Plant Physiol. (1997) 113: 1303–1308

Three Different Polygalacturonases Are Expressed in Tomato Leaf and Flower Abscission, Each with a Different Temporal Expression Pattern[1]

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Abscission, or organ separation, is accompanied by a marked increase in hydrolases, which are responsible for the degradation of the middle lamella and the loosening of the primary cell wall surrounding cells in the separation layer. We recently reported on the cloning of a tomato (*Lycopersicon esculentum*) polygalacturonase (PG) cDNA, TAPG1, expressed during leaf and flower abscission. In addition to TAPG1, we have cloned two more PG cDNAs (TAPG2 and TAPG4) that are also expressed during leaf and flower abscission. The peptide sequences for the three abscission PGs are relatively similar (76–93% identity) yet different from the those of tomato fruit PG (38–41% identity). None of the three abscission PG mRNAs are expressed in fruit, stems, petioles, or anthers of fully open flowers. An RNase protection assay revealed that all three PGs are expressed in leaf and flower abscission zones and in pistils of fully open flowers. *TAPG4* mRNA is detected much earlier than *TAPG1* and *TAPG2* mRNA during both leaf and flower abscission.

Abscission is the mechanism used by plants to shed organs. Abscission allows the plant to shed flowers, fruit, or leaves in response to developmental cues (e.g. infertile flowers or ripe fruit) or to adapt to various environmental stresses, including frost, drought, and limited sunlight, or as part of a defense response when attacked by bacteria, fungi, or herbivores (Sexton, 1995). Moreover, abscission is not limited to leaves, flowers, and fruit. Organs that can be abscised include vegetative buds, branches, roots, trichomes, sepals, petals, anthers, ovaries, etc. (Addicott, 1982). Abscission is a complex process that varies between species and the organs that are to be shed (Addicott, 1982). For example, the abscission of petals or flowers is very rapid in comparison with the abscission of leaves or branches (Sexton and Roberts, 1982). Nevertheless, a common feature in many, if not most, abscission events is the synthesis of hydrolases, which are responsible for the degradation of the middle lamella and the loosening of the primary cell wall of separation layer cells (Sexton, 1995).

Lashbrook et al. (1994) showed that mRNA for two different cellulases were expressed during tomato (*Lycopersicon esculentum*) flower abscission, and recently del Campillo and Bennett (1996) cloned a third cellulase mRNA that is also expressed in tomato flower abscission. The importance of multiple cellulases in abscission is not well understood at this time. Each may have a unique substrate specificity or cell-specific expression within the abscission zone.

In addition to cellulase, PG activity has been shown to increase in the abscission zones of tomato (Tucker et al., 1984; Roberts et al., 1989), peach (Bonghi et al., 1992), and *Sambucus nigra* (Taylor et al., 1993). Recently, we cloned a tomato PG mRNA (*TAPG1*), in which the expression correlates with the abscission of leaves and flowers (Kalaitzis et al., 1995). The transcript for the tomato abscission *TAPG1* was not detected in stems or fruit (Kalaitzis et al., 1995). This is in contrast to the tomato cellulase mRNAs discussed by Lashbrook et al. (1994), which were not only detected in flower abscission zones but also at varying levels in fruit and stems. It appears that *TAPG1* expression is more specific to tomato abscission than the current number of cellulase genes, in which the transcripts have been cloned and expression patterns determined for abscission zones, fruit, and stems.

Southern-blot analysis showed that *TAPG1* is a member of a small subfamily of genes in tomato, suggesting that additional gene products may contribute to the PG activity measured in tomato abscission zones (Tucker et al., 1984; Roberts et al., 1989). We report here the cloning of two more putative PG mRNAs expressed in tomato leaf and flower abscission. The temporal expression patterns in leaf and flower abscission were determined for each of these abscission PG mRNAs, as well as the *TAPG1* mRNA cloned earlier (Kalaitzis et al., 1995). In addition, we examined their expression in both the anthers and pistils of fully open tomato flowers.

[1] This work was supported by grant 92-37301-7680 from the U.S. Department of Agriculture.

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Abbreviations: PFPG, peach fruit PG; PG, polygalacturonase; RACE, rapid amplification of cDNA ends; TAPG, tomato abscission PG; TFPG, tomato fruit PG.

MATERIALS AND METHODS

Plant Material Preparation

Tomato (*Lycopersicon esculentum* cv Rutgers) plants were grown in the greenhouses at the U.S. Department of Agriculture (Beltsville, MD) under standard conditions. Leaf abscission zones were prepared from the explants of 3-month-old tomato plants. Each explant included 15 to 20 cm of stem tissue with three to four petioles with the leaf blades excised. For flower abscission zones, explants were prepared from 5-month-old tomato plants. Flowering explants were cut to 10 to 15 cm, with one to three inflorescences per explant and several flowers in each. Special care was taken to remove any flowers that showed signs of senescence. This was done to eliminate flowers that may have already begun an abscission program. All explants were surface-sterilized in 10% commercial bleach (final concentration, 0.5% sodium hypochlorite) for 3 min, rinsed with several volumes of water, and placed upright in beakers of water. The beakers with the explants were then placed in a 33-L darkened chamber and exposed to 25 μL/L ethylene in air at a flow rate of 2 L/min.

Leaf abscission zones at the base of the petiole were collected by cutting 2 mm at each side of the abscission fracture. Flower abscission zones included 1.5 mm of tissue at each side of the abscission fracture. Pedicel tissue sections (4 mm) were harvested from the proximal and distal parts of the pedicel 1 to 2 mm away from the abscission zone. Anthers and pistils (stigmas and styles only) were collected from nonsenescent, fully open flowers. Harvested tissues were immediately frozen in liquid nitrogen and stored at −70°C.

RNA Extraction and cDNA Library Construction and Screening

Frozen tissue (1–2 g) from a −70°C freezer was ground into a fine powder in liquid nitrogen with a mortar and pestle, and polysomal RNA extracted as previously described by Jackson and Larkins (1976). RNA extracted from leaf abscission zones from tomato explants that were exposed to ethylene for 72 h was used to prepare a cDNA library in λgt10, as described by Kalaitzis et al. (1995). The TAPG1 cDNA insert (Kalaitzis et al., 1995) was used to screen approximately 100,000 plaque-forming units from the unamplified cDNA library. Hybridization was performed at moderately low-stringency conditions at 42°C in 5× SSPE, 5× Denhardt's solution, 0.1% SDS, 20% formamide, and 150 μg/mL denatured salmon sperm DNA (Sambrook et al., 1982). Nitrocellulose filters were washed at 45°C in 0.2× SSPE, 0.1% SDS, and 0.1% pyrophosphate (Sambrook et al., 1982).

5′-RACE and Sequencing

5′-RACE was performed essentially as described by Frohman et al. (1988). The sequences for the cDNA clones identified from the cDNA library described above were aligned to highlight the regions in the sequences having a limited identity with each other. Sequences from these regions were used to prepare the gene-specific primers required for the 5′-RACE reactions to avoid annealing the primers to the other PG transcripts. Polysomal RNA extracted from flower abscission zones treated with ethylene for 24 h was used as a template for the 5′-RACE reaction to obtain the 5′ end of the *TAPG2* transcript. Preliminary results indicated that *TAPG4* mRNA accumulates earlier in flower abscission than *TAPG1* and *TAPG2* mRNA. Therefore, polysomal RNA was extracted from the flower abscission zones that were treated with ethylene for only 6 h and used as a template for the 5′-RACE reaction to obtain the 5′ end of the *TAPG4* mRNA.

The sequences of the cDNA clones were determined using the subclones of the overlapping endonuclease restriction fragments and synthesized oligonucleotide primers according to standard procedures (Sambrook et al., 1982). The sequences for the 5′-RACE products were determined using oligonucleotide primers only. Sequences were analyzed with the University of Wisconsin Genetics Computer Group program (Devereux et al., 1984).

RNase Protection

The cDNA sequences that were used to prepare RNA-specific probes for each of the three PG clones were based on regions of low sequence identity determined by optimal alignment of the nucleotide sequences for the three PG cDNAs. These low-identity sequences were amplified with PCR using specific primers (TAPG1: 5′ primer, AAAGGGATCCTCATAAGGGCTCGG, 3′ primer, AGTGTGGTACCGCTACATGGATTAC; TAPG2: 5′ primer, AAAGGGATGCTCATAAGGGCTCGG, 3′ primer, AGTGTGGTACCGCTACATGGTTCA; TAPG4: 5′ primer, ACAATCTAGACAAATGTTCCATATTT, 3′ primer, GTTTCCCGGGCCAATAGATATACAA). The PCR-amplified inserts for TAPG1 (116 bp), TAPG2 (120 bp), and TAPG4 (174 bp) were subcloned into the T3/T7α-19 vector (GIBCO-BRL) at the appropriate restriction sites. Plasmid DNAs for the in vitro transcription reactions were prepared using a CsCl/ethidium bromide plasmid purification protocol (Sambrook et al., 1982). The RNA probes were prepared in vitro from DNA inserts using the Riboprobe Gemini System kit from Promega. Approximately 1 μg of linearized plasmid was used for in vitro synthesis of [^{32}P]CTP-labeled RNA transcripts in the antisense orientation following the manufacturer's instructions.

The hybridization reactions were performed using the RNase Protection Assay system (Promega). Labeled antisense RNA was hybridized to 5 μg of target polysomal RNA. After hybridization, single-stranded RNA was digested with 10 units of RNase ONE (Promega) at 30°C for 1 h. The samples were suspended in a gel-loading buffer, denatured, and loaded onto an 8 M urea/5% polyacrylamide gel in 1× Tris-borate-EDTA buffer following the kit's instructions. The labeled products were detected by overnight autoradiography of the dried gel.

RESULTS

Identification of Two Additional PG cDNA Clones (TAPG2 and TAPG4)

The same abscission cDNA library that was used to identify the TAPG1 clone, as described by Kalaitzis et al. (1995), was screened again using the TAPG1 cDNA as a probe. Several clones were identified and partially sequenced to identify unique PG transcripts. Two unique cDNA clones, pTAPG2 and pTAPG4, were identified and further characterized. TAPG2 and TAPG4 are cDNA clones of 748 and 937 bp, respectively (Figs. 1 and 2). Northern-blot analysis showed that both TAPG2 and TAPG4 cDNAs hybridized to mRNAs of approximately 1.5 kb, indicating that they are partial cDNA clones (data not shown). To

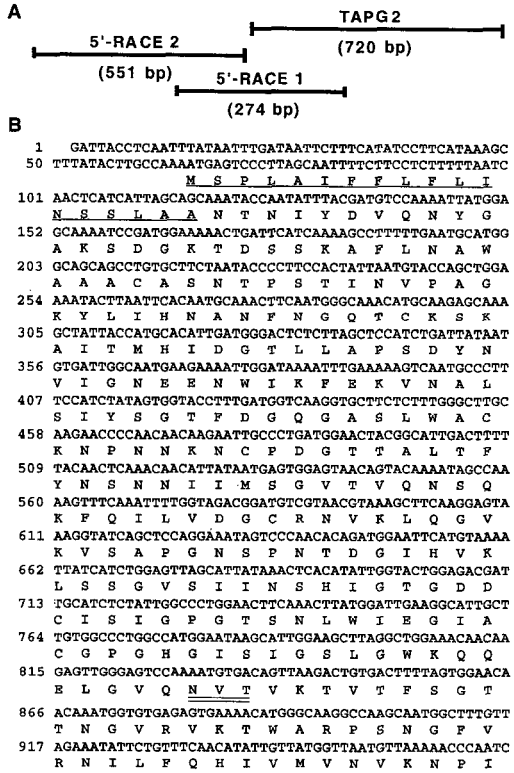

Figure 1. DNA and deduced amino acid sequence of the TAPG2 cDNA clone and the two 5′-RACE products. A, Diagram shows the length and relative overlap of each clone. B, Composite sequence for TAPG2, 5′-RACE1, and 5′-RACE2. The deduced amino acid sequence is shown below the nucleotide sequence in a single-letter amino acid code. The predicted signal peptide is underlined to the cleavage site after amino acid 18. Two potential Asn glycosylation sites (N-X-S/T) are double-underlined at nucleotide positions 830 to 839 and 1143 to 1152.

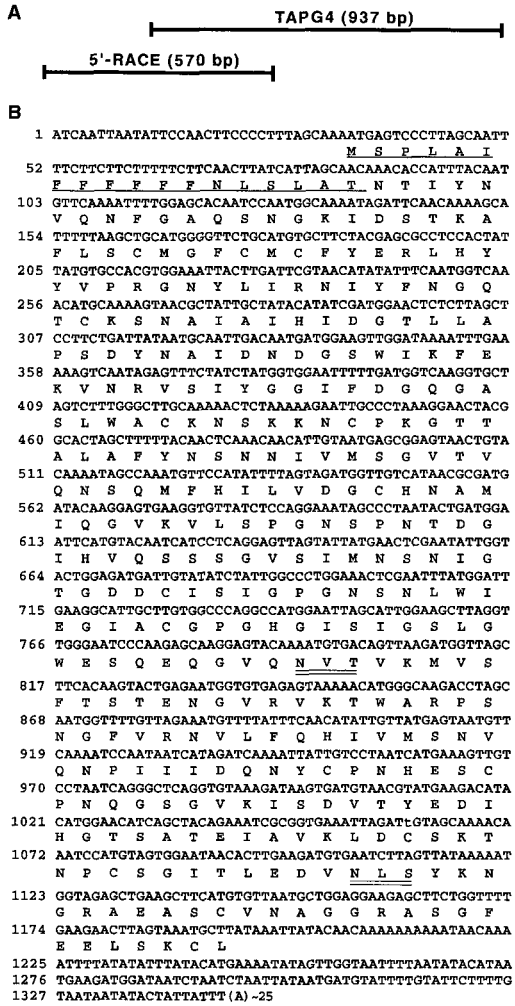

Figure 2. DNA and deduced amino acid sequence of the TAPG4 cDNA clone and the 5′-RACE product. A, Diagram shows the length and relative overlap of each clone. B, Composite sequence for TAPG4 and 5′-RACE clone. The deduced amino acid sequence is shown below the nucleotide sequence in a single-letter amino acid code. The predicted signal peptide is underlined to the cleavage site after amino acid 18. Two potential Asn glycosylation sites (N-X-S/T) are double-underlined at nucleotide positions 793 to 801 and 1113 to 1121.

obtain the 5′ end of the *TAPG2* and *TAPG4* mRNAs, a PCR approach, 5′-RACE, was used (Frohman et al., 1988).

To obtain the sequence for the full open reading frame in the *TAPG2* mRNA, we performed two sequential 5′-RACE reactions, 5′-RACE1 and 5′-RACE2 (Fig. 1). The composite cDNA sequence of the pTAPG2 and two 5′-RACE products is 1267 bp long (Fig. 1). An open reading frame beginning with an ATG starting at nucleotide 65 extends 1176 nucleotides (392 amino acids). The 3′-untranslated region of this cDNA clone is 27 bp long (Fig. 1). The TAPG2 cDNA includes an approximately 150-bp poly(A) tail at the 3′ end. The first 18 amino acids of the deduced amino acid sequence includes a hydrophobic core of amino acids and a cleavage site indicative of a signal peptide (von Heijne, 1983). The mature peptide (i.e. after removal of the putative

Table I. *Percent identities between peptide and nucleotide sequences for TAPGs (TAPG1, TAPG2, and TAPG4), TFPG (Della Penna et al., 1986), and PFPG (Lester et al., 1994)*

Percent identities for the alignments of peptide sequences are shown in boldface.

	TAPG1	TAPG2	TAPG4	TFPG	PFPG
TAPG1	100	**93**	**76**	**41**	**61**
TAPG2	95	100	**76**	**41**	**61**
TAPG4	80	81	100	**38**	**58**
TFPG	50	49	52	100	**40**
PFPG	63	65	62	47	100

signal peptide) has a predicted molecular mass of 40.1 kD, identical to that of the TAPG1 peptide, and a pI of 8.08.

The composite cDNA sequence of the pTAPG4 and 5′-RACE product is 1339 bp (Fig. 2). An open reading frame starts at nucleotide 34 with an ATG and extends to nucleotide 1188 (385 amino acids). A putative signal peptide makes up the first 17 amino acids of the deduced amino acid sequence, including a hydrophobic core of amino acids and a cleavage site (von Heijne, 1983). The mature peptide (i.e. after removal of the putative signal peptide) has a predicted molecular mass of 39.8 kD and a pI of 6.9. The deduced amino acid sequence of the TAPG4 cDNA clone shares 76% identity with the TAPG1 and TAPG2 sequences.

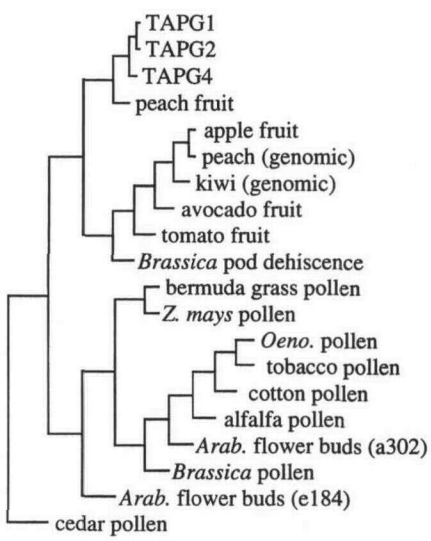

Figure 3. Dendrogram prepared from the comparison of deduced amino acid sequences for plant, fungal, and bacterial PG cDNA and genomic clones. The dendrogram was constructed using the University of Wisconsin Genetics Computer Group software (Madison, WI). The GenBank accession numbers or literature references for the plant PG cDNA and genomic clones are: TAPG1, U23053; TAPG2, U70480; TAPG4, U70481; peach fruit, X76735; apple fruit, L27743; peach (genomic), X77231; kiwi (genomic), L12019; avocado fruit, L06094; tomato fruit, X04583; *Brassica* pod dehiscence, X95800; bermuda grass, A28056; *Zea mays* pollen, S66022; *Oenothera* pollen (Brown and Crouch, 1990); tobacco pollen, X71017; cotton pollen, U09717; alfalfa pollen, U20431; Arabidopsis (*Arab.*) flower buds (a302), X73222; *Brassica* pollen, L19879; Arabidopsis (*Arab.*) flower buds (e184), X72291; and cedar pollen, D29772.

Relationship of the TAPGs with Other PGs

Table I shows the relative percent identities for the nucleotide and peptide sequences for the three TAPGs (TAPG1, TAPG2, and TAPG4), TFPG (Della Penna et al., 1986), and PFPG (Lester et al., 1994). The TAPGs are clearly different from the TFPGs (38–41% amino acid sequence identities), putting them into a separate subfamily of tomato PG genes. This is further supported in the dendrogram shown in Figure 3. The three TAPGs are grouped

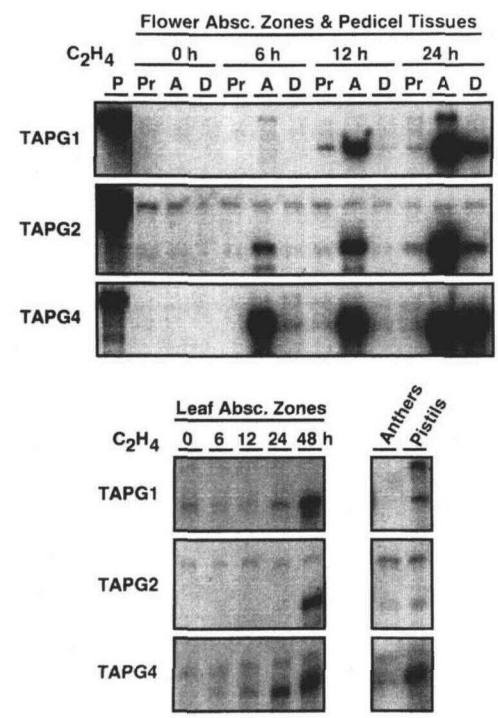

Figure 4. Expression patterns for three different PG genes in tomato abscission. Polysomal RNA was extracted from flower abscission zones (A), tissue proximal (Pr) and distal (D) to the abscission zones of flower pedicels, and abscission zones of leaf explants exposed to 25 μL/L ethylene for the indicated intervals of time. RNA was also extracted from anthers and pistils (stigma and styles only) of fully open tomato flowers. Labeled RNA probes were prepared for each of the three PG clones (TAPG1, TAPG2, and TAPG4) and hybridized with 5 μg of the RNA samples. After hybridization, samples were incubated with RNase ONE (Promega), separated on acrylamide gels, and exposed to x-ray film. An undigested probe (P) was loaded onto each gel to show the size of probe that escaped RNase digestion in the treated samples.

together with the PFPG but in a separate group from other fruit, pollen, and dehiscence PGs from several different plant species (Fig. 3).

RNase Protection Analysis

Northern-blot analysis (data not shown) showed that *TAPG2* and *TAPG4* mRNAs have similar tissue-specific expression patterns to *TAPG1* mRNA (Kalaitzis et al., 1995). In other words, all three showed strong hybridization bands in RNA from flower and leaf abscission zones and no hybridization to RNA from stems, petioles, or fruit. However, because the three *TAPG* mRNAs share a high degree of sequence identity, it is possible that where mRNA was detected on the northern blots the probes might not have been specific for the respective mRNA. To overcome the problem of cross-hybridization, an RNase protection assay (Ausubel et al., 1989) was used to investigate the temporal expression of each of the three *TAPG* transcripts in leaf and flower abscission and in pistils and anthers.

Abscission of tomato flowers and leaves is nearly 100% complete after 24 and 72 h of exposure, respectively, of explants to 25 μL/L ethylene. In an earlier report in which we used northern blot analysis rather than RNase protection, we showed that mRNA hybridizing to a *TAPG1* probe accumulated to fairly high levels in the abscission zones of flowers and leaves after 24 and 48 h of exposure to ethylene, respectively (Kalaitzis et al., 1995). To get a better measure of when the different PG mRNAs are first expressed in abscission, we extracted polysomal RNA from flower pedicel abscission zones and proximal and distal pedicel tissues after 0, 6, 12, and 24 h and from leaf abscission zones of tomato explants exposed to ethylene for 0, 6, 12, 24, and 48 h.

Figure 4 shows that the PG mRNAs are not detectable in the RNA from abscission zones of the fully open flowers that were not treated with ethylene and did not show signs of senescence. However, in flower abscission zones collected after only 6 h of ethylene treatment the mRNA levels of *TAPG4* increased markedly and remained at that level through 24 h of the ethylene treatment. In contrast, the *TAPG2* mRNA, although detectable after 6 h of ethylene treatment, was much less abundant compared with the *TAPG4* mRNA and continued to increase through 24 h of ethylene treatment, reaching levels similar to *TAPG4*. The *TAPG1* mRNA was detectable after 12 h of ethylene treatment and increased through 24 h, following an expression pattern similar to the *TAPG2* transcript. It is interesting that the relatively high levels of mRNA for all three *TAPG* genes are detected in the distal pedicel tissue after 24 h of ethylene treatment (Fig. 4). However, the mRNA levels of all three PGs are very low in proximal pedicel tissue after 24 h of ethylene treatment (Fig. 4).

In leaf abscission zones *TAPG4* transcript was detected in samples taken after 12 h of exposure to ethylene (Fig. 4). *TAPG1* and *TAPG2* mRNAs, on the other hand, were detected in samples taken after 24 h of ethylene exposure. All three PG mRNAs increased through 48 h of ethylene treatment. The amount of RNA, labeled probe, and exposure time are equal for all of the lanes and panels included in Figure 4. The relative abundance of the PG transcripts shown in Figure 4 are, therefore, directly comparable. PG mRNA concentrations are severalfold higher in flower abscission zones than in leaf abscission zones (Fig. 4).

In addition to leaf and flower abscission, we also examined the expression of the three PG transcripts in the anthers and pistils (stigma and styles only) of fully open flowers. Low levels of mRNA for all three *TAPG* genes were detected in the RNA samples from pistils; however, the relative abundance of the three PG transcripts varied. Accumulation of transcript for *TAPG1* and *TAPG4* in the pistils was notably greater than for *TAPG2* (Fig. 4). No significant amount of any of the PG transcripts accumulated in the anthers from fully open tomato flowers (Fig. 4).

DISCUSSION

We have shown that at least three different PG genes are expressed in tomato flower and leaf abscission and in mature pistils. The *TAPG* gene products from tomato most likely encode endopolygalacturonases due to their high percent identity with the PFPG (62% identity), which was shown to encode an endopolygalacturonase (Lester et al., 1994). Moreover, in the dendrogram shown in Figure 3 the fruit and abscission PGs are grouped separately from the pollen PGs. Tebbutt et al. (1994) suggested that the PG genes expressed during pollen maturation encode exopolygalacturonases. Although *TAPG* mRNA was detected in pistils, the lack of expression in mature anthers suggests that the *TAPG* mRNAs are in a separate subfamily from the PG genes expressed during pollen maturation (Brown and Crouch, 1990; Niogret et al., 1991; Allen and Lonsdale, 1993). The separation of the abscission and fruit PGs from the pollen PGs in the dendrogram may reflect a functional difference of endo- and exoenzyme activity between these two groups.

One of the major events in tomato abscission is the increase in activity of cellulase and PG during abscission (Tucker et al., 1984). Lashbrook et al. (1994) and del Campillo and Bennett (1996) showed that mRNAs for tomato cellulases *Cel1*, *Cel2*, and *Cel5* are all expressed in flower abscission zones but are expressed differently in pedicel tissues proximal and distal to the flower abscission zone. *Cel1* was expressed abundantly both in the flower abscission zone and in the tissue distal to the abscission zone in abscised flowers, whereas *Cel2* and *Cel5* were expressed most abundantly in the abscission zone but also at a much lower level in the pedicel tissue proximal to the abscission zone and at barely detectable levels in the distal pedicel tissue. In contrast, none of the tomato PGs had an appreciable accumulation in the proximal pedicel tissue, but did show a fairly high accumulation in the distal tissue after 24 h of exposure to ethylene (Fig. 4). Although all three PGs appear to be expressed in the same regions in the abscission zone, their temporal expression patterns during abscission are quite different. *TAPG1* and *TAPG2* have fairly similar temporal expression patterns and similar primary sequences (93% identity). The primary sequence for

TAPG4, on the other hand, is more dissimilar from those of the other two (76% identity), and its mRNA accumulates to a high level much earlier than *TAPG1* and *TAPG2*.

In bean, where genomic blots indicated that the abscission cellulase gene, *BAC1*, is not highly similar to any other cellulase genes (Tucker and Milligan, 1991), the *BAC1* cellulase mRNA was shown using in situ hybridization to be expressed in all cell types across the separation layer of leaf abscission zones and in cells up to 4 mm distal to the separation layer in the vascular tissue of the abscission zone (Tucker et al., 1991). However, in tomato, where several cellulase and PG genes have been shown to be expressed during abscission, it is possible that each of the cellulase and three PG genes may have different cell-specific expression patterns within the leaf and flower abscission zones.

Thompson and Osborne (1994) demonstrated, using surgical manipulation of the bean abscission zones, that the stele is in some way responsible for sending a signal to the surrounding cortex to begin cell separation. They speculated that an oligosaccharide released from the cell walls of the stele might act as the signal for cortical cells to begin the process of cell separation. It is possible that the early expression of *TAPG4* in tomato leaf and flower abscission may play a role in a similar signaling process in tomato. In our experience with several different abscission systems, the accumulation of *TAPG4* mRNA, relative to the appearance of a fracture line, is the earliest of all of the hydrolase mRNAs we have examined (Tucker et al., 1988; Kalaitzis and Tucker, 1994; Kemmerer and Tucker, 1994). The significance of the early expression of the *TAPG4* mRNA is unknown at this time but is of interest for future experiments.

Received September 20, 1996; accepted January 7, 1997.
Copyright Clearance Center: 0032-0889/97/113/1303/06.
The GenBank accession numbers for the sequences described in this article are U70480 (TAPG2) and U70481 (TAPG4).

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