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# Expression of a Chimeric Polygalacturonase Gene in Transgenic *rin* (Ripening Inhibitor) Tomato Fruit Results in Polyuronide Degradation but not Fruit Softening

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**Tomato fruit ripening is accompanied by extensive degradation of pectic cell wall components. This is thought to be due to the action of a single enzyme, polygalacturonase, whose activity is controlled, at least in part, at the level of gene expression. At the onset of tomato fruit ripening, polygalacturonase enzyme activity, mRNA levels, and relative rate of gene transcription all increase dramatically. To elucidate the role of polygalacturonase during tomato fruit ripening, we utilized a pleiotropic genetic mutation, *rin*, that blocks many aspects of ripening, including the activation of polygalacturonase gene transcription. The polygalacturonase structural gene was ligated to a promoter that is inducible in mature *rin* fruit and inserted into the fruit genome, and plants were regenerated. This allowed expression of the polygalacturonase gene in transgenic *rin* fruit at a time corresponding to ripening in wild-type fruit. Expression of this gene resulted in the accumulation of active polygalacturonase enzyme and the degradation of cell wall polyuronides in transgenic *rin* fruit. However, no significant effect on fruit softening, ethylene evolution, or color development was detected. These results indicate that polygalacturonase is the primary determinant of cell wall polyuronide degradation, but suggest that this degradation is not sufficient for the induction of softening, elevated rates of ethylene biosynthesis, or lycopene accumulation in *rin* fruit.**

## INTRODUCTION

Ripening is the final phase of fruit development when the promotion of both catabolic and anabolic processes results in sharp metabolic changes. In tomato fruit, a model system for the analysis of ripening, specific changes include increased respiration and ethylene production, chlorophyll degradation, carotenoid synthesis, production of essential oils, and softening (Rhodes, 1980). In many cases, these transitions have been shown to reflect the appearance of new enzyme activities (Brady et al., 1987), which in turn, result from the regulated accumulation of specific mRNAs (Speirs et al., 1984; Mansson et al., 1985; Biggs et al., 1986; DellaPenna et al., 1986; Lincoln et al., 1987).

The cell wall of tomato fruit is similar to other plant cell walls, and softening is thought to result from cell wall modifications that occur during ripening (Tigchelaar et al., 1978). Although numerous subtle changes in tomato fruit cell wall structure occur during ripening, a major structural change is the degradation of polyuronides (Huber, 1983b). This has been observed as an increase in water- or EDTA-soluble polyuronides, a decrease in the degree of polymerization of polyuronides, and the loss of the darkly staining polyuronide-rich middle lamella in electron micrographs (Huber, 1983a; Crookes and Grierson, 1983). Thus, it has been proposed that polyuronide degradation is the primary determinant of tomato fruit softening. A single cell wall enzyme, polygalacturonase (poly(1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15), has been implicated as the primary agent of polyuronide degradation in ripening tomato fruit (Wallner and Bloom, 1977; Themmen et al., 1982; Huber, 1983a) and hence implicated as the primary enzyme regulating tomato fruit softening (Hobson, 1964; Brady et al., 1982; Huber, 1983b). Considerable evidence has accumulated supporting this hypothesis (for review see, Bennett and DellaPenna, 1987a). First, coincident with the onset of fruit softening there is a dramatic increase in polygalacturonase enzyme activity (Tucker, et al., 1980; Brady, et al., 1982), mRNA concentration (DellaPenna et al., 1986, Lincoln et al., 1987), and relative rate of gene transcription (Sheehy et al., 1988; D. DellaPenna, J. E. Lincoln, R. L. Fischer, and A. B. Bennett, manuscript submitted for publication). Second, there is a rough correlation between levels of polygalacturonase enzyme activity and the rate of tomato fruit softening in different cultivars and in ripening-impaired mutants (Tigchelaar et al., 1978; Brady et al., 1983). Third, there is little correlation between the activity of several other cell wall degrading

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enzymes (cellulase, β-1,3-glucanase, pectinesterase) and rate of tomato fruit softening (Hobson, 1968; Wallner and Walker, 1975; Tigchelaar et al., 1978).

However, other results indicate that softening may not be regulated exclusively by polygalacturonase. First, there are exceptions to the correlation between polygalacturonase activity and the rate of tomato fruit softening (C. Brady, personal communication). Second, other changes in cell wall composition, such as hemicellulose degradation, also occur during tomato fruit ripening and may contribute to softening (Huber, 1983a). Third, there are examples of fruits other than tomato that soften in the absence of dramatic increases in polygalacturonase activity (Huber, 1983b). Thus, the action of other enzymes or factors, such as substrate accessibility, also may be important determinants of tomato fruit softening.

In addition to the proposed role of polygalacturonase in polyuronide degradation and fruit softening, it has been proposed that polygalacturonase-dependent polyuronide degradation may release pectic fragments that regulate other components of the ripening process (Brady et al., 1987; Bennett and DellaPenna, 1987a; Baldwin and Pressey, 1988). This proposal has been supported by reports of cell wall fragments being capable of stimulating ethylene biosynthesis when applied to cultured cells (Tong et al., 1986), tomato pericarp tissue (Brecht and Huber, 1986), or leaves (VanderMolen et al., 1983). Similarly, it has been reported that infiltration of purified polygalacturonase protein into mature green tomato fruit stimulates ethylene production (Baldwin and Pressey, 1988). Finally, pectic polysaccharides of plant cell walls have been shown to activate phytoalexin (Davis et al., 1986) and proteinase inhibitor I (Walker-Simmons and Ryan, 1986) biosynthesis. Thus, numerous lines of evidence support a role for polygalacturonase in polyuronide degradation, fruit softening, and perhaps in regulating other components of the ripening process.

To investigate further the role of polygalacturonase in polyuronide degradation, tomato fruit softening, and other parameters of ripening, we have developed a strategy to modify polygalacturonase gene expression in vivo and assess its physiological function using the pleiotropic tomato mutant, *rin* (ripening inhibitor). *rin* fruit do not soften, produce only basal levels of ethylene, do not accumulate carotenoid pigments, and have greatly reduced levels of polygalacturonase, relative to wild-type fruit (Tigchelaar, 1978; DellaPenna et al., 1987). The *rin* mutation does not represent a lesion in the polygalacturonase gene, as the *rin* and polygalacturonase loci are located on different chromosomes (S. Tanksley, personal communication). Rather, the failure of *rin* fruit to activate polygalacturonase gene expression results from a block at the level of gene transcription (DellaPenna et al., 1987; D. DellaPenna, J. E. Lincoln, R. L. Fischer, and A. B. Bennett, manuscript submitted for publication). Our strategy has been to induce polygalacturonase gene expression in *rin* fruit and to examine its effect on polyuronide degradation, fruit softening, and other parameters of ripening.

To this end, we have constructed a chimeric gene consisting of the polygalacturonase structural gene fused to the regulatory sequences of another ripening-associated gene of unknown function, *E8* (Deikman and Fischer, 1988). Our rationale for using E8 regulatory sequences is as follows. In wild-type tomato plants, polygalacturonase and E8 gene expression are tightly coordinated. That is, both E8 and polygalacturonase mRNAs are abundant in ripe tomato fruit, but are not detected in other organs such as leaf, root, or stem (Lincoln and Fischer, 1988a; J. Giovannoni and R. Fischer, unpublished results). Furthermore, in wild-type fruit, E8 and polygalacturonase mRNA levels and relative rates of gene transcription increase coincidentally (D. DellaPenna, J. E. Lincoln, R. L. Fischer, and A. B. Bennett, manuscript submitted for publication). However, the regulation of polygalacturonase and E8 gene expression differs in two important aspects. First, whereas polygalacturonase gene transcription is inhibited severely in *rin* fruit, the relative rate of E8 gene transcription is 60% of the wild-type level (D. DellaPenna, J. E. Lincoln, R. L. Fischer, and A. B. Bennett, manuscript submitted for publication). Second, E8 gene transcription, and not polygalacturonase, is activated by ethylene in both unripe wild-type and *rin* fruit (Lincoln and Fischer, 1988b). Thus, our strategy has been to utilize E8 regulatory sequences to induce expression of a chimeric E8-polygalacturonase gene in *rin* fruit. We report here the physiological consequences of its expression.

## RESULTS

### Structure of Polygalacturonase and E8 Genes

To isolate a polygalacturonase gene, libraries of tomato DNA were screened by hybridizing plaques with labeled polygalacturonase cDNA clones. As shown in Figure 1A, two overlapping clones, λPG12 and λPG11.5 were recovered by these procedures. To analyze the structure of the polygalacturonase gene, a restriction endonuclease site map was constructed (Figure 1A), and restriction fragments that hybridized with labeled polygalacturonase coding sequences were identified. The cloned restriction fragments corresponded exactly to those observed in genomic DNA gel blot experiments, suggesting that polygalacturonase is encoded by a single-copy gene. The polygalacturonase transcription initiation site was defined by S1-nuclease protection and primer extension experiments (data not shown). The location determined using these techniques coincided with the 5′ end of a full-length polygalacturonase cDNA clone (Grierson et al., 1986). The structure of the E8 gene, shown in Figure 1B, was determined in Deikman and Fischer (1988).

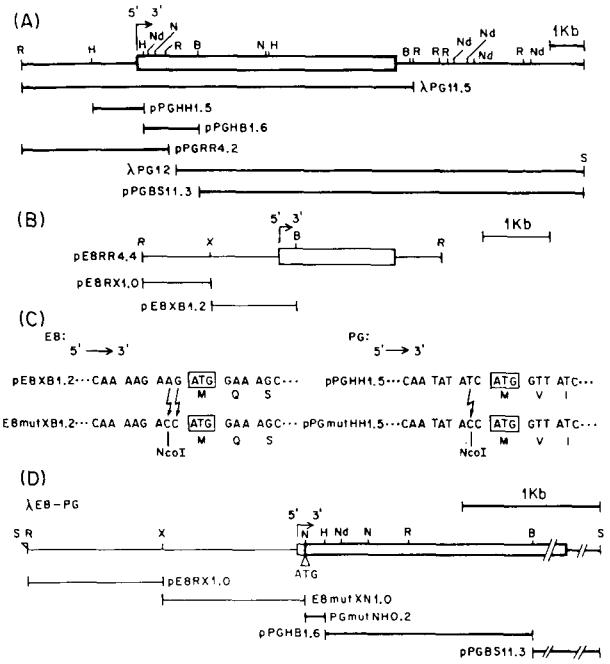


**Figure 1.** Construction of a Chimeric E8-Polygalacturonase Gene.

E8 gene and flanking sequences are shown in thin lines, and polygalacturonase gene and flanking sequences are drawn in thick lines. B, BamHI; H, HindIII; N, NcoI; Nd, NdeI; R, EcoRI; S, SalI; X, XbaI.

**(A)** Polygalacturonase gene structure. λPG12 was obtained by screening a library of tomato (cv VFNT Cherry) genomic DNA in the Charon 35 vector by plaque hybridization with the labeled polygalacturonase cDNA clone pE41 (Lincoln et al., 1987). λPG11.5 was isolated in a similar manner, except that a library of tomato (cv T6) genomic DNA cloned in the Charon 4 vector was screened with the labeled polygalacturonase cDNA clone pPG1.9 (Bennett and DellaPenna, 1987b). pPGHH1.5, pPGHB1.6, pPGRR4.2, and pPGBS11.3 represent restriction fragments from the genomic clones inserted into pUC118 plasmid vector. The name of each subclone indicates its flanking restriction sites and its molecular mass (in kilobase pairs). The box indicates transcribed sequences. Restriction endonuclease sites were deduced from single and double digestions.

**(B)** E8 gene structure. pE8RR4.4 encodes the E8 gene plus flanking sequences (Deikman and Fischer, 1988). Restriction fragments from pE8RR4.4 that span the 5′-flanking sequences were subcloned into the pUC18 vector to generate pE8RX1.0 and pE8XB1.2. The box indicates transcribed sequences.

**(C)** Generation of NcoI restriction endonuclease sites at translation initiation codons. Translation initiation codon for the polygalacturonase gene was determined by comparing the DNA sequence from the 3′ end of pPGHH1.5 to the DNA sequence of a full-length polygalacturonase cDNA clone (Grierson et al., 1986). The translation initiation codon for the E8 gene, encoded by pE8XB1.2, was determined in an analogous fashion (Deikman and Fischer, 1988). pE8XB1.2 and pPGHH1.5 were used as targets for synthetic oligonucleotide-mediated site-directed mutagenesis to generate pE8mutXB1.2 and pPGmutHH1.5, which encode an NcoI site at the ATG translation initiation codon of the E8 and polygalacturonase genes, respectively. DNA sequences that immediately flank translation initiation sites are shown. Translation initiation codons for the E8 and polygalacturonase genes are boxed, and predicted amino acid sequences are shown in single letter code.

**(D)** Construction of chimeric gene. pE8mutXB1.2 and pPGmutHH1.5 were digested with NcoI, and restriction fragments E8mutXN1.0 and PGmutNH0.2, respectively, were purified by agarose gel electrophoresis. The E8-polygalacturonase gene was then constructed as follows. E8mutXN1.0 was ligated to PGmutNH0.2 and subcloned into XbaI/HindIII-digested pUC118. The resulting 1.2-kb XbaI/HindIII restriction fragment was ligated at the XbaI site to the pE8RX1.0 insert and cloned into pUC118. The resulting 2.2-kb EcoRI/HindIII restriction fragment was ligated at the HindIII site to the pPGHB1.6 insert and cloned into pUC118. The resulting 3.8-kb EcoRI/BamHI restriction fragment was ligated at the BamHI site to the pPGBS11.3 insert. The resulting 15.1-kb EcoRI/SalI restriction fragment was ligated to gel-purified λEMBL3 right and left arms terminating in EcoRI and SalI restriction endonuclease sites, respectively. DNA was then packaged in vitro and phage, designated λE8-PG, containing an E8-polygalacturonase chimeric gene were recovered.

## Construction of a Chimeric E8-Polygalacturonase Gene with the Fusion Point at the Translation Initiation Codon and Its Insertion into the *rin* Genome

To facilitate construction of a chimeric gene, NcoI restriction endonuclease sites were generated at the ATG translation initiation codons of the polygalacturonase and E8 genes, respectively (Figure 1C). It is important to note that no nucleotides in the polygalacturonase protein coding region were altered, ensuring that the polygalacturonase enzyme encoded by the chimeric gene would be identical to that produced by the normal polygalacturonase gene. Figure 1D describes the construction of the chimeric E8-polygalacturonase gene, λE8-PG. This gene contains 2.0 kb of E8 5′-flanking sequences, the E8 site of transcription initiation, and the 34-bp E8 untranslated mRNA leader sequence fused at the ATG translation initiation site to 7.5 kb of the polygalacturonase structural gene, followed by 5.5 kb of 3′-polygalacturonase flanking sequences. DNA sequence analysis verified that the fusion was made at the ATG translation initiation site, and the integrity of the chimeric gene was checked by extensive mapping of restriction endonuclease sites (data not shown). The E8-polygalacturonase chimeric gene was subcloned into pMLJ1 and the resulting plasmid, pMLJ1(E8/PG), was transferred to the *rin* tomato genome using disarmed Ti plasmid vectors from *Agrobacterium tumefaciens* as described in "Methods." Three plants were recovered and are designated *rin*(E8/PG)-1, *rin*(E8/PG)-2, *rin*(E8/PG)-3. Blot hybridization experiments indicated that each plant genome contained a single chimeric E8-polygalacturonase gene (data not shown). In addition, a control *rin* plant, designated *rin*(C)-1, was transformed with plasmid pMLJ1.

### Expression of E8-Polygalacturonase Fusion Gene in Transformed *rin* Fruit

To distinguish among E8, polygalacturonase, and chimeric E8-polygalacturonase gene expression, mRNA was isolated from wild-type, *rin*, and transformed *rin*(E8/PG)-2 fruit and analyzed by the S1-nuclease protection assay. Figure 2A shows that, as expected, E8 mRNA was detected in wild-type, *rin*, and *rin*(E8/PG)-2 fruit, whereas mRNA encoded by the endogenous polygalacturonase gene (Figure 2B) was detected in wild-type fruit, but not in *rin* or *rin*(E8/PG)-2 fruit. Curiously, mRNA encoded by the E8-polygalacturonase gene (Figure 2B) was not detected in air-treated *rin*(E8/PG)-2 fruit. This result was unexpected because the E8 promoter of the endogenous E8 gene is active in *rin* fruit (Figure 2A; Lincoln and Fischer, 1988b). However, because E8 gene expression is activated by ethylene in both wild-type and *rin* fruit (Lincoln and Fischer, 1988b), expression of the E8-polygalacturonase gene was activated by treating fruit with an ethylene analog, propylene. Propylene was chosen because we wished to measure ethylene evolution in response to the action of polygalacturonase in later experiments. The effects of ethylene and propylene on fruit ripening are very similar, except that higher concentrations of propylene must be used (Burg and Burg, 1967; McMurchie et al., 1972). As expected, propylene did not significantly stimulate endogenous polygalacturonase gene expression (Figure 2B) in *rin*(E8/PG)-2 or control *rin* fruit. However, expression of the E8-polygalacturonase gene was stimulated in propylene-treated *rin*(E8/PG)-2 fruit. These results indicate that chimeric E8-polygalacturonase gene expression, but not endogenous polygalacturonase gene expression, occurs in propylene-treated *rin*(E8/PG)-2 fruit.

To assay for production of polygalacturonase protein, fruit pericarp cell wall proteins from 35-day fruit exposed continuously to either air or propylene for 3 to 30 days were isolated and analyzed by immunoblotting. Figure 3 shows that propylene-treated fruit from three independently transformed plants, *rin*(E8/PG)-1, *rin*(E8/PG)-2, and *rin*(E8/PG)-3 accumulated polygalacturonase protein coincident with the appearance of E8-polygalacturonase mRNA in *rin*(E8/PG)-2 fruit (Figure 2B). The immunologically detectable polygalacturonase protein was identical in size to polygalacturonase isolated from wild-type fruit. Very little polygalacturonase protein was detected in air-treated fruit from the three transformed plants, consistent with the fact that E8-polygalacturonase mRNA did not accumulate in air-treated *rin*(E8/PG)-2 fruit (Figure 2B). As expected, only basal levels of immunologically detectable polygalacturonase protein were observed in air- or propylene-treated *rin* and transformed control *rin*(C)-1 fruit. We conclude that, as the result of E8-polygalacturonase gene expression, cell walls from propylene-treated *rin*(E8/PG)-1, *rin*(E8/PG)-2, and *rin*(E8/PG)-3 fruit accumulate significant levels of polygalacturonase protein relative to wild-type fruit.

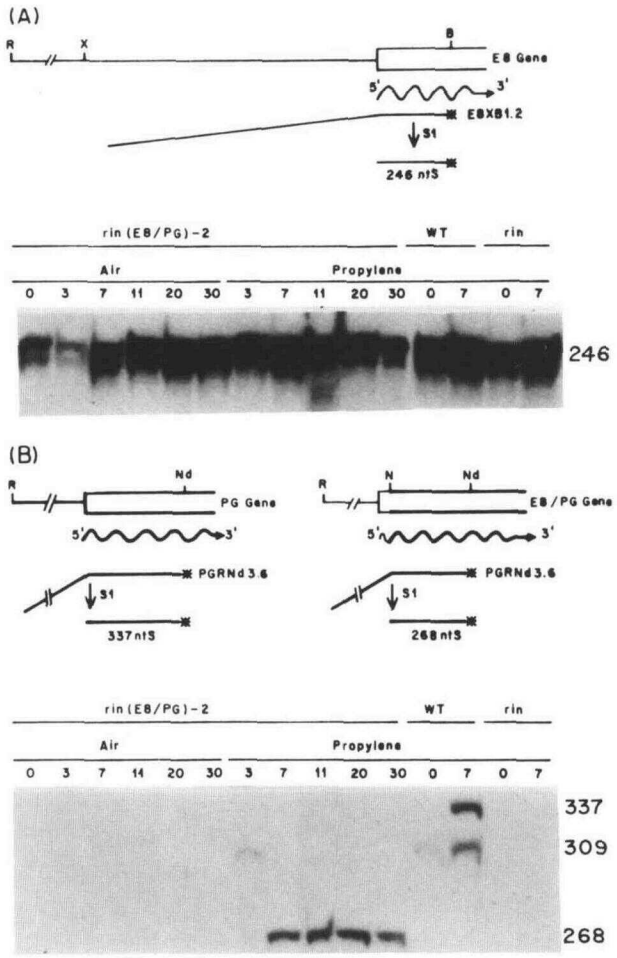


**Figure 2.** S1-Nuclease Analysis of E8, Polygalacturonase, and Chimeric E8-Polygalacturonase Gene Expression in Wild-Type, *rin*, and Transgenic *rin* Fruit.

Three fruit (35 days after anthesis) were treated with either air or propylene for the indicated period of time, and total RNA was isolated, hybridized with the indicated 5′ end-labeled probe, subjected to S1-nuclease digestion, and analyzed by acrylamide gel electrophoresis. Thin lines refer to E8 gene and flanking regions, thick lines refer to polygalacturonase gene and flanking regions, and boxes indicate transcribed sequences. ⇝, RNA sequences; ——*, $^{32}P$ end-labeled DNA.

**(A)** E8 RNA accumulation. The structure of the 5′ end-labeled probe (E8XB1.2; see Figure 1B) that hybridizes with E8 RNA, and the predicted 246 nucleotide S1-resistant fragment, are shown above the actual S1-resistant products.

**(B)** Polygalacturonase and E8-polygalacturonase RNA accumulation. The structure of the 5′ end-labeled probe (3.6-kb EcoRI/NdeI restriction fragment from pPGRR4.2; see Figure 1A) used to hybridize with polygalacturonase and chimeric E8-polygalacturonase RNAs, and their predicted S1-resistant digestion products, 337 and 268 nucleotides, respectively, are shown above the actual S1-resistant products. The 309-nucleotide S1-resistant fragment probably represents S1 cleavage at an AT-rich sequence ($ATA_9TAA$) located in the 5′-untranslated leader of the polygalacturonase RNA.

### Polygalacturonase Activity in *rin* Transgenic Fruit

To determine whether the polygalacturonase protein produced in transgenic *rin* fruit is active, cell wall protein extracts were assayed for the ability to degrade polygalacturonic acid in vitro. Figure 4A shows that protein extracts isolated from air-treated *rin*, *rin*(C)-1, and *rin*(E8/PG)-2 fruit possessed essentially no polygalacturonase activity, whereas wild-type fruit extracts contained significant levels of polygalacturonase enzyme activity after 7 days of air treatment. As shown in Figure 4B, treatment of *rin* and *rin*(C)-1 fruit with propylene had little effect on levels of polygalacturonase enzyme activity. In contrast, propylene treatment of *rin*(E8/PG)-2 fruit resulted in a significant increase in polygalacturonase activity to 60% of maximal wild-type levels after 30 days.

To ascertain whether the polygalacturonase enzyme produced in transgenic *rin* fruit resulted in polyuronide degradation in vivo, the level of EDTA-soluble uronic acid in fruit cell walls was determined. Figure 5A shows that the amount of EDTA-soluble uronic acid recovered from air-treated *rin*(E8/PG)-2 fruit did not differ significantly from that found in air-treated *rin* and *rin*(C)-1 fruit, whereas air-treated wild-type fruit contained approximately threefold more EDTA-soluble uronic acid at 20 days. However, in the presence of exogenous propylene, an increase of EDTA-soluble uronic acid was observed in *rin*(E8/PG)-2 fruit but not in *rin* or *rin*(C)-1 fruit (Figure 5B). Treating *rin*(E8/PG)-2 fruit with propylene for 11 days resulted in a level of EDTA-soluble uronic acid similar to that found in propylene-treated wild-type fruit. We conclude that the protein product of the E8-polygalacturonase chimeric gene in propylene-treated *rin*(E8/PG)-2 fruit functions in vivo to degrade cell wall polyuronides.

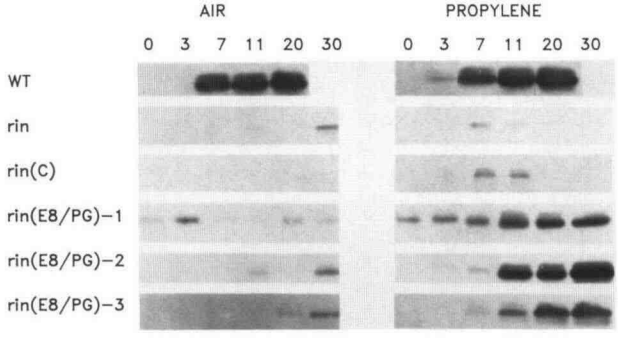


**Figure 3.** Protein Gel Blot Analysis of Cell Wall Protein Extracts from Wild-Type, *rin*, and Transgenic *rin* Fruit.

Three fruit (35 days after anthesis) were exposed to either propylene or air for the indicated period of time. Cell wall protein extracts were isolated, fractionated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose, and polygalacturonase protein was detected using anti-polygalacturonase serum. WT, wild-type; *rin*(C), *rin*(C)-1.

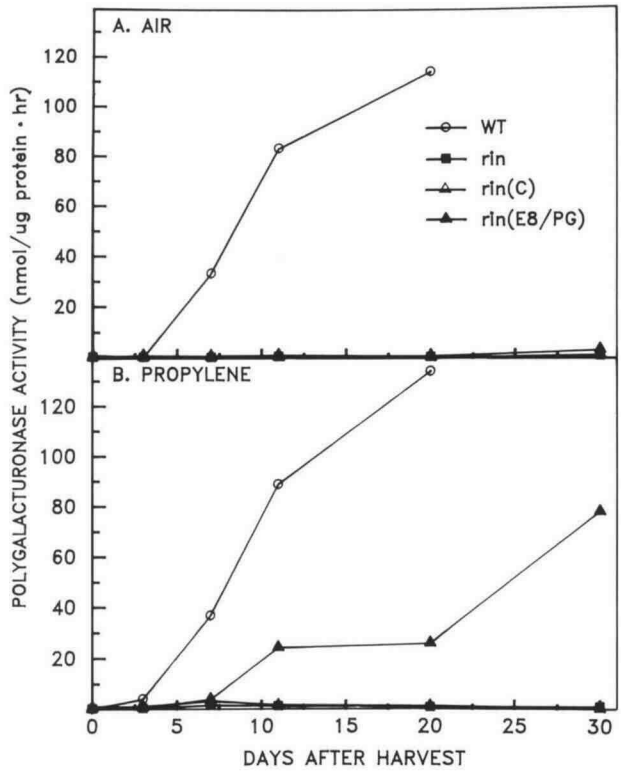


**Figure 4.** Polygalacturonase Enzyme Activity in Wild-Type, *rin*, and Transgenic *rin* Fruit.

Three fruit (35 days after anthesis) were exposed continuously to either air **(A)** or propylene **(B)** for the indicated period of time. Cell wall protein extracts were isolated and levels of polygalacturonase enzyme activity were determined. Each point represents the mean of duplicate assays. WT, wild-type; *rin*(C), *rin*(C)-1; *rin*(E8/PG), *rin*(E8/PG)-2.

### Physiological Consequences of Polygalacturonase Activity in Transgenic *rin* Fruit

To determine whether polygalacturonase enzyme activity in the cell wall of transgenic *rin* fruit results in softening, fruit from wild-type, *rin*, *rin*(C)-1, and *rin*(E8/PG)-2 plants were exposed continuously to air or propylene for 3 to 30 days and assayed for compressibility (see "Methods"). As shown in Figure 6A, compressibility of *rin*, *rin*(C)-1, and *rin*(E8/PG)-2 fruit changed little over the course of air treatment, whereas wild-type fruit exhibited a fourfold increase in compressibility. Figure 6B shows that exposure of *rin*(E8/PG)-2 fruit to exogenous propylene, which elicited significant levels of polygalacturonase activity and wild-type levels of polyuronide degradation after 11 days (see Figure 5B), had no discernible effect on fruit compressibility relative to either *rin* or *rin*(C)-1 fruit even after a 30-day treatment. Similar results were observed in two other independently transformed plants, *rin*(E8/PG)-1 and *rin*(E8/PG)-2 (data not shown).

To ascertain whether polygalacturonase enzyme activity affects other aspects of tomato fruit ripening, we measured ethylene evolution from wild-type, *rin*, *rin*(C)-1, and *rin*(E8/PG)-2 fruit treated with either air or propylene. Figure 7 shows that *rin*, *rin*(C)-1, and *rin*(E8/PG)-2 fruit all produce low levels of ethylene in the presence of either air or exogenous propylene. The slight rise in ethylene production in propylene-treated *rin*(E8/PG)-2 at 25 days was due to elevated ethylene production by a single fruit. This returned to basal ethylene production level by 30 days. The reason for its increase is unclear, but is probably not related to polygalacturonase activity, as all other transgenic fruit from two other transformed plants, *rin*(E8/PG)-1 and *rin*(E8/PG)-3, failed to produce elevated levels of ethylene (data not shown). Finally, Figure 8 shows no significant differences in the color of 30-day propylene-treated *rin* and *rin*(E8/PG)-2 fruit. We conclude that poly-

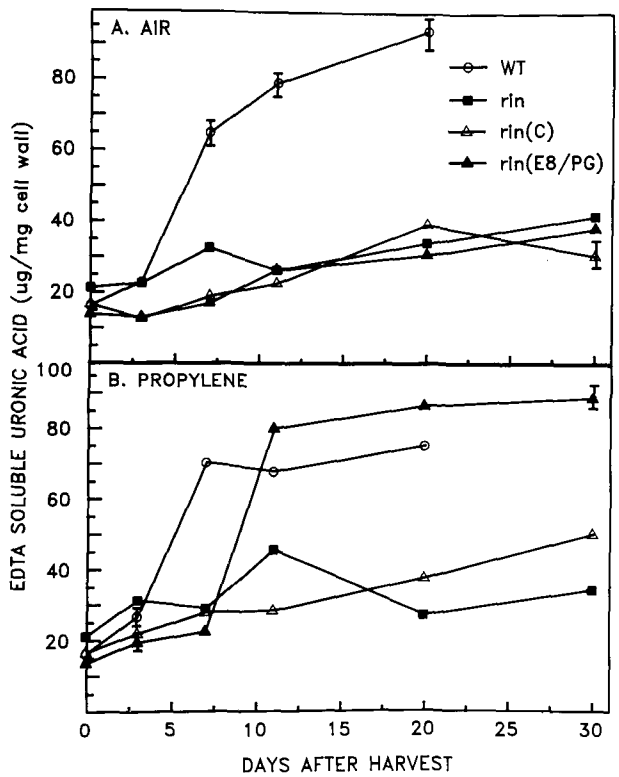


**Figure 5.** Levels of Soluble Uronides in Wild-Type, *rin*, and Transgenic *rin* Fruit.

Three fruit (35 days after anthesis) were exposed continuously to either air **(A)** or propylene **(B)** for the indicated period of time. Polyuronides were isolated and the level of EDTA-soluble uronic acid was determined. Data points represent the mean of at least three determinations. Error bars represent standard deviations. Where error bars are not shown, the standard deviation was no greater than the size of the symbol. WT, wild-type; *rin*(C), *rin*(C)-1; *rin*(E8/PG), *rin*(E8/PG)-2.

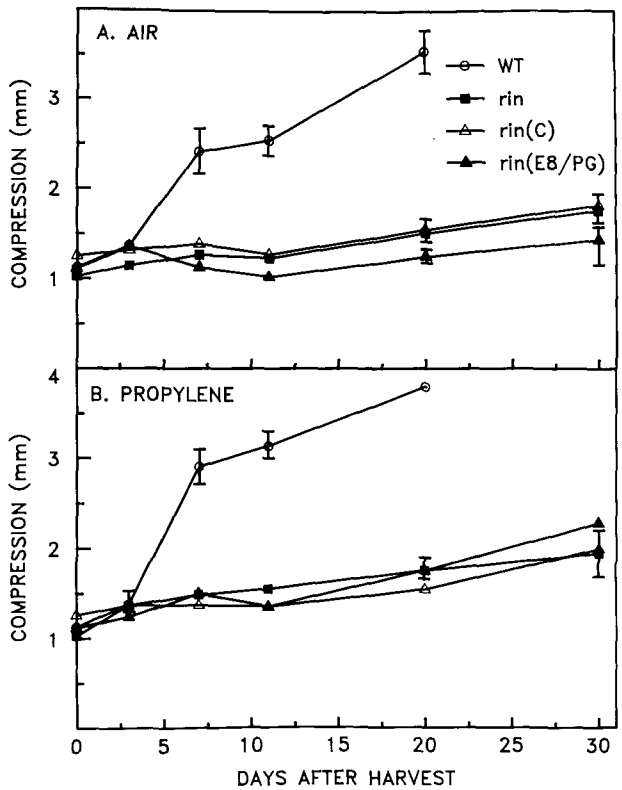


**Figure 6.** Compressibility of Wild-Type, *rin*, and Transgenic *rin* Fruit.

A minimum of five fruit (35 days after anthesis) were exposed to either air **(A)** or propylene **(B)** for the indicated period of time and fruit compressibility was determined. Error bars represent standard deviations. Where error bars are not shown, the standard deviation was no greater than the size of the symbol. WT, wild-type; *rin*(C), *rin*(C)-1; *rin*(E8/PG), *rin*(E8/PG)-2.

galacturonase activity in E8-polygalacturonase transformed *rin* fruit degrades polyuronides at near wild-type levels as shown by the production of EDTA-soluble uronic acid in transgenic *rin* cell walls. However, this activity had no detectable effect on softening, ethylene evolution, or color development.

## DISCUSSION

A major change in cell wall structure during tomato fruit ripening is the degradation of polyuronides. This is thought to reflect the action of polygalacturonase, an enzyme whose activity, mRNA concentration, and gene transcription all increase at the onset of ripening. To assess the physiological function of polygalacturonase during tomato

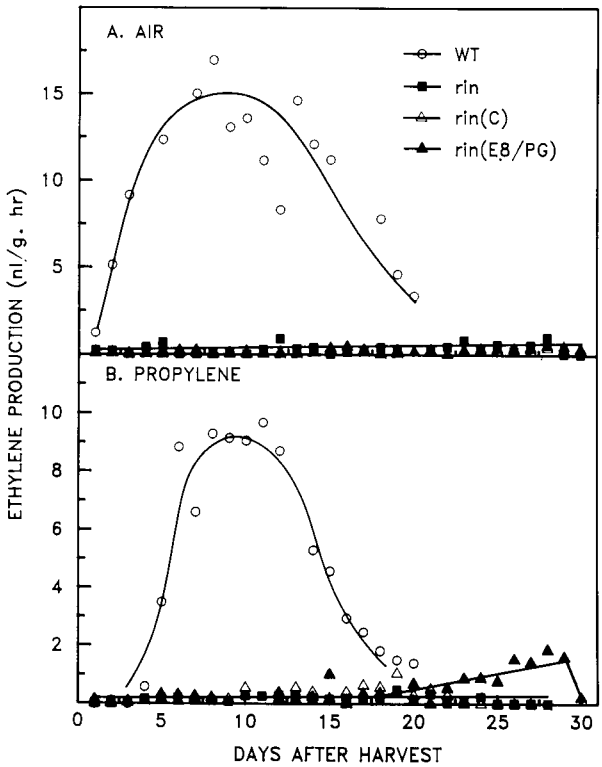


**Figure 7.** Evolution of Ethylene Gas by Wild-Type, *rin*, and Transgenic *rin* Fruit.

Fruit (35 days after anthesis) were exposed continuously to either air **(A)** or propylene **(B)** and at the indicated time the level of ethylene evolution was determined by the procedure of Su et al. (1984). Each point represents the mean of duplicate assays from a minimum of two fruit. WT, wild-type; *rin*(C), *rin*(C)-1; *rin*(E8/PG), *rin*(E8/PG)-2.

fruit ripening, we have utilized a pleiotropic genetic mutation, *rin*, that inhibits many aspects of ripening, including softening, ethylene production, color development, and polygalacturonase gene expression. Our strategy has been to construct a chimeric polygalacturonase gene that is expressed in *rin* fruit during the developmental period corresponding to wild-type fruit ripening. Insertion of the chimeric gene into the *rin* genome has allowed us to analyze the effect of polygalacturonase enzyme activity on cell wall polyuronide degradation, fruit softening, and other aspects of ripening.

### Chimeric Polygalacturonase Gene Expression

A chimeric gene was constructed by ligating the 5′-flanking and leader sequences of the E8 gene to the coding sequences of the polygalacturonase gene (Figure 1), and a single copy of the chimeric gene was introduced into the *rin* genome. However, expression of the chimeric gene was not completely as predicted. That is, although E8 mRNA was detected in air- and propylene-treated *rin*(E8/PG)-2 fruit (Figure 2A), E8-polygalacturonase mRNA was detected only in propylene-treated *rin*(E8/PG)-2 fruit (Figure 2B). This pattern of expression was observed also in two other independently transformed plants, *rin*(E8/PG)-1 and *rin*(E8/PG)-3 (Figure 3). One possible explanation is that the chimeric E8-polygalacturonase gene is not transcribed in air-treated *rin*(E8/PG)-2 fruit. Perhaps important regulatory sequences for E8 gene transcription in air-treated *rin* fruit reside either within the E8 gene or in 3′-flanking sequences and were therefore discarded when the E8-polygalacturonase chimeric gene was constructed. Alternatively, the defect in E8-polygalacturonase gene expression in air-treated *rin* fruit might be at the post-transcriptional level. That is, the E8-polygalacturonase gene might be transcribed, but the mRNA it encodes might not be transported to the cytoplasm, or be stable. In this regard it is important to note that ethylene has been shown to stimulate gene expression during fruit development at both the transcriptional and post-transcriptional levels (Lincoln and Fischer, 1988a, 1988b). To determine the level at which propylene acts, nuclear run-on transcription procedures will be utilized to measure the relative rate of E8-polygalacturonase gene transcription in air- and propylene-treated *rin*(E8/PG)-2 fruit.

In the presence of propylene, mRNA containing polygalacturonase coding sequences isolated from transgenic *rin* fruit was shown by S1-nuclease protection analysis to be derived solely from the E8-polygalacturonase chimeric gene (Figure 2B). Expression of the chimeric gene resulted in production of polygalacturonase protein that: (1) was electrophoretically indistinguishable from native polygalacturonase (Figure 3), (2) appeared to be targeted properly and localized in the cell wall, and (3) possessed enzymatic activity when assayed in vitro (Figure 4). This provided a transgenic system for analyzing the physiological function of polygalacturonase.

### Role of Polygalacturonase in Cell Wall Polyuronide Degradation

To determine whether the expression of the polygalacturonase gene in transgenic *rin* fruit resulted in degradation of cell wall polyuronides, we assayed for the presence of EDTA-soluble uronic acid in cell wall preparations of control and *rin*(E8/PG) fruit. Our results showed that the levels of EDTA-soluble polyuronides in *rin*(E8/PG) fruit when held in propylene were comparable with levels in wild-type fruit (Figure 5), in spite of the fact that polygalacturonase in vitro activity levels were 60% of wild-type (Figure 4). This result indicates that polygalacturonase is the primary determinant of polyuronide degradation in tomato fruit and suggests further that levels of polygalacturonase in wild-

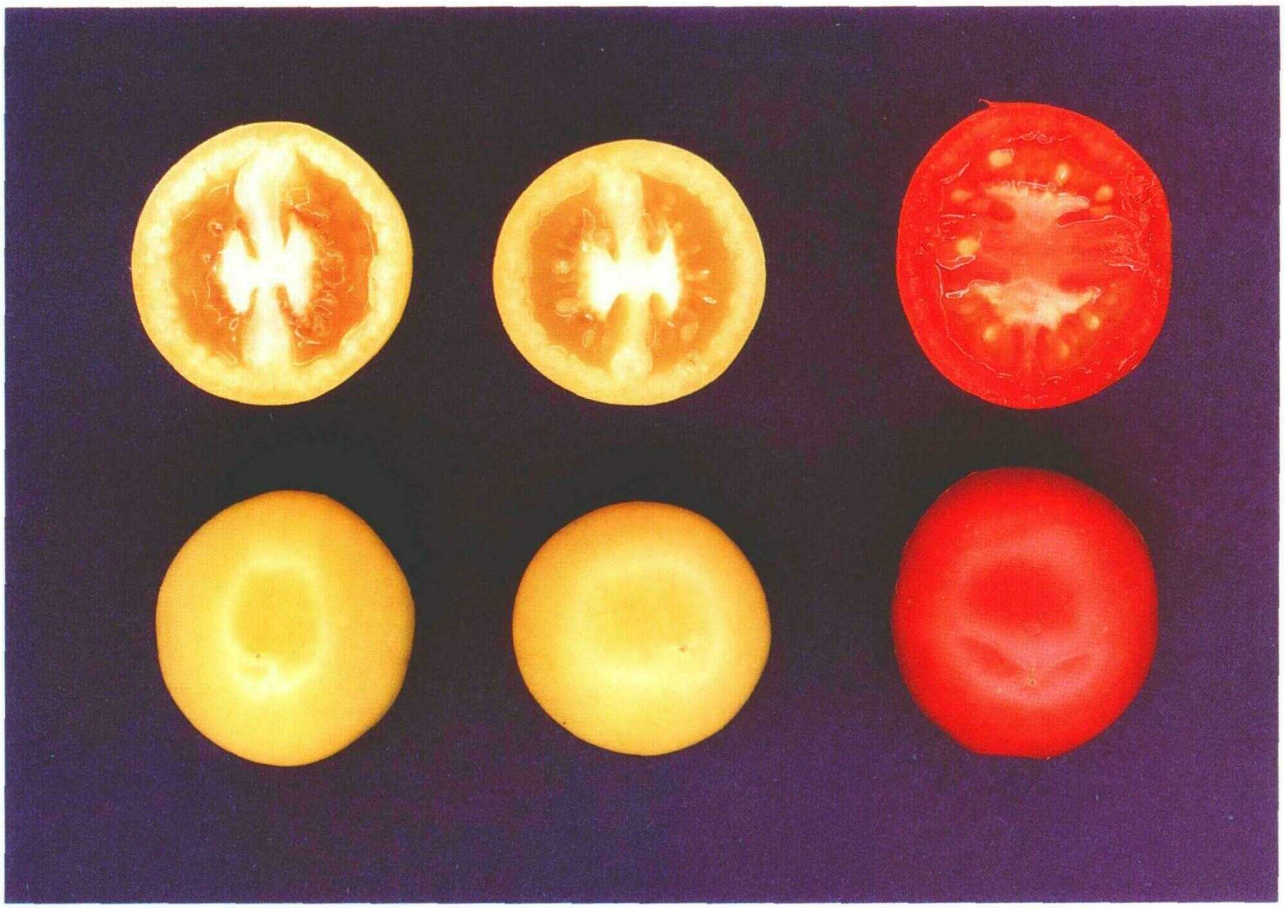

**Figure 8.** Transgenic *rin*, *rin*, and Wild-Type Fruit after Propylene Treatment.

Thirty-five-day fruit were exposed to propylene for 30 days. Fruit on the left is *rin*(E8/PG)-2, middle is *rin*, right is wild-type.

type fruit are in excess of that required to achieve maximal levels of EDTA soluble polyuronides. Indeed, in transgenic *rin*(E8/PG)-2 fruit, maximal production of EDTA-soluble polyuronides was observed at 11 days of propylene treatment, when the polygalacturonase enzyme level was less than 20% of the maximal level found in wild-type fruit. Although our results indicate a primary role for polygalacturonase in polyuronide degradation, further characterization of the size distribution of polyuronide fragments will be required to assess fully this function of polygalacturonase.

### Role of Polygalacturonase in Tomato Fruit Softening

Historically, the level of polygalacturonase enzyme activity and polyuronide degradation have been correlated roughly with an elevated rate of tomato fruit softening, and on this basis polygalacturonase was proposed to be a major determinant of tomato fruit softening. Our results demonstrate that high levels of polygalacturonase enzyme activity and polyuronide degradation are not sufficient to induce softening in transgenic *rin*(E8/PG)-2 fruit (Figure 6). Similar results in fruit from two other transformed plants, *rin*(E8/PG)-1 and *rin*(E8/PG)-3, were observed (data not shown).

Explanations for the lack of softening in propylene-treated *rin*(E8/PG) fruit include the possibility that insufficient levels of polygalacturonase enzyme were generated by expression of the E8-polygalacturonase chimeric gene. However, several results argue against this hypothesis. First, although the level of polygalacturonase in vitro activity in propylene-treated *rin*(E8/PG)-2 fruit was less than that found in wild-type fruit, the degree of polyuronide degradation observed was approximately the same, although delayed somewhat. That is, wild-type and *rin*(E8/PG)-2 fruit held for 7 and 11 days, respectively, contained similar levels of polygalacturonase enzyme activity (Figure 4B) and equivalent maximal levels of polyuronide degradation (Figure 5B). However, at these time points, significant softening had occurred in wild-type, but not *rin*(E8/

PG)-2 fruit (Figure 6B). Second, the levels of polygalacturonase activity and soluble polyuronides in 30-day propylene-treated *rin*(E8/PG)-2 fruit that do not soften are equivalent to those in 11-day propylene-treated wild-type fruit that do soften. Third, levels of polygalacturonase enzyme activity have been reduced to 10% of wild-type levels by the expression of polygalacturonase antisense genes (Smith et al., 1988; Sheehy et al., 1988) without detectable changes in tomato softening (Smith et al., 1988). These results suggest that propylene treatment induces sufficient levels of polygalacturonase activity and polyuronide degradation in transgenic *rin* fruit to bring about any polygalacturonase-associated effects on softening.

Another possible reason for the observed lack of softening is that the physiologically active form(s) of polygalacturonase associated with softening are not being made when the chimeric E8-polygalacturonase gene is expressed. In this regard it is important to note that the E8-polygalacturonase gene was constructed so that there would be no alterations in the polygalacturonase amino acid sequence (Figure 1). Also, non-denaturing polyacrylamide gel electrophoresis of cell wall protein extracts revealed that the three polygalacturonase isoforms in wild-type fruit (PG1, PG2A, PG2B; Ali and Brady, 1982) are present in the 30-day propylene-treated *rin*(E8/PG)-2 fruit (data not shown). This result makes it unlikely that the proper form of polygalacturonase is not being produced.

Other explanations for the lack of softening in the *rin*(E8/PG) fruit cannot be ruled out at this time; however, they can be addressed experimentally in the future. For example, little is known about how the *rin* mutation inhibits tomato fruit ripening, and it is possible that it somehow blocks or masks the effects of polyuronide degradation in the *rin*(E8/PG)-2 fruit. This issue can be addressed by the introduction of chimeric polygalacturonase genes into other genetic backgrounds, including wild-type tomato plants and other ripening-impaired mutants. The possibility also exists that polygalacturonase in transgenic *rin* fruit is not being synthesized in the appropriate pericarp cell type(s). In situ experiments designed to localize normal and chimeric polygalacturonase mRNA and protein can be used to answer this question. We are examining also alternative methods for measuring fruit softening that rely on parameters other than fruit compressibility. Finally, ripening in detached fruits may differ in subtle ways from ripening in attached fruits, and experiments are in progress in which attached *rin*(E8/PG)-2 fruit are being treated with ethylene.

### Polygalacturonase and Other Parameters of Fruit Ripening

No significant differences were found between propylene-treated *rin*(E8/PG)-2, *rin*(C)-1, and untransformed *rin* fruit with respect to ethylene evolution (Figure 7) or color development (Figure 8). This result contrasts with results reported by Baldwin and Pressey (1988) in which introduction of polygalacturonase into *rin* fruit by vacuum infiltration resulted in ethylene production and some lycopene pigment accumulation. However, it is possible that vacuum infiltration localizes polygalacturonase in regions not normally accessed via the natural protein secretory pathway. Polygalacturonase activity in areas in which it is absent normally may be perceived by the plant as a wound, thus resulting in ethylene evolution. The possibility also exists that the polygalacturonase infiltrate contains other cell wall degrading enzymes that might elicit ethylene biosynthesis (Fuchs and Anderson, 1987).

In summary, polygalacturonase protein has been introduced into *rin* tomato fruit pericarp cell walls by means of an E8-polygalacturonase chimeric gene. Propylene induction of chimeric gene expression in transgenic *rin* fruit resulted in the accumulation of polygalacturonase protein, a dramatic increase in polygalacturonase enzyme activity, and the in vivo degradation of polyuronides. However, the rate of softening, ethylene production, and color development did not increase appreciably. We conclude that polygalacturonase activity is necessary for polyuronide degradation, but is not sufficient for the induction of softening, ethylene production, or lycopene accumulation in transgenic *rin* fruit.

## METHODS

### Plant Material

Wild-type tomato seed (cv. Ailsa Craig) and seed isogenic for the *rin* (ripening inhibitor; Smith and Ritchie, 1983) mutation were obtained from the Glasshouse Crops Research Institute. Wild-type, *rin*, and transformed *rin* plants were all grown under standard greenhouse conditions. Tagged fruit were harvested at 35 d postanthesis and were at the mature green 2 to 3 stage (Lincoln et al., 1987) at the time of harvest.

### Plant Transformation

The 15.5-kb SalI restriction fragment from λE8-PG was subcloned into the intermediate vector pMLJ1 and transferred into the disarmed *Agrobacterium* pGV3850 Ti plasmid vector by the procedure of Van Haute et al. (1983). Sterile cotyledon pieces from *rin* plants were incubated on tobacco feeder cells and infected with *Agrobacterium* with the pGV3850:pMLJ1E8-PG cointegrate plasmid, or control pGV3850:pMLJ1, and transformants were selected with 50 mg/liter kanamycin by the procedure of Fillatti et al. (1987).

### Exposure of Fruit to Gasses

Thirty-five-day postanthesis fruit were placed in a 10-L glass chamber and exposed to 20 L/hr of humidified propylene in air (500 μl/L) or to humidified air alone for up to 30 days.

### Isolation and Analysis of Nucleic Acids

Total plant RNA was isolated from pericarp tissue as described in DellaPenna et al. (1986) and tomato leaf genomic DNA was isolated as described previously (Deikman and Fischer, 1988). DNA sequences were determined using the dideoxy chain termination method (Sanger et al., 1977). Synthetic oligonucleotide-mediated site-directed mutagenesis was performed using material and procedures supplied by Boehringer Mannheim (Indianapolis, IN). S1-nuclease protection analysis of total pericarp RNAs was performed as described in Deikman and Fischer (1988). Twenty-five micrograms of total pericarp RNA were used in each protection experiment.

### Protein Extraction and Polygalacturonase Enzyme Assays

Cell wall protein extracts and polygalacturonase activity assays were performed as described elsewhere (DellaPenna et al., 1987). Reducing sugars were measured by the arsenomolybdate method (Nelson, 1944) using $\alpha$-D-galacturonic acid as a standard.

### Protein Gel Electrophoresis and Protein Gel Blotting

Cell wall proteins were fractionated by SDS-polyacrylamide gel electrophoresis as described in DellaPenna et al. (1987). Preparation of biotinylated molecular weight standards, polygalacturonase antibodies, electrophoretic blotting to nitrocellulose, and immunological detection methods were as described (DellaPenna et al., 1986).

### Isolation of Pectic Polysaccharides and Uronic Acid Assay

Tomato pericarp cell walls were isolated from 10 g of frozen pericarp tissue, and EDTA-soluble uronic acids were isolated from 50 mg of dry cell wall material as described (Huber 1983a). The level of EDTA-soluble uronic acid was determined by the metahydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973).

### Measurement of Fruit Softening

Fruit softening was measured by extent of compression of the fruit when subjected to a 500-g weight for 15 sec as described previously (Brady et al., 1983).

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