an average fresh weight 25% that of MG; IG2 fruit weight was ∼50% that of MG. Internal anatomy of IG1 and IG2 fruit was surveyed to confirm immaturity of seed and locules. Overripe fruit was defined as fruit held 4 days past the attainment of full red color. In all fruit samples, seeds and locular material were removed, and pericarp tissue was frozen in liquid nitrogen prior to storage at −80°C for subsequent analysis.

Floral explants harvested within a day of anthesis were bright yellow with no evidence of flower or pedicel senescence. Floral explants consisting of a stem bearing one to three flowers were trimmed of leaves and buds and then placed in water-filled tubes and treated in air or ethylene as described above. For time course experiments, abscission zones (2 to 2.5 mm) and equivalently sized distal and proximal tissue sections located 2 to 2.5 mm away from the abscission zone were harvested and frozen in liquid nitrogen and then stored at −80°C. Abscission was scored on an absolute basis following the inflection of each floral pedicel at its abscission zone.

For experiments with 2,4-norbornadiene (NBD; bicycloheptadiene; Aldrich Chemicals), fruits or flowers were placed in sealed 20-L bottles fitted with septum-covered ports into which appropriate amounts of liquid NBD and/or 100% ethylene were introduced to yield specified final concentrations. Solid NaOH served as a CO$_2$ scavenger.

RNA Isolation

Total RNA was isolated from floral or vegetative tissues or samples of at least 100 abscission zones or noncontiguous flanking tissue sections as previously described (Chomczynski and Sacchi, 1987) with two modifications. Water-hydrated polyvinylpolypyrrolidone was present in the initial grinding buffer at 0.06 g of thick slurry per mL and was subsequently removed by centrifugation prior to phenol extraction of the aqueous medium. In addition, LiCl precipitation (2 M LiCl final concentration) was added at the end of the procedure to remove carbohydrates and other A_{230}-absorbing contaminants. RNA purity was assessed by ratios of A_{260} to A_{280}, which routinely exceeded 1.8. Ratios of A_{260} to A_{230} routinely exceeded 2.0.

Total RNA from fruit was isolated from 5 to 10 g fresh weight of frozen pericarp. Tissue ground in an electric coffee grinder containing dry ice chips was added to a solution containing one part ultrapure phenol saturated with 1 M Tris, pH 9, one part chloroform-isoamyl alcohol (24/1 [v/v]), and two parts 1 M Tris, pH 9, 5% mercaptoethanol (v/v) at a ratio of 0.25 g fresh weight of tissue per mL, homogenized

for 1 min at high speed with a Tissuemizer (Tekmar Inc., Cincinnati, OH), and centrifuged for 10 min at 1000g in a benchtop centrifuge or at 12,000g in a Sorvall centrifuge (Du Pont Co., Wilmington, DE); the aqueous phase was retained. Following back extraction of the organic phase, nucleic acids in the pooled aqueous phases were precipitated with sodium acetate and ethanol. After resuspension in 0.1 M Tris, 0.1% SDS, pH 8, the crude RNA was extracted with phenol–chloroform (saturated in 0.1 M Tris, pH 8) and then chloroform; extraction was followed by sequential precipitation with 2 M LiCl and NH₄Ac–EtOH; resuspension was in 300 µL of RNase-free water. One-half volume of absolute ethanol (150 µL) was added, and the suspension was incubated 30 min on ice before microcentrifugation at full speed for 10 min. This yielded a pellet of polysaccharide contaminants, which was discarded. The ethanolic supernatant, containing total RNA, was precipitated when 5 M NH₄Ac (140 µL) and additional absolute ethanol (900 µL) were added. Following resuspension of the pellet in 300 µL of H₂O, the polysaccharide removal procedure was repeated. Total RNA was resuspended in 300 µL of diethyl pyrocarbonate–treated water and was quantified spectroscopically. Ratios of A_{260} to A_{280} routinely exceeded 1.8, and ratios of A_{260} to A_{230} typically exceeded 2.0.

Genomic DNA Gel Blot Analysis

Genomic DNA isolated as previously described (Murray and Thompson, 1980; modified by Bernatzky and Tanksley, 1986) was digested with the indicated restriction enzymes, electrophoresced on 0.8% agarose gels, and capillary blotted (Southern, 1975) to HyBond-N membranes (Amersham). Probes were radiolabeled with ³²P-dATP by random priming. Hybridization at 20°C below the melting temperature for Cel1 (T_m −20°C for Cel1) was for 48 hr at 30°C in 50% formamide, 6 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.2% SDS, and 0.05 M sodium phosphate containing 100 µg mL⁻¹ base-denatured salmon sperm DNA. Blots were sequentially washed at T_m −20°C for Cel1 (2 × SSPE, 0.1% SDS, 0.01% sodium pyrophosphate, 54°C) and T_m −5°C for Cel1 (1 × SSPE, 0.1% SDS, 0.01% sodium pyrophosphate, 64°C). Hybridization was detected by autoradiography on preflashed film.

mRNA Quantitation

Cel1 and Cel2 mRNA transcript levels in 5 to 20 µg of total RNA were quantified by RNase protection assays (RPA II kit; Ambion, Austin, TX) with two modifications: RNase digestion was performed at 30°C instead of 37°C and gels of protected fragments were fixed for 30 min in 15% ethanol, 10% acetic acid and dried on a gel dryer (Bio-Rad) prior to autoradiography on preflashed film. Signals within the linear range of a standard curve were quantified by densitometry using the BioImage program (Millipore, Ann Arbor, MI) attached to a Sun Sparc Workstation (Sun Microsystems Inc., Mountain View, CA).

Cel1 and Cel2 sense strand transcripts used to generate standard curves for RNase protection assays were synthesized in vitro via the incorporation of ³H-UTP using T3 or T7 RNA polymerase. Shorter ³²P-labeled Cel1 and Cel2 antisense probes were transcribed from subclones in pBluescript (SK− and KS+, respectively) vectors.

ACKNOWLEDGMENTS

REFERENCES

Ahmed, A.E., and Labavitch, J.M. (1980). Cell wall metabolism in ripening fruit. I. Cell wall changes in ripening "Bartlett" pears. Plant Physiol. 65, 1009–1013.

Babbitt, J.K., Powers, M.J., and Patterson, M.E. (1973). Effects of growth-regulators on cellulase, polygalacturonase, respiration, color and texture of ripening tomatoes. J. Am. Soc. Hort. Sci. 98, 77–81.

Beguin, P. (1990). Molecular biology of cellulose degradation. Annu. Rev. Microbiol. 44, 219–248.

Bernatzky, R., and Tanksley, S.D. (1986). Genetics of actin-related sequences in tomato. Theor. Appl. Genet. 72, 314–321.

Brecht, J.K., and Huber, D.J. (1988). Products released from enzymically active cell wall stimulate ethylene production and ripening in preclimacteric tomato (Lycopersicon esculentum Mill.) fruit. Plant Physiol. 88, 1037–1041.

Brummell, D.A., Lashbrook, C.C., and Bennett, A.B. (1994). Plant endo-1,4-β-D-glucanases: Structure, properties and physiological function. Am. Chem. Soc. Symp. Ser., in press.

Campbell, A.D., and Labavitch, J.M. (1991). Induction and regulation of ethylene biosynthesis and ripening by pectic oligomers in tomato pericarp discs. Plant Physiol. 97, 706–713.

Cass, L.G., Kirven, K.A., and Christoffersen, R.E. (1990). Isolation and characterization of a cellulase gene family member expressed during avocado fruit ripening. Mol. Gen. Genet. 223, 76–86.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Christoffersen, R.E., Tucker, M.L., and Laties, G.G. (1984). Cellulase gene expression in ripening avocado fruit: The accumulation of cellulase mRNA and protein as demonstrated by cDNA hybridization and immunodetection. Plant Mol. Biol. 3, 385–391.

DellaPenna, D., Alexander, D.C., and Bennett, A.B. (1986). Molecular cloning of tomato fruit polygalacturonase: Analysis of polygalacturonase mRNA levels during ripening. Proc. Natl. Acad. Sci. USA 83, 6420–6424.

DellaPenna, D., Kates, D.S., and Bennett, A.B. (1987). Polygalacturonase gene expression on Rutgers, rin, nor, and Nr tomato fruits. Plant Physiol. 85, 502–507.

DellaPenna, D., Lashbrook, C.C., Toenjes, K., Giovannoni, J.J., Fischer, R.L., and Bennett, A.B. (1990). Polygalacturonase isozymes and pectin depolymerization in transgenic rin tomato fruit. Plant Physiol. 94, 1881–1886.

Dickinson, D.B., and McCollum, D.P. (1964). Cellulase in tomato fruits. Nature 203, 525.

Durbin, M.L., and Lewis, L.N. (1988). Cellulases in Phaseolus vulgaris. Methods Enzymol. **160**, 342–351.

Farkas, V., and Maclachlan, G. (1988). Stimulation of pea 1,4-β-glucanase activity by oligosaccharides derived from xyloglucan. Carbohydr. Res. **184**, 213–219.

Fischer, R.L., and Bennett, A.B. (1991). Role of cell wall hydrolases in fruit ripening. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42**, 675–703.

Giovannoni, J.J., DellaPenna, D., Bennett, A.B., and Fischer, R.L. (1989). Expression of a chimeric polygalacturonase gene in transgenic rin (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. Plant Cell **1**, 53–63.

Gross, K.C., and Wallner, S.J. (1979). Degradation of cell wall polysaccharides during tomato fruit ripening. Plant Physiol. **63**, 117–120.

Hall, C.B. (1963). Cellulase in tomato fruits. Nature **200**, 1010–1011.

Hall, C.B. (1964). Cellulase activity in tomato fruits according to portion and maturity. Bot. Gaz. **125**, 156–157.

Hanahan, D., and Meselson, M. (1980). Plasmid screening at high colony density. Gene **10**, 63–67.

Hatfield, R., and Nevins, D.J. (1986). Characterization of the hydrolytic activity of avocado cellulase. Plant Cell Physiol. **27**, 541–552.

Hayashi, T., Wong, Y.-S., and Maclachlan, G. (1984). Pea xyloglucan and cellulose. II. Hydrolysis by pea endo-β-1,4-glucanases. Plant Physiol. **75**, 605–610.

Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L., and Mornon, J.-P. (1989). Cellulase families revealed by hydrophobic cluster analysis. Gene **81**, 83–95.

Hobson, G.E. (1968). Cellulase activity during the maturation and ripening of tomato fruit. J. Food Sci. **33**, 588–592.

Huber, D.J. (1983). Polyuronide degradation and hemicellulose modifications in ripening tomato fruit. J. Am. Soc. Hort. Sci. **108**, 405–409.

Jensen, T.E., and Valdovinos, J.G. (1967). Fine structure of abscission zones. I. Abscission zones of the pedicels of tomato flowers at anthesis. Planta **77**, 298–318.

Kramer, M., Sanders, R.A., Sheehy, R.E., Melis, M., Kuehn, M., and Hiatt, W.R. (1990). Field evaluation of tomatoes with reduced polygalacturonase by antisense RNA. In Horticultural Biotechnology, A.B. Bennett and S.D. O'Neill, eds (New York: Alan R. Liss), pp. 347–355.

Lewis, L.N., Linkins, A.E., O'Sullivan, S., and Reid, P.D. (1974). Two forms of cellulase in bean plants. In Proceedings of the 8th International Conference of Plant Growth Substances (Tokyo: Hirokawa Publishing Co.), pp. 708–718.

Maclachlan, G., and Brady, C.J. (1992). Multiple forms of 1,4-β-glucanase in ripening tomato fruits include a xyloglucanase activatable by xyloglucan oligosaccharides. Aust. J. Plant Physiol. **19**, 137–146.

McDougall, G.J., and Fry, S.C. (1988). Inhibition of auxin-stimulated growth of pea stem segments by a specific nonasaccharide of xyloglucan. Planta **175**, 412–416.

Murray, M., and Thompson, W.F. (1980). Rapid isolation of high molecular weight DNA. Nucl. Acids Res. **8**, 4321–4325.

Navarro, A., Chebrou, M.-C., Beguin, P., and Aubert, J.-P. (1991). Nucleotide sequence of the cellulase gene celf of Clostridium thermocellum. Res. Microbiol. **142**, 927–936.

Roberts, J.A., Schindler, C.B., and Tucker, G.A. (1984). Ethylene-promoted tomato flower abscission and the possible involvement of an inhibitor. Planta **160**, 159–163.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463–5467.

Sato, T., Kusaba, S., Nakagawa, H., and Ogura, N. (1985). Polygalacturonase mRNA of tomato: Size and content in ripe fruits. Plant Cell Physiol. **26**, 211–214.

Schuch, W., Kanczler, J., Hobson, G., Tucker, G., Grierson, D., Bright, S., and Bird, C. (1991). Fruit quality characteristics of transgenic tomato fruit with altered polygalacturonase activity. HortScience **26**, 1517–1520.

Sexton, R., Lewis, L.N., Trewavas, A.J., and Kelly, P. (1985). Ethylene and abscission. In Ethylene and Plant Development, J.A. Roberts and G. Tucker, eds (London: Butterworths), pp. 173–196.

Sexton, R., Del Campillo, E., Duncan, D., and Lewis, L.N. (1990). The purification of an anther cellulase (β(1:4)4-glucan hydrolase) from Lathyrus odoratus L. and its relationship to the similar enzyme found in abscission zones. Plant Sci. **67**, 169–176.

Sisler, E.C., and Yang, S.F. (1984). Anti-ethylene effects of cis-2-butene and cyclic olefins. Phytochemistry **23**, 2765–2768.

Smith, C.J.S., Watson, C.F., Morris, P.C., Bird, C.R., Seymour, G.B., Gray, J.E., Arnold, C., Tucker, G.A., Schuch, W., Harding, S., and Grierson, D. (1990). Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Mol. Biol. **14**, 369–379.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**, 503–517.

Speirs, J., Lee, E., Brady, C.J., Robertson, J., and McGlasson, W.B. (1989). Endopolygalacturonase: Messenger RNA, enzyme and softening in the ripening fruit of a range of tomato genotypes. J. Plant Physiol. **135**, 576–582.

Su, L.Y., McKeon, T., Grierson, D., Cantwell, M., and Yang, S.F. (1984). Development of 1-aminocyclopropane-1-carboxylic acid synthase and polygalacturonase activities during the maturation and ripening of tomato fruit. HortScience **19**, 576–578.

Tong, C.B.S., Labavitch, J.M., and Yang, S.F. (1986). The induction of ethylene production from pear cell culture by cell wall fragments. Plant Physiol. **81**, 929–930.

Tucker, G.A., Schindler, C.B., and Roberts, J.A. (1984). Flower abscission in mutant tomato plants. Planta **160**, 164–167.

Tucker, M.L., and Milligan, S.B. (1991). Sequence analysis and comparison of avocado fruit and bean abscission cellulases. Plant Physiol. **95**, 928–933.

Tucker, M.L., Durbin, M.L., Clegg, M.T., and Lewis, L.N. (1987). Avocado cellulase: Nucleotide sequence of a putative full-length cDNA clone and evidence for a small gene family. Plant Mol. Biol. **9**, 197–203.

Tucker, M.L., Sexton, R., del Campillo, E., and Lewis, L.N. (1988). Bean abscission cellulase: Characterization of a cDNA clone and regulation of gene expression by ethylene and auxin. Plant Physiol. **88**, 1257–1262.

U.S. Department of Agriculture (1975). Color classification requirements in United States standards for grades of fresh tomatoes. U.S. Department of Agriculture visual aid (poster) TM-L-1.

York, W.S., Darvill, A.G., and Albersheim, P. (1984). Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. Plant Physiol. **75**, 295–297.