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Polygalacturonase Isozymes and Pectin Depolymerization in Transgenic *rin* Tomato Fruit[1]

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ABSTRACT

We have previously described the construction and expression of a chimeric gene that allows developmentally regulated expression of tomato (*Lycopersicon esculentum*) polygalacturonase in ripening-impaired, mutant (*rin*) tomato fruit (JJ Giovannoni, D DellaPenna, AB Bennett, RL Fischer [1989] The Plant Cell 1: 53–63). We now show that expression of the chimeric polygalacturonase gene in *rin* tomato fruit resulted in the accumulation of all three polygalacturonase isozymes (PG1, PG2A, and PG2B). Polyuronide solubilization and polyuronide depolymerization both reached their maximal levels in transgenic *rin* fruit prior to the appearance of PG2 isozymes. These results demonstrate that PG1, PG2A, and PG2B all arise by differential processing of a single gene product and further suggest that the PG1 isozyme is sufficient to carry out both polyuronide solubilization and depolymerization *in vivo*.

Tomato fruit ripening is characterized by a number of coordinated biochemical and physiological changes, which collectively alter fruit color, flavor, and texture. Biochemical and genetic analysis (6, 7) has indicated that many of the physiological processes associated with ripening are regulated at the level of gene expression, a view firmly supported by recent analysis of gene expression during tomato fruit ripening (1, 8, 11, 14, 17, 28).

Among the ripening-associated enzymes studied in tomato, the cell wall-degrading enzyme polygalacturonase has been analyzed in the greatest detail (2, 4, 5, 8, 9, 12, 13, 24–26). The site of action of polygalacturonase is the cell wall, where it hydrolyzes polyuronides, or pectins. The increase in the level of chelator-soluble polyuronides during ripening and their corresponding decrease in molecular size have been well documented and are attributed to the action of polygalacturonase (15, 22, 23). Polygalacturonase activity isolated from ripe fruit is comprised of three closely related isozymes, PG1, PG2A, and PG2B (4, 5, 10, 18–20). The PG2 isozymes (PG2A and PG2B) are comprised of a single catalytic polygalacturonase subunit and differ from one another by degree of glycosylation (10). PG1 is comprised of a single catalytic polygalacturonase subunit (PG2A or PG2B) associated with a 41 kD glycoprotein ancillary subunit derived from a separate, uncharacterized gene (18). Thus, the catalytic subunit of all three PG isozymes appears to be derived from a single gene, with PG1 arising from further assembly of the catalytic subunit with an ancillary polypeptide.

The role of each PG isozyme has been controversial. It has been suggested that PG1 is an artifact of extraction, with PG2A and PG2B being the only isozymes present *in vivo* (20). However, Knegt *et al.* (16) have presented data contrary to this view and propose that the association of PG2 isozymes with the noncatalytic subunit to form PG1 is required for proper anchoring in the cell wall and that PG1 is therefore the physiologically active isozyme. Recent analyses of transgenic tomato fruit expressing an antisense polygalacturonase gene suggest that different PG isozymes are responsible for distinct aspects of pectin degradation, with PG1 acting to solubilize pectin from the cell wall and PG2 isozymes responsible for depolymerization of pectic polymers (27).

In order to investigate critically the physiological role of polygalacturonase in the ripening process, we developed a molecular genetic strategy to modify the expression of polygalacturonase *in vivo* in the tomato ripening mutant *ripening-inhibitor* (*rin*). We have previously reported the construction and transfer into *rin* plants of a chimeric polygalacturonase gene containing a promoter that could be activated in mature fruit by exogenous application of ethylene or its structural analog propylene (12). Mature transgenic *rin* fruit carrying the chimeric gene (referred to herein as *rin*[E8/PG] fruit) were found to accumulate polygalacturonase mRNA and enzymically active polygalacturonase protein upon exposure to propylene. Our previous results indicated that polygalacturonase was produced in *rin*[E8/PG] fruit at a developmental time corresponding to ripening in wild-type fruit and that the protein was properly processed and targeted to the cell wall. The expression of polygalacturonase in *rin*[E8/PG] fruit resulted in an increase in the levels of chelator-soluble polyuronides comparable to that in ripening wild-type fruit but failed to influence softening or other ripening parameters (12). The determination of chelator-soluble polyuronides provides a measure of polyuronides that have been enzymically cleaved by the action of polygalacturonase but remain ionically bound in the cell wall by calcium cross-linkage to other acidic cell wall polymers. Although this determination is often employed as an indication of *in vivo* polygalacturonase activity, it does not reflect the extent of polyuronide depolymerization, an

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additional measure of *in vivo* polygalacturonase activity. Additional factors such as the presence, levels, and timing of appearance of PG1 and PG2 isozymes might also influence activity and hence the physiological action of polygalacturonase in *rin*[E8/PG] fruit. Here we have analyzed the accumulation of polygalacturonase isozymes and the depolymerization of pectic polymers in *rin*[E8/PG] fruit as a means to evaluate the contribution of specific polygalacturonase isozymes to pectin degradation in ripening tomato fruit.

MATERIALS AND METHODS

Plant Material

Details of the production and initial characterization of polygalacturonase-expressing transgenic *rin* tomato (*Lycopersicon esculentum*) (designated herein as *rin*[E8/PG] fruit) have been previously reported (12). A control *rin* plant, designated *rin*C, was transformed with the intermediate vector pMLJ1 alone and the fruit from this plant were used as a control in the experiments reported. Wild-type, *rin*C and *rin*[E8/PG] plants were grown under standard conditions in a greenhouse and fruit harvested at the mature green stage (35 DAF). Fruit were held under continuous exposure to 500 ppm propylene for up to 30 d and at the appropriate time fruit were sectioned, locular material was removed, and pericarp tissue was quick frozen in liquid nitrogen prior to being stored at −80°C until use.

Polygalacturonase Isozyme Analysis

Cell wall proteins from pericarp tissue were extracted and assayed for polygalacturonase activity as described (9). Polygalacturonase isozyme composition was determined using nondenaturing polygalacturonase activity gels, performed as described (9) using the gel system of Reisfeld *et al.* (21).

Isolation and Column Chromatography of Pectic Polysaccharides

Acetone-insoluble cell wall materials were isolated from pericarp tissue as described by Huber (15) and Seymour *et al.* (23). Care was taken to fully disperse cell wall material during the phenol:acetic acid:water treatment (2:1:1; w:v:v) to ensure complete inactivation of endogenous pectic enzymes (23). EDTA-soluble pectic polysaccharides were extracted from the acetone-insoluble cell wall material by incubation for three hours at room temperature in 50 mM sodium acetate, 40 mM EDTA (pH 4.5) as described (15). The extraction volumes used typically yielded a final concentration of approximately 1.0 mg/mL of uronic acid equivalents, which was then adjusted to 0.5 mg/mL. Uronic acids were measured by the metahydroxydiphenyl assay (3) using polygalacturonic acid as a standard. Gel filtration of isolated EDTA-soluble polyuronides was performed at 20°C using a Sepharose CL 4B, Sephacryl S-300, Sephacryl S-400 or Sephacryl S-500 (Pharmacia) column (70 × 1.5 cm) in a column buffer of 100 mM sodium acetate, 20 mM EDTA (pH 6.5). One mg of EDTA-soluble polyuronides was applied to the column in a volume of 2.0 mL and chromatographed at a flow rate of 21 mL/h. Fractions of 2.1 mL were collected and analyzed for uronic acid content. Yields from gel filtration columns were always more than 90%.

RESULTS

Polygalacturonase Isozymes

The composition of polygalacturonase isozymes from wild-type and transgenic *rin* fruit was analyzed by activity staining of isolated cell wall proteins following native gel electrophoresis. As shown in Figure 1, PG1 was first detectable after 3 d of propylene treatment in wild-type fruit and after 7 d in *rin*[E8/PG] fruit, and was then followed by the accumulation of PG2 isozymes after 11 and 30 d of propylene treatment in wild-type and *rin*[E8/PG] fruit, respectively. This pattern of polygalacturonase isozyme accumulation is similar to that observed previously in wild-type tomato fruit (4, 5, 30). Accumulation of polygalacturonase in *rin*[E8/PG] fruit is delayed relative to wild-type fruit but follows a similar pattern, resulting in the appearance of PG1 followed by PG2A and PG2B (Fig. 1, panel B). We have previously demonstrated that the polygalacturonase mRNA that accumulates in *rin*[E8/PG] fruit is derived exclusively from the chimeric E8/PG gene (12). Thus, the accumulation of PG1, PG2A, and PG2B in *rin*[E8/PG] fruit confirms a previous proposal that all three polygalacturonase isozymes are derived by differential processing of a single gene product (10).

Comparison of the polygalacturonase isozyme composition (Fig. 1A, lower panel) with the extent of polyuronide solubilization (Fig. 1A, upper panel) in wild-type fruit indicates that near maximal levels of polyuronide solubilization occurred prior to the appearance of PG2 isozymes. The slower accumulation of polygalacturonase in *rin*[E8/PG] fruit expanded the period when PG1 but not PG2 isozymes were present in tomato fruit. Comparison of the polygalacturonase isozyme composition (Fig. 1B, lower panel) with the extent of polyuronide solubilization (Fig. 1B, upper panel) in *rin*[E8/PG] fruit indicates that near maximal levels of polyuronide solubilization occurred at least 9 days prior to the appearance of PG2 isozymes.

Polyuronide Depolymerization

Because polygalacturonase activity results in the depolymerization of polyuronide polymers, as well as in polyuronide solubilization, we examined the size of chelator soluble polyuronides in wild-type and *rin*[E8/PG] fruit using gel filtration chromatography (15, 22, 23). Previous procedures using gel filtration to fractionate polyuronides have differed in the chromatographic medium used with one report using Ultrogel AcA34 (15) and others using Sephacryl S-400 (22, 23). Because of the significant differences in chromatographic properties of Ultragel AcA34 and Sephacryl S-400 we evaluated the suitability of several gel filtration chromatographic media to determine which was best suited for discriminating size fractions of polyuronides from mature green and ripe wild-type tomato fruit. Initial attempts to resolve polyuronide polymer fragments by chromatography on Ultrogel AcA34 as previously described (15) were unsuccessful, with all polyuronide polymer fragments from mature green and ripe fruit eluting in the void volume (data not shown). Polyuronides

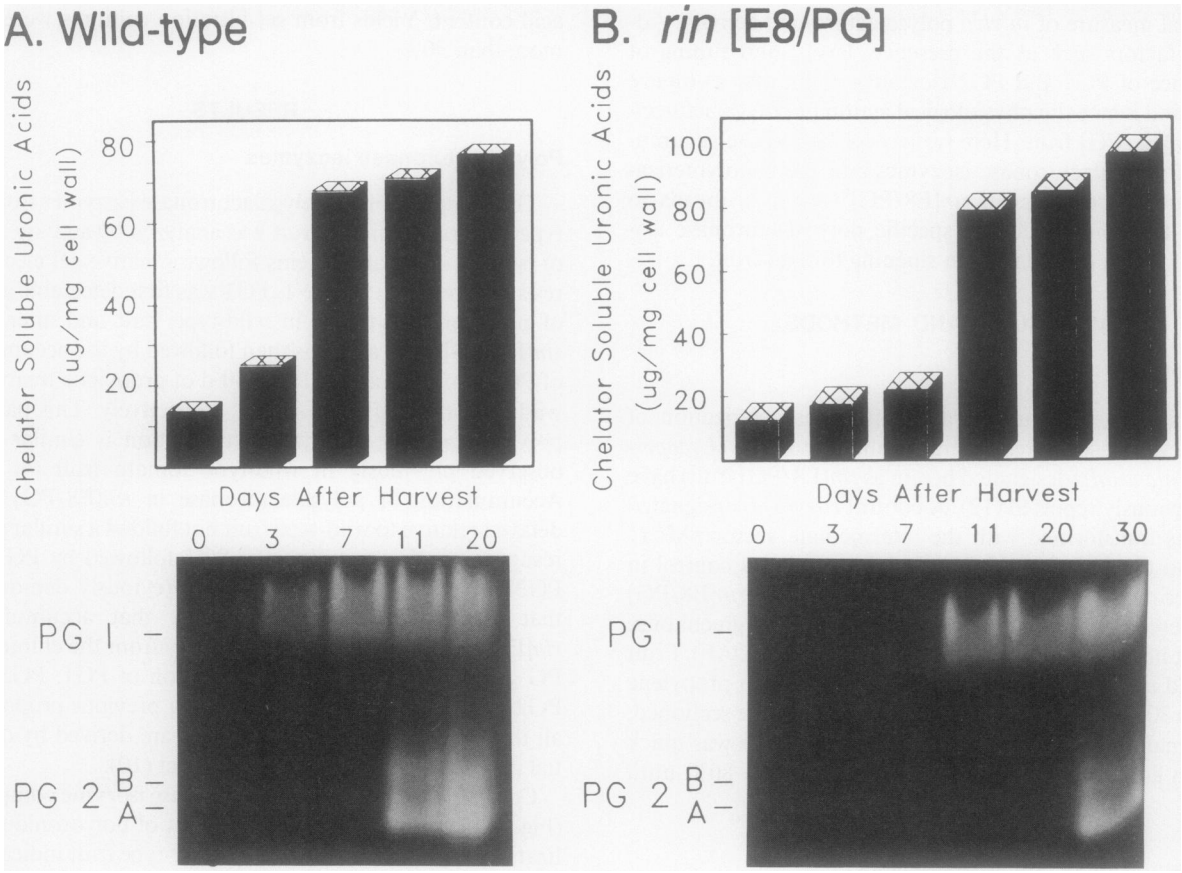

Figure 1. Polygalacturonase isozyme composition and release of chelator soluble uronic acids during ripening of wild-type fruit (A) and over the same period in *rin*[E8/PG] fruit (B). After reaching the mature green stage, fruit of both genotypes were harvested and held in 500 ppm propylene to induce chimeric polygalacturonase gene expression and sampled at the indicated time points. Protein was extracted and polygalacturonase isozyme composition analyzed by nondenaturing gel electrophoresis (lower panels). Data for chelator soluble uronic acids (upper panels) are replotted from Giovannoni *et al.* (12) to facilitate direct comparison to the isozyme composition at each time point.

from mature green and ripe fruit eluted in or near the void volume of Sephacryl S-300 (Fig. 2A). However, polyuronides from mature green and ripe fruit gave distinct chromatographic profiles on Sephacryl S-400 and Sepharose CL4B, indicating the appearance of smaller polyuronide fragments upon fruit ripening (Fig. 2B, D). Polyuronides from both mature green and ripe fruit were entirely included on Sephacryl S-500 (Fig. 2C). Chromatographic profiles of polyuronide fragments on Sephacryl S-400 and Sepharose CL4B were very similar to those described by Seymour *et al.* (22, 23) who also used Sephacryl S-400, suggesting that these matrices provide a useful chromatographic separation to analyze ripening-associated changes in tomato fruit polyuronide fragments. Sepharose CL4B gave consistently reproducible results and was used for further analysis.

Polyuronides isolated from propylene-treated *rin*C fruit eluted in or near the column void volume, indicating a high degree of polymerization, and this pattern did not appreciably change after 11 and 30 d of exposure to propylene (Fig. 3A), consistent with the low level of endogenous polygalacturonase activity in this genotype (9). Polyuronides isolated from air-treated *rin*C and air-treated *rin*[E8/PG] fruit (which also have low levels of endogenous polygalacturonase activity) exhibited polyuronide chromatographic profiles similar to *rin*C fruit (results not shown). Polyuronides isolated from mature green (0 d, Fig. 3B) wild-type fruit showed a chromatographic profile similar to *rin* fruit. However, after ripening of wild-type fruit for 7 d, a substantial portion of chelator-soluble polyuronides showed a decrease in size, as evidenced by their elution in the included volume of the column (Fig. 2B, fractions 43–80). The size distribution of chelator-soluble polyuronides isolated from overripe wild-type fruit (20 d of propylene treatment) was similar to that observed after 7 d (Fig. 2B), indicating that the substantial increase in polygalacturonase accumulation between 7 and 20 d (Fig. 1A) did not result in further polyuronide depolymerization.

Analysis of the extent of depolymerization of chelator-soluble polyuronides from propylene treated *rin*[E8/PG] fruit indicated that, as in wild-type fruit, a substantial portion of chelator-soluble polyuronides exhibited a decrease in size that was first observed after 11 d of exposure to propylene (Fig. 3C). The levels of polygalacturonase activity in *rin*[E8/PG] fruit and wild-type fruit were approximately equal after 11 and 7 d, respectively (12). As with wild-type fruit, the substantial increase in polygalacturonase activity in *rin*[E8/PG] fruit between 11 and 30 d of propylene treatment failed to

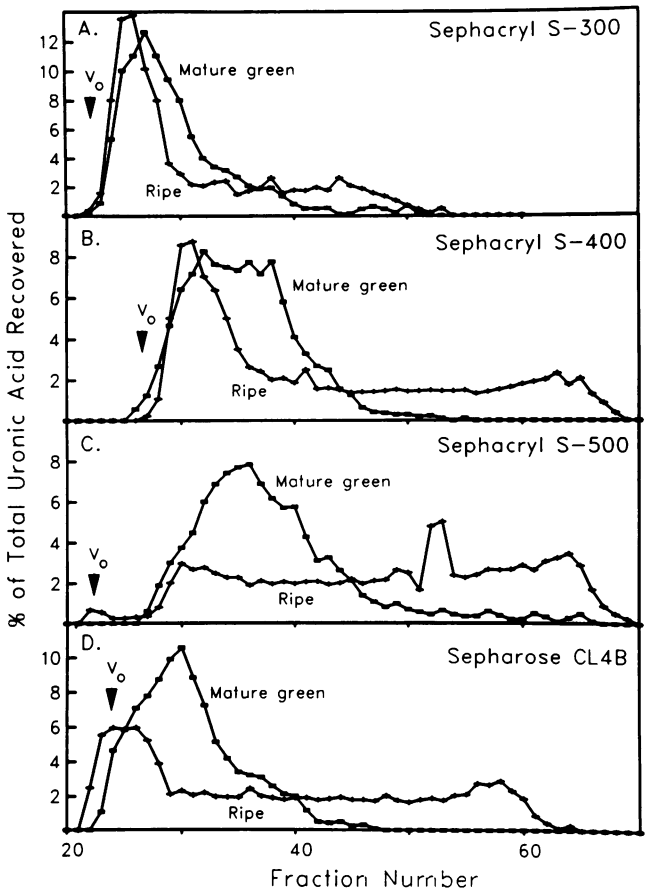

Figure 2. Gel filtration chromatographic analysis of chelator soluble polyuronides isolated from mature green and ripe wild-type-tomato fruit. One milligram of chelator soluble uronic acid was chromatographed on columns of Sephacryl S-300 (A), Sephacryl S-400 (B), Sephacryl S-500 (C) or Sepharose CL 4B (D) as described in "Materials and Methods." Each fraction was assayed for uronic acid content and is expressed as the percentage of total recovered from the column. In all cases recovery exceeded 90% of the uronic acid applied. The void volume of each column is indicated (V_o).

produce further polyuronide depolymerization. The quantity of small polyuronide fragments (column fractions 43–80) produced in propylene-treated *rin*[E8/PG] fruit was considerably higher than in *rin*C fruit and was 67% of that produced by wild-type fruit. It is interesting to note that the maximal level of polyuronide depolymerization, as determined by size fractionation of chelator-soluble polyuronides (Fig. 3), occurs prior to the accumulation of PG2 isozymes in both wild-type and *rin*[E8/PG] fruit (Fig. 1). This is especially apparent in *rin*[E8/PG] fruit where there is a longer delay between the appearance of the PG1 and PG2 isozymes.

DISCUSSION

The production of transgenic plants with altered polygalacturonase levels has provided a way to assess the role of polygalacturonase isozymes in cell wall degradation. Smith *et al.* (27) demonstrated that tomato plants carrying one or two copies of an antisense polygalacturonase gene accumulated greatly reduced levels of polygalacturonase protein. In fruit with reduced polygalacturonase, the remaining polygalacturonase was present as the PG1 isozyme. In these same fruit polyuronide solubilization (production of chelator soluble uronic acids) was not affected but polyuronide depolymerization was inhibited. This led the authors to propose that PG1 may account for polyuronide solubilization but PG2 isozymes were required to promote polyuronide depolymerization (27). The interpretation of Smith *et al.* (27) implies that polyuronide solubilization and depolymerization are distinct degradative processes rather than reflecting two measurements of the same catalytic process.

Our experiments differed from those described above, in that we constructed a chimeric polygalacturonase gene that was expressed in *rin* fruit, a genotype that normally fails to accumulate polygalacturonase. In these fruit, polygalacturonase accumulates more slowly than in wild-type but eventually all three polygalacturonase isozymes are produced. This

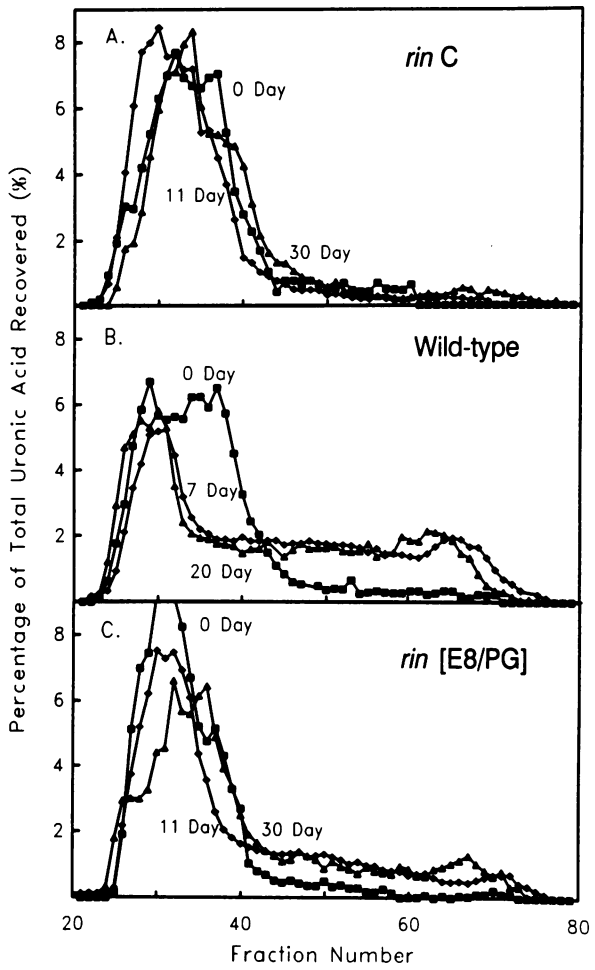

Figure 3. Gel filtration chromatographic analysis of chelator soluble polyuronides isolated from various ripening stages of *rin*C (A), wild-type (B), or *rin*[E8/PG] fruit (C). One milligram of chelator soluble uronic acid isolated from fruit of the indicated genotype and days after harvest was chromatographed on a Sepharose CL4B column as described in "Materials and Methods." Panel A, C: (■), 0 d; (♦), 11 d; (▲), 30 d. Panel B: (■), 0 d; (♦), 7 d; (▲), 20 d.

clearly indicates that all three polygalacturonase isozymes arise from differential processing of a single gene product because a single chimeric polygalacturonase gene was transferred into the *rin* tomato plant. The decreased rate of polygalacturonase accumulation relative to wild-type fruit resulted in a period of at least 13 d when the only polygalacturonase isozyme present in the fruit was PG1. During this period maximum polyuronide solubilization and maximum polyuronide depolymerization occurred, suggesting that PG1 carries out both aspects of polyuronide degradation that is associated with fruit ripening. Our interpretation is consistent with the proposal of Knegt *et al.* (16) that PG1 is the physiologically active form of polygalacturonase *in vivo,* and further suggests that polyuronide solubilization and depolymerization represent two manifestations of the same catalytic process.

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