Transcriptional Analysis of Polygalacturonase and Other Ripening Associated Genes in Rutgers, rin, nor, and Nr Tomato Fruit

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ABSTRACT

We have studied the transcription of polygalacturonase (PG) and several other ripening-associated genes in wild-type tomato (Lycopersicon esculentum) fruit and three ripening-impaired mutants, rin, nor, and Nr. In wild-type fruit, the PG gene becomes transcriptionally active early in ripening and remains transcriptionally active during the ripening process. Fruit of the three ripening-impaired mutants, which have reduced levels of PG mRNA, have correspondingly reduced PG transcription rates. Other ripening-associated genes showed diverse patterns of expression in the ripening-impaired mutant backgrounds. These results indicate that transcriptional activation of the PG gene is an important control point regulating the expression of PG during ripening in wild-type fruit and that PG expression in rin, nor, and Nr fruit is blocked at the level of transcription. A comparison of PG transcription rates and mRNA levels with those of other ripening-associated genes suggests that posttranscriptional processes may also contribute to the large accumulation of PG mRNA during ripening.

Fruit ripening is a complex, developmentally regulated process resulting from the coordination of numerous biochemical and physiological changes within the fruit tissue (6, 7). In ripening tomato fruit, these changes include increased ethylene biosynthesis, lycopene production, Chl degradation, alterations in acidity and aroma, and softening of the fruit tissue. The softening of tomato fruit during ripening has been the focus of considerable research and a single cell wall enzyme, PG, 2 has been implicated as the primary enzyme mediating this process (for review, see ref. 4). PG activity and immunologically detectable protein increase dramatically during ripening (8), and studies with PG cDNA clones have shown that during this period the steady state levels of PG mRNA increase more than 2000-fold, accounting for as much as 2% of the mRNA mass in ripe fruit (3, 9, 10).

Further studies of gene expression in tomato fruit using in vitro translation and RNA blot analysis have shown increases in the steady state levels of other mRNAs during ripening (5, 12–14, 19, 23, 25, 26). Application of exogenous ethylene to mature green tomato fruit, a treatment that hastens the onset of ripening, stimulates the accumulation of some of these ripening-associated mRNAs (19, 24) and also increases the transcription rates of several of the corresponding genes (20). Conversely, exposure of tomato fruit to norbornadiene, an inhibitor of ethylene action and ripening, represses the accumulation of several ripening-associated mRNAs (19). These observations support the hypothesis that alterations in gene expression play an important role in the ripening process.

One advantage in using tomato fruit as a system for studying gene expression during ripening is the existence of several single-gene mutations which impair the ripening process. Normal changes such as lycopene production, increased ethylene production, and the appearance of PG activity are all repressed to varying degrees by the mutations (28). These mutations have been useful tools in comparative studies with normally ripening genotypes (10, 20). We have previously characterized the steady state PG mRNA levels in wild-type and three ripening-impaired mutant genotypes of tomato and found that, in contrast to the high level of PG mRNA expression in wild-type fruit, fruit of the mutant genotypes showed greatly reduced but distinct patterns of PG mRNA accumulation (10). The regulation of PG gene expression during fruit ripening may occur at many levels including gene transcription, mRNA processing and transport, mRNA stability, and translation (11). To extend our understanding of the mechanisms regulating gene expression during tomato fruit ripening we have determined the relative transcription rates and steady state mRNA levels of PG and other ripening-associated genes in wild-type and mutant genotypes.

MATERIALS AND METHODS

Plant Materials

Two tomato (Lycopersicon esculentum) genotypes were used in our analysis of transcriptional activity of PG. VFNT cherry fruit were used to examine early induction of PG expression since this genotype has been previously characterized with regard to gene expression in the earliest stages of ripening (19). Rutgers fruit were used to examine PG transcription in wild-type and ripening-impaired mutant fruit because isogenic mutants are available in this genetic back-
Nuclei isolation and in Vitro \[^{32}P\]nRNA Synthesis

Nuclei from VFNT cherry fruit were isolated, and \[^{32}P\]nRNA synthesis was carried out as described (20). Nuclei from Rutgers, \textit{rin}, \textit{nor}, and \textit{Nr} genotypes were isolated following published procedures (20, 30) with the following modifications. After phenol extractions, the aqueous phases were pooled, and an equal volume of 10% (w/v) TCA containing 30 mM NaH2PO4 was added and the solution incubated on ice for 30 min. The precipitated \[^{32}P\]nRNA was collected by vacuum filtration onto a 0.45 μm Millipore filter and washed twice with 5 mL of ice-cold, 5% (w/v) TCA containing 30 mM NaH2PO4. The filter was then placed into a silanized glass liquid scintillation vial containing 60 μg of RNase-free DNase in 1 mL of 10 mM Tris (pH 7.6), 10 mM MgCl2 and was incubated at 37°C. After 30 min, EDTA and SDS were added to 15 mM and 1% (w/v), respectively, and incubation continued at 65°C. After 10 min the liquid (containing released \[^{32}P\]nRNA) was removed and replaced with 1 mL of 10 mM Tris (pH 7.6), 10 mM EDTA containing 1% SDS and incubated at 65°C. After 10 min the liquid was removed. This process was repeated a total of three times after which the aqueous samples were pooled and the \[^{32}P\]nRNA was ethanol precipitated. The \[^{32}P\]nRNA was then extracted with cetyltrimethylammonium bromide to remove polysaccharides, ethanol precipitated, and used for hybridizations.

DNA Gel Blotting and \[^{32}P\]nRNA Hybridization

Each plasmid was digested with the appropriate restriction enzyme to liberate the cDNA insert. Five μg of DNA from each digestion was separated by agarose gel electrophoresis and blotted to nitrocellulose. Prehybridization and hybridization were for 24 and 48 h, respectively, as described (20). Each hybridization contained 6 × 10⁶ cpm of purified \[^{32}P\]nRNA. Under these conditions of DNA excess, the amount of \[^{32}P\]nRNA hybridized reflects the rate of transcription of the respective gene (16, 17). After hybridization and washing, the blots were treated with RNase as described (30). After autoradiography the radioactivity associated with each cDNA insert was determined by scintillation counting. Hybridization to vector sequences was undetectable.
RESULTS

Run-Off Transcription in Isolated Tomato Fruit Nuclei

The yield of nuclei was similar for all genotypes and ages and, assuming a DNA content of 1.5 pg/nucleus, ranged from 4 x 10^8 to 1 x 10^9 nuclei per g fresh tissue (Table I). Nuclei prepared as described incorporated [32P]UTP into mRNA for up to 90 min, and incorporation was proportional to the number of nuclei in the reaction (results not shown). The nuclei were nearly identical in their ability to incorporate [32P]UTP when expressed as a function of DNA content (Table I). These results indicated that nuclei isolated from all developmental stages and all genotypes were transcriptionally active and were comparable to other isolated plant nuclei (2, 15, 18, 20, 21).

Transcription of the PG Gene in Normal and Ripening-Impaired Mutant Fruit

We have previously shown that during tomato fruit ripening there is a dramatic increase in steady state PG mRNA levels with PG mRNA first becoming detectable early in ripening after a slight but detectable rise in ethylene production by the fruit (9). To determine whether this mRNA increase results from activation of PG gene transcription, we analyzed the relative transcription rates and steady state PG mRNA levels during the early stages of ripening in VENT cherry fruit, the genotype in which gene expression at the earliest stages of ripening has been well characterized (19). These data, as well as those for the constitutively expressed gene D21, are shown in Figure 1. Immature green fruit were approximately 20 d old, MG1 fruit approximately 35 d old, and the transition from MG1 to MG4 occurs over a period of approximately 3 to 5 d. We did not detect PG gene transcription in immature, MG1, or MG2 fruit. Transcription of the PG gene first became detectable at the MG3 stage, when a rise in ethylene production occurred, and continued to increase in MG4 fruit, a stage when lycopene synthesis first becomes detectable. As shown in Figure 1, the steady state PG mRNA levels closely paralleled this increase in PG transcription. In contrast, both the transcription rate and mRNA level of the constitutively expressed cDNA clone D21 were relatively constant during this period. These results indicate that transcriptional activation of the PG gene is an important factor in the induction of PG gene expression during the early stages of tomato fruit ripening.

PG gene transcription was also studied during later stages of ripening in Rutgers fruit, a genotype in which well characterized isogenic lines of the ripening-impaired mutants are available (Fig. 2). At 45 d after flowering (maturity stage equivalent to MG2 of VENT cherry) PG gene transcription and mRNA in Rutgers were near background levels. At 55 d the fruit were fully ripe and showed maximal ethylene production rates. PG transcription rates at this time had increased at least 50-fold over their level at 45 d, while PG mRNA levels had increased over 2000-fold. By 67 d Rutgers fruit were overripe and showed decreased ethylene production rates. The PG transcription rate had decreased 90% by this time while PG mRNA levels decreased approximately 80%. D21 transcription levels varied less than 2-fold during the same 3-week period while D21 mRNA levels showed a slow and steady decline. These results suggest that transcriptional processes play an important role in both the initiation (Fig. 1) and maintenance (Fig. 2) of PG expression throughout the ripening process.

We have previously shown that PG mRNA levels in the ripening mutants rin, nor, and Nr are greatly reduced compared to the levels in wild-type fruit of similar age (10). As shown in Figure 2, the relative transcription rates of PG in the mutants were also greatly reduced relative to wild-type levels. PG transcription rates in rin and nor at 55 d were less than 3% of the corresponding wild-type transcription rates, while PG mRNA levels in these mutants were less than 1% of wild-type levels. Nr fruit showed a delayed and attenuated PG transcription rate reaching 20% of the maximal wild-type rate by 67 d. PG mRNA in Nr fruit reached 8% of the maximal wild-type level by this time. D21 transcription rates

Table I. Yield of Nuclei and [32P]UTP Incorporation for the Indicated Tomato Genotypes and Fruit Developmental Stages

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Developmental Stage</th>
<th>Yield of Isolated Nuclei</th>
<th>Incorporation of [32P]UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAF</td>
<td>μg DNA/g tissue</td>
<td>CPM/μg DNA x 10^6</td>
</tr>
<tr>
<td>Rutgers</td>
<td>45</td>
<td>1.05</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>0.9</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>1.26</td>
<td>2.6</td>
</tr>
<tr>
<td>rin</td>
<td>45</td>
<td>0.58</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>0.76</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>1.36</td>
<td>2.0</td>
</tr>
<tr>
<td>nor</td>
<td>45</td>
<td>0.86</td>
<td>3.7</td>
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</tr>
<tr>
<td></td>
<td>67</td>
<td>1.04</td>
<td>3.3</td>
</tr>
<tr>
<td>Nr</td>
<td>45</td>
<td>1.32</td>
<td>3.3</td>
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<td></td>
<td>55</td>
<td>1.08</td>
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<td></td>
<td>67</td>
<td>1.22</td>
<td>2.2</td>
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* Incorporation analysis was performed in a 20 μL reaction volume containing 40 μCi of [32P]UTP (>3000 Ci/mum) using the conditions of Walling et al. (30).
E8, and J49 was affected differently by each of the ripening mutations (Fig. 3). E4, like PG, showed near complete transcriptional repression in all three ripening-impaired mutants (Fig. 3, panel A). However, E4 was the only gene examined whose expression was affected by the mutations in the same manner as PG.

E8 mRNA levels in wild-type fruit closely paralleled transcription rates indicating that, as with PG, transcriptional

in mutant fruit were similar to those observed in wild type, varying less than 2-fold, while D21 mRNA levels decreased with age.

Transcription Rates and mRNA Levels of Other Ripening-Associated Genes in Normal and Mutant Fruit

To extend our understanding of the mechanisms controlling gene-expression during ripening, we analyzed the transcription and mRNA accumulation of several other ripening-associated genes in wild-type and mutant genotypes. The four ripening-associated clones E4, E17, E8, and J49 have been characterized elsewhere (19, 20). Transcriptional analysis of these genes has only been performed during early ripening stages (MG1 through MG4) in wild-type and rin fruit (20). The data presented here have extended this analysis to later ripening time points and have been expanded to include wild-type, rin, nor, and Nr genotypes.

The expression of the ripening-associated genes, E4, E17,
regulation is an important control point in the expression of E8 in wild-type fruit. E8 transcription was partially repressed in all three mutants (Fig. 3, panel B). In rin and nor, E8 transcription, though reduced, followed the same pattern as in wild-type fruit (i.e. peaking at 55 d), while in Nr E8 transcription steadily increased with age. E8 mRNA continued to accumulate with time in the three ripening-impaired mutants and did not follow transcription rates closely. E8 mRNA continued to accumulate in 67 d rin and nor fruit despite decreased transcription rates, suggesting that posttranscriptional processes may contribute to determining E8 mRNA levels in 67 d rin and nor fruit.

Lincoln and Fischer (20) reported that E17 transcription was only slightly reduced by the rin mutation whereas E17 mRNA levels were significantly reduced. They concluded that posttranscriptional processes were responsible for the failure of E17 mRNA to accumulate in the rin mutant. Our results for E17 expression in 45 and 55 d old rin fruit agree with this conclusion. Furthermore, E17 expression in nor and Nr also appeared to be posttranscriptionally affected, resulting in a failure of E17 mRNA to accumulate in 55 d nor and Nr fruit, despite transcriptional activation of the gene (Fig. 3, panel C). By 67 d all three mutants had partially overcome this posttranscriptional block in expression and had begun to accumulate E17 mRNA, though at a reduced level relative to wild-type fruit.

The rin and Nr mutations had little effect on the expression of J49 (Fig. 3, panel D). J49 transcription rates and mRNA levels in rin and Nr were similar to wild-type levels. In contrast, the nor mutation had a dramatic effect on J49 expression at both the transcriptional and steady state mRNA levels. J49 was transcriptionally activated in nor fruit, reaching a rate 10-fold higher than maximal wild-type levels with its mRNA accumulating to similarly elevated levels.

### Relationship of Relative Transcription Rates to mRNA Abundance

The relative transcription rates of PG and two other genes in ripe (55 d) Rutgers fruit were analyzed and compared to the abundance of their corresponding mRNAs (Fig. 4). At this developmental time point the PG mRNA level was 6-fold greater than that of D21, yet the transcription rates of the PG and D21 genes were approximately equal. Comparison of PG and E8 mRNA levels and transcription rates is even more striking. The PG mRNA level was 3.5-fold higher than the E8 mRNA level despite the transcription rate of the E8 gene being over 6-fold greater than that of the PG gene (Fig. 4). The relationship between mRNA levels and the relative transcription rates of each gene can most readily be seen by calculating the ratio of the mRNA levels to the relative transcription rate (Fig. 4). The high PG mRNA level, relative to its moderate transcription rate, suggests that posttranscriptional processes (29), such as mRNA stability or cytoplasmic entry rates, also contribute to the relatively high accumulation of PG mRNA during tomato fruit ripening.

### Discussion

We have previously shown (9) that during ripening of wild-type tomato fruit PG mRNA first becomes detectable after a small rise in ethylene production and continues to accumulate to high levels in ripe fruit. Polygalacturonase activity and immunologically detectable protein closely parallel this rise in PG mRNA indicating that PG expression during ripening is primarily regulated by the level of PG mRNA rather than by a translational or posttranslational mechanism. To determine the role that transcription plays in controlling this dramatic increase in PG mRNA during normal fruit ripening, we have analyzed transcription of the PG gene in nuclei isolated from wild-type fruit of various ripening stages. We have shown that the PG gene is transcriptionally active in immature, MG1, and MG2 fruit and becomes transcriptionally activate in MG3 fruit, the stage at which ethylene production by the fruit increases. Transcription of the PG gene increases by the MG4 stage to 20% of the transcription rate of D21 (Fig. 1) and continues to increase at later stages in ripening to a rate approximately equal to that of D21 (Fig. 2, panel B, 55 d Rutgers). The transcription rate of D21 remains relatively constant during this time. The kinetics and magni-
tude of the changes in PG mRNA accumulation during ripening parallel the changes in transcriptional activity of the PG gene, indicating that transcriptional control plays an important role in both the initiation (Fig. 1) and maintenance (Fig. 2) of PG expression during ripening in wild-type fruit.

In the ripening mutants rin, nor, and Nr, PG steady state mRNA levels are greatly reduced relative to wild-type (10). The greatest reduction is observed in rin and nor (approximately 100-fold less than wild-type) while Nr fruit eventually accumulate PG mRNA to 10 to 20% of wild-type levels (see Fig. 2). We have shown here that the rate of PG gene transcription in all three mutant genotypes is greatly reduced relative to wild-type fruit of the same age. Rin and nor fruit show the most severe reduction in PG gene transcription while Nr fruit show a delayed activation and reduced rate of PG gene transcription relative to wild type. The steady state PG mRNA levels in the ripening mutants parallel PG transcription rates, providing further evidence for the importance of transcriptional control in the regulation of PG mRNA levels during ripening. While transcriptional activation of the PG gene is apparently important in determining the developmental timing of PG expression, the high level of PG mRNA (2–4% of the mRNA mass) relative to its modest transcription rate suggests that posttranscriptional processes may also contribute to the high accumulation of PG mRNA in ripening fruit.

The transcription of other ripening-associated genes in the mutant genotypes did not, in general, parallel that of PG. This indicates that the mutations do not generally repress transcription of ripening-associated genes, but rather, they affect the transcriptional activity of these genes in diverse manners. In addition, the expression of some ripening-associated genes in the mutant genotypes was also affected at the posttranscriptional level. Overall the results presented here suggest that multiple mechanisms operate to regulate gene expression during ripening in wild-type fruit and that the three ripening mutations, rin, nor, and Nr exert distinct effects on these mechanisms.

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LITERATURE CITED