Peach (Prunus persica) Endopolygalacturonase cDNA Isolation and mRNA Analysis in Melting and Nonmelting Peach Cultivars

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Two distinct partial cDNAs, PRF1 and PRF3, similar in sequence to previously described polygalacturonases, were amplified from ripe peach (Prunus persica L. Batsch cv Flavorcrest) fruit cDNA by the polymerase chain reaction. PRF1-related RNA was present in fruit from early ripening at levels not detected by northern analysis. PRF3-related RNA was readily detectable in ripe fruit by northern analysis. PRF3 was used to isolate a cDNA with a complete open reading frame, PRF5, from a λZAP II cDNA library prepared from poly(A)+ RNA of ripe peach fruit. PRF5 coded for a predicted protein of 393 amino acids with a molecular mass of 41,500 D. The derived amino acid sequence of PRFS included a putative leader sequence of 23 amino acids, followed by a sequence that matched the N terminus of endopolygalacturonase protein purified from ripe peach fruit. By northern analysis, PRF3-related RNA was undetectable in firm, unripe Flavorcrest fruit. It appeared at low levels as a 1.7-kb transcript in fruit that had begun to ripen and soften and was very abundant in ripe fruit that had undergone the "melting" stage of softening. The marked increase in PRF3-related RNA levels took place over a period of less than 2 d at 20°C and coincided with the climacteric peak in ethylene evolution. Levels of 1-aminocyclopropane-1-carboxylate oxidase-related RNA increased during ripening at a much earlier stage than levels of PRF3-related RNA. Lower levels of 1.7-kb RNA transcript were detected by PRF3 in ripe fruit of the melting cultivar Fragar, which are firmer than Flavorcrest fruit. In ripe fruit of the nonmelting cultivar Carolyn, PRF3 detected a 1.45-kb RNA transcript that was present at low levels. Transcripts of a peach polygalacturonase-related genomic sequence were not detected in ripening fruit.

Soft, juicy flesh is a feature of ripe peach (Prunus persica L. Batsch) fruit. In fruit of fresh eating varieties softening occurs in two stages. During early ripening, tissue firmness decreases slowly and progressively. Toward the end of ripening, loss of tissue firmness is rapid. This second stage of softening is called the "melting" stage (Bailey and French, 1949; Sherman et al., 1990; Fishman et al., 1993). After melting, fruit are very susceptible to physical injury and can be stored for a few days only.

Fruit of peach varieties used for canning do not have a melting phase of softening. Ripe fruit remain relatively firm and maintain their shape throughout processing. These fruit have good keeping qualities but have not been generally favored for fresh consumption because of their relatively dense flesh. Breeding projects that aim to produce a nonmelting peach desirable for fresh eating are currently underway (Sherman et al., 1990).

The melting flesh character is dominant and segregates in a Mendelian fashion (Bailey and French, 1949). It is tightly linked to the freestone character, which is also reported to be controlled by a single locus (Bailey and French, 1949).

Fruit of melting varieties show an increase in activity of endoPG (EC 3.2.1.15) during ripening (Pressey and Avants, 1978). EndoPG activity increases slightly during initial softening and markedly during the melting stage of softening in fruit of the freestone variety Flavorcrest (Orr and Brady, 1993). No significant endoPG activity has been detected in nonmelting fruit (Pressey and Avants, 1978). PG has long been thought to contribute to fruit softening through its action on intercellular and cell wall pectins, although recent transformation experiments establish that, in tomatoes, there is no simple relationship between endoPG action and fruit firmness (Fischer and Bennett, 1991). In peaches, endoPG activity may be necessary for melting of fruit flesh to occur. Notable differences in the physical and chemical properties of pectins in melting and nonmelting peach fruit occur at the later stages of ripening (Fishman et al., 1993).

A number of PG gene and cDNA sequences from plants have been described (Grierson et al., 1986; Sheehy et al., 1987; Bird et al., 1988; Brown and Crouch, 1990; Lee et al., 1990; Niogret et al., 1991; Dopico et al., 1993; Kutsumai et al., 1993; R.G. Atkinson and R.C. Gardner, GenBank accession number L12019; D.M. Lonsdale, GenBank accession number X71019). Comparison at the amino acid level reveals a high level of conservation in certain regions of these sequences.

Expression of a peach PG gene (Lee et al., 1990) could not be demonstrated in fruit by methods that included the use of PCR (D.R. Lester, unpublished data). Screening peach fruit cDNA libraries for ripening-related genes and for genes cross-hybridizing with tomato endoPG cDNA failed to isolate a peach endoPG cDNA (Callahan et al., 1993).

In this work, PG sequence conservation was exploited in a cloning strategy employing PCR to isolate two distinct partial cDNAs that show PG sequence similarity from ripe Flavor-

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crest peach fruit. A cDNA with a complete open reading frame corresponding to one of the partial clones was isolated from a XZAP cDNA library. Sequence analysis showed a match between part of the amino acid sequence derived from this clone and the N-terminal sequence of the mature endoPG protein purified from ripe peach fruit.

Both partial cDNA clones were used to characterize gene expression in relation to ripening in fruit of the melting variety Flavorcrest and Fragar and of the nonmelting variety Carolyn. Northern analysis of the ripening-related ACC oxidase gene was also performed.

MATERIALS AND METHODS

Plant Material

Peach (Prunus persica L. Batsch) fruit of the melting flesh cultivars Flavorcrest and Fragar and the nonmelting cultivar Carolyn were used in these experiments. Flavorcrest fruit were collected from early ripening to the commercial harvest stage from Arcadia, New South Wales, Australia. Fragar fruit at the commercial harvest stage were collected from Bathurst, New South Wales, Australia. Ripening Carolyn fruit were obtained from Stanhope, Queensland, Australia. Flesh firmness of fruit was measured by removing a small disc of skin at the commercial harvest stage from Arcadia, New South Wales, Australia. Fragar Carolyn fruit were monitored until ethylene production peaked.

Measuring Ethylene Evolution of Fruit

Fruit were collected at commercial harvest stage (approximately 6 kg firmness), weighed, and placed in sealed 800-mL jars ventilated with humidified air at flow rates of 1 to 1.2 L/h at 20°C. Ethylene concentrations were measured in duplicate 1-mL samples of air taken from outlet tubes of continuously ventilated jars. Ethylene in the air was measured after separation on a Loenco model 15 CFX gas chromatograph fitted with a 1.52-m × 0.32-cm column of 60- to 80-mesh alumina and flame ionization detection. Ethylene concentration of Flavorcrest fruit was monitored daily for 12 d after harvest and three fruit with typical ethylene readings were sacrificed every 2 d for RNA analysis. Fragar and Carolyn fruit were monitored until ethylene production peaked.

RNA Preparation

RNA was extracted from fruit tissue by the following protocol, which was based on that of Callahan et al. (1989). Fresh frozen fruit mesocarp was powdered in a coffee grinder, and 1 g was added to 20 mL of 100 mM Tris-HCl, pH 9, 100 mM NaCl, 1% (w/v) SDS, 1% (w/v) PVP-360, 1% (v/v) β-mercaptoethanol, 100 μg/mL proteinase K (Boehringer Mannheim) and left to stand for 5 min at room temperature. Cellulose was removed by centrifugation at 15,000 g for 10 min. The solution was extracted with phenol equilibrated with 10 mM Tris-HCl, pH 7.5, then with phenol: chloroform (1:1), and finally with chloroform:isoamyl alcohol (24:1). The aqueous phase was placed on ice and 0.1 volume of 3 M sodium acetate, pH 4.8, 0.01 volume of 10% SDS, and 0.1 volume of 5 M NaCl were added. After incubation on ice for 2 h, the precipitate was removed by centrifugation at 13,000 g for 20 min. An equal volume of 6 M LiCl was added and the solution was incubated at 4°C overnight. The RNA was pelleted by centrifugation at 27,000 g for 30 min and resuspended in 400 μL of water. The RNA was precipitated in 0.1 M NaCl with 2.5 volumes of 95% ethanol and pelleted by centrifugation. The RNA pellet was washed with 70% ethanol, dried at room temperature and pressure, and resuspended in 100 μL of water. The concentrations of RNA solutions were calculated from absorption readings taken at 260 nm.

Total RNA was passed through a cellulose column to remove carbohydrates (Palmeter, 1973) and then over oligo(dT)-cellulose for poly(A)⁺ selection (Aviv and Leder, 1972).

Oligonucleotide Sequence and Design

The sequences of oligonucleotides (5'-3') used as PCR primers and the conserved PG amino acid sequences they matched were: AA(T/C)ACIGA(T/C)GGI(A/G)TICA (NTDG1H) (primer 1); CCATGTCTTGATCTAATCCTV (GVRKTW) (primer 2); and GCCGATGATCCTCCTTCT-G (GDDCVSLG) (primer 3). Primers 1 and 3 are in the sense orientation; primer 2 is in the antisense orientation. The sequence of primer 4 was the same as that of the adaptor primer described in the protocol for rapid amplification of cDNA ends (Frohman et al., 1988). Primer 1 included all the permutations of DNA sequence coding for the amino acid sequence given, with the nucleotide base I used at positions that were totally degenerate. Primer 2 was synthesized as a sequencing primer for a peach PG genomic clone (Lee et al., 1990), and primer 3 was designed according to sequence of the PCR product of primers 1 and 2; therefore, these do not show degeneracy.

First-Strand cDNA Synthesis and PCR

First-strand cDNA synthesis and PCR (Saiki et al., 1985) reactions are based on the rapid amplification of cDNA ends method (Frohman et al., 1988). First-strand cDNA was synthesized from 2 μg of poly(A)⁺ RNA from Flavorcrest fruit at <0.5 kg firmness at 42°C for 1 h in 20 μL of 1× PCR buffer with 1 mM deoxynucleotide triphosphates (1.8 mM MgCl₂), 20 units of human placental ribosomal RNAse inhibitor (Promega), and 4 units of avian myeloblastosis virus reverse transcriptase (Promega) primed by 100 pmol of oligonucleotide with the sequence (5'-3') GACTCGAGATCGACAT-GA(T)₁₇.

PCR reactions were carried out in a volume of 50 μL with 1× PCR buffer, 1.8 mM MgCl₂, 0.4 mM deoxynucleotide triphosphates, 0.4 μM primers, 10 μL of the cDNA synthesis reaction, and 1.25 units of Taq polymerase (Perkin-Elmer Cetus). Reactions were cycled 35 times at 94°C for 1 min, 55°C for 1 min, and 72°C for 40 s.

PCR products were cloned into T-tailed (Marchuk et al., 1991) pBluescript KS vector (Strategene) and sequenced by
the dideoxynucleotide method (Sanger et al., 1977) using Sequenase (United States Biochemical).

The sequence of a 255-bp fragment produced from PCR with primers 1 and 2 had similarity to previously described PG sequences and was designated PRF1. PCR with primers 3 and 4 was designed to amplify the 3' cDNA end of PRF1; however, this was unsuccessful and, instead, produced a distinct sequence, PRF3, which also showed PG sequence similarity.

**λZAP cDNA Library Construction**

A cDNA library was made from 5 μg of poly(A)+ RNA prepared from Flavorcrest soft (<0.5 kg) ripe fruit using a ZAP-cDNA synthesis kit (Stratagene) in the λ phage vector Uni-ZAP XR (Stratagene) according to methods described by the manufacturer. Phage was packaged in Gigapack Gold (Stratagene).

**Library Screening and Analysis of Positive Clones**

Duplicate plaque lifts of 40,000 primary recombinants on Biotrace NT membrane (Gelman, Ann Arbor, MI) were screened with insert DNA prepared from PRF1 and PRF3. Insert DNA (100 ng) was labeled to high activity with [α-32P]-dATP using a Bresatec (Adelaide, South Australia) random-labeling kit. Membranes were hybridized for 1 h at 65°C in 0.5 M sodium phosphate, 1 mM EDTA, 7% SDS, 1% BSA, pH 7.2, and washed in 40 mM sodium phosphate, 5% SDS and in 20 mM sodium phosphate, 1% SDS at 65°C according to Gelman recommendations, with radioactive probes at a concentration of 1 x 10⁶ cpm/mL. Positive plaques were purified to a single species, and pBluecript KS phagemid DNA with insert was excised in the presence of helper phage R408 and rescued as plasmid DNA according to Stratagene methods.

Positive cDNA clones with the largest inserts were sequenced (Sanger et al., 1977). A cDNA, PRF5, containing a complete open reading frame was identified. The complete sequence of both strands of PRF5 was obtained from nested deletions generated by the Erase-A-Base system (Promega).

**Northern Analysis**

RNA was fractionated on formamide/formaldehyde denaturing gels (Fourney et al., 1988) with 10 μg of total RNA from each sample loaded. RNA was transferred to a Zeta-probe membrane (Bio-Rad) by methods described by the manufacturer. Filters were probed with [α-32P]-dATP-labeled insert DNA from PRF1 and PRF3. PRF3 was used as a probe rather than PRF5 because it was shorter and, therefore, less likely to hybridize nonspecifically.

A cDNA with sequence related to ACC oxidase, also known as ethylene-forming enzyme, was isolated from a peach fruit cDNA library on the basis of cross-hybridization with the tomato cDNA pTOM13 (Holdsworth et al., 1987). The coding sequence of this clone, PAO1, was identical to that described by Callahan et al. (1992). Insert DNA of PAO1 was used to perform northern analysis on this gene. Duplicate sets of filters were prepared for northern analyses with PRF3 and PAO1.

**RESULTS**

**Isolation of Partial PG-Related cDNAs**

Comparison of the PCR product generated by primers 1 and 2, PRF1 (Fig. 1A), with GenBank sequences resulted in a highest match with Oenothera organensis PG, with 66% identity over 80 amino acid residues. The sequence of the 800-bp PCR product, PRF3, from primers 3 and 4 was distinct from that of PRF1 but also showed PG sequence similarity.

**Construction and Screening of the Flavorcrest λZAP cDNA Library**

A total of 120,000 primary recombinants were obtained in the λZAP cDNA library, of which over 90% contained inserts. The screen with PRF1 produced no positive plaques. The screen with PRF3 produced approximately 400 positive plaques. One clone, PRF5, with an insert of 1496 bp, contained sequence identical to that of PRF3 from bases 711 to 1496.

**Sequence Analysis of PRF5**

Sequence analysis of PRF5 revealed an open reading frame that codes for a polypeptide of 393 amino acids (Fig. 1B) with a predicted molecular mass of 41,500 Da. Highly conserved regions of PG sequences were present in the sequence. Comparison of PRF5 with GenBank sequences resulted in a highest match with kiwifruit PG, showing 41% overall identity at the amino acid level. The sequences encoded by PRF1 and PRF5 are compared with those encoded by a previously characterized peach genomic sequence and conserved sequences in other higher plant PG cDNAs in Figure 2.

The N-terminal sequence of the mature endoPG protein isolated from ripe peach fruit was determined to be TPVTYN-VASLGAKADGKTDSTAPFLS. A corresponding sequence was found in the amino acid sequence of the predicted protein encoded by PRF5, 24 amino acids from the putative translation commencement point.

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N-Terminal Sequence

The endoPG enzyme was purified from ripe Flavorcrest fruit as described by Orr and Brady (1993). The M, 44,000 band was transferred to a polyvinylidene difluoride membrane (Millipore) as described by Matsudaira (1987). The membrane was washed in water, briefly stained with 0.1% Coomassie blue R, destained, and rinsed with water. The stained membrane segment was used to obtain the N-terminal amino acid sequence using an Applied Biosystems model 470A gas phase sequencer equipped with a modified reaction cartridge (Krieger and Doloman, 1988).

**Sequence Comparison**

Similarities between the derived amino acid sequences of PRF1, PRF5, and previously described PG sequences were determined using the FASTA program (Pearson and Lipman, 1988) and the GenBank data base.

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The coding sequence of this clone, PAO1, was identical to that described by Callahan et al. (1992).
Analysis of RNA Related to PRF1, PRF3, and PAO1

The advance of softening was one parameter against which RNAs in peach fruit related to PRF1, PRF3, and PAO1 were studied. Northern analysis detected no RNA hybridizing with PRF1-related sequence in peach fruit of 6 kg firmness when filters were exposed for 3 d. Results from exposure of filters for 24 h (Fig. 3A) showed that a massive increase in PRF3-related RNA occurred as fruit lost tissue firmness from 4 to <0.5 kg.

Figure 1. A, Nucleotide and derived amino acid sequence of PRF1. PCR primer sequence is represented by italics. B, Nucleotide and derived amino acid sequence of PRF3. A potential glycosylation site is described by Lee et al. (1991), with three peach accession numbers (Kutsumai et al., 1991, CenBarik accession number X77231, PRF5 accession number X71019), tomato (Crierscin et al., 1993), kiwifruit (Nio-kiwifruit accession number PAP1, 1991), and Oenothera (Brown and Crouch, 1993). The comparison is with maize (Nio) and 4 kg fruit of Flavorcrest, Fragar, and Carolyn fruit at any stage of softening. PCR using primers 1 and 2 amplified a product with the same size as PRF1 in cDNA prepared from Flavorcrest fruit of 4 and <0.5 kg firmness but not in fruit of >12 or 6 kg firmness. The possibility that PRF1 represented contaminating peach genomic DNA sequence was ruled out because PCR with the same primers on a template of genomic DNA gave a product of a different size (500 bp). Southern analysis confirmed that PRF1 represents sequence in the peach genome (data not shown).

In unripe Flavorcrest fruit of firmness >12 kg, no RNA hybridizing with PRF3 was detected by northern analysis. A PRF3-related sequence of 1.7 kb was detected in fruit of 6 and 4 kg firmness when filters were exposed for 3 d. Results from exposure of filters for 24 h (Fig. 3A) showed that a massive increase in PRF3-related RNA occurred as fruit lost tissue firmness from 4 to <0.5 kg.
Figure 3. Northern analysis with PRF3 (A) and PAO1 (B) probes of 10 μg of total RNA from Flavorcrest fruit at progressive stages of softening with exposure of filters for 24 h at -70°C. Fruit softening is measured in kg of force required for a constant deformation.

PAO1-related RNA was present as a 1.6-kb transcript at low levels in fruit of 12 kg firmness and at higher levels in fruit of 6 and 4 kg firmness. It was abundant in ripe fruit of less than 0.5 kg firmness (Fig. 3B).

Levels of RNA related to PRF3 and PAO1 during ripening were studied according to the parameter of ethylene evolution by Flavorcrest fruit during a postharvest period (Fig. 4, A–C). PAO1-related RNA was present at low levels when ethylene evolution from fruit was <10 μL kg⁻¹ h⁻¹. In these fruit, PRF3 detected RNA only after a 3-d exposure of the filters. For both PRF3 and PAO1, a sudden increase in related RNA levels occurred in the 1 or 2 d before the climacteric peak associated with the final stages of ripening and remained high for 6 d afterwards. Melting of fruit flesh occurred in the days when these increases were observed.

Figure 4. Northern analysis with PRF3 (A) and PAO1 (B) probes of 10 μg of total RNA from Flavorcrest fruit taken at intervals of 2 d in a postharvest period of 12 d. Filters were exposed for 16 h at -70°C. Ethylene production (C) is the mean rate for three fruit.

Figure 5. Northern analysis with PRF3 (A) and PAO1 (B) probes of 10 μg of total RNA from Flavorcrest, Fragar, and Carolyn climacteric fruit. Filters were exposed for 24 h at -70°C.

Fragar and Flavorcrest fruit showed a drop in penetrometer readings from approximately 4 to 0.5 kg or less over a period of 2 d, consistent with the melting phenotype. After the melting phase, Fragar fruit remained firmer by subjective assessment than Flavorcrest fruit, but the limited sensitivity of the penetrometer meant that this difference was not quantitated. Ripe fruit of the nonmelting variety Carolyn were firm relative to both Fragar and Flavorcrest, with a minimum penetrometer reading of 6 kg. In the Fragar and Carolyn fruit, levels of RNA that hybridized with PRF3 were found to peak around the climacteric peak (data not shown). Comparison of climacteric fruit of the different varieties (Fig. 5A) showed much lower levels of 1.7-kb RNA detected by PRF3 in Fragar than those in Flavorcrest. In Carolyn fruit PRF3 detected levels of mRNA below those found in Flavorcrest and Fragar. There was an obvious difference in size of the hybridizing transcript in Carolyn compared with that in Flavorcrest and Fragar. It was estimated to be 250 bases shorter.

PAO1 RNA was present as a 1.6-kb transcript in climacteric fruit of all three varieties (Fig. 5B). Carolyn had the highest levels of PAO1 message and Fragar had levels slightly lower than Flavorcrest. There was no relationship between relative levels of ACC oxidase-related RNA in the three varieties and their relative ethylene readings. Ethylene readings for the Flavorcrest, Fragar, and Carolyn fruit were 40, 55, and 65 μL kg⁻¹ h⁻¹, respectively.

DISCUSSION

In this study we present evidence that a cDNA isolated from ripe Flavorcrest peach fruit, PRF5, encoded the endoPG protein found in Flavorcrest fruit during ripening. This conclusion was based on the presence of known PG consensus sequences and on a match between part of the deduced amino acid sequence of PRF5 and the N-terminal sequence of the purified protein.

The unprocessed protein predicted from PRF5 nucleotide sequence was 393 amino acids long and had a molecular mass of 41,500 D. It contained one potential N-glycosylation site at a similar position to one found in tomato endoPG. The putative leader sequence of peach endoPG contained the characteristic hydrophobic regions (von
Heijne, 1983). It was 23 amino acids long, similar in length to that of tomato, but the peach sequence lacked the additional N-terminal extension that is present in tomato (Grierson et al., 1986; Sheehy et al., 1987). From the PRF5 sequence, the predicted molecular mass of endoPG after processing was 39,000 D, which compares with an M, of 44,000 reported for the native protein.

The accumulation of RNA that hybridized to a partial clone of PRF5, PRF3, in relation to softening of Flavorcrest fruit occurred in a pattern similar to that of increases in endoPG activity (Orr and Brady, 1993). Low levels of both were associated with the gradual softening of early ripening. A marked increase in both PRF3-related RNA and endoPG activity was associated with the melting stage of softening. There was a relationship between levels of RNA that hybridized to PRF3 and the degree of softness of ripe fruit between cultivars in Flavorcrest, Fragar, and Carolyn.

The smaller RNA transcript detected by PRF3 in fruit of the nonmelting variety Carolyn is of particular interest. Significant endoPG activity was not detected in nonmelting fruit, which led to the theory that absence of the enzyme accounted for the nonmelting phenotype (Pressey and Avants, 1978). From northern analysis of Carolyn fruit, it appears that transcription of the endoPG gene may still occur in nonmelting fruit. The smaller size of the RNA transcript in Carolyn fruit may reflect a sequence aberration that affects translation and/or production of active enzyme.

Maize and O. organensis have multiple PG genetic sequences showing high conservation levels among coding regions of >99 and 89%, respectively (Brown and Crouch, 1990; Allen and Lonsdale, 1992; Barakate et al., 1993). Overlapping regions of PRF1, PRF3, and the genomic PG clone (Lee et al., 1990) display relatively low conservation of 37%. The divergence may reflect differences in functional roles of the PGs produced by the three genes. Genes represented by PRF1 and PRF5 are both expressed in fruit, but the gene encoding PRF5 appears to have a more significant role because it seems to represent the prevalent endoPG enzyme in fruit. PRF1 RNA is associated with ripening but is present at very low levels. The role in fruit of the gene represented by PRF1 is not clear from this work. Possibly it represents the gene encoding the exoPG present in ripening fruit (Downs et al., 1992). Expression of the peach PG gene (Lee et al., 1990) may occur in tissues other than fruit such as pollen or abscission zones. The hypothesis that endoPG enzyme in peach fruit confers the melting flesh phenotype is currently being tested using restriction fragment length polymorphism analysis.

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The EMBL/GenBank/DDBJ accession number for the nucleotide sequence of PRF5 is X76735. The nucleotide sequences for PAO1 and the PG genomic sequence will appear in the data bases under the accession numbers X77232 and X77231, respectively.

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