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Comparison of Pch313 (pTOM13 Homolog) RNA Accumulation during Fruit Softening and Wounding of Two Phenotypically Different Peach Cultivars

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ABSTRACT

Pch313 was isolated as a cDNA whose RNA accumulated during the softening period of peach (*Prunus persica* L. Batsch) fruit development. To better understand the role of the gene, we compared the amount of pch313-related RNA detected during fruit softening and tissue wounding between cultivars with different softening characteristics. The cultivar that softened faster, "Bailey," had a significantly higher amount of pch313-related RNA accumulate during softening than the slower-softening cultivar, "Suncrest." Pch313 was sequenced and found to be related to a tomato fruit cDNA clone, pTOM13, which has been shown to encode the ethylene-forming enzyme. The derived amino acid sequence of pch313 is 74 to 83% identical to the pTOM13-related sequences. A pch313-3′ noncoding region probe was used to demonstrate that pch313 is related to both a wound-induced RNA transcript and the major fruit-softening transcript. The relationship of pch313 RNA accumulation and ethylene evolution was examined upon wounding and appeared to be both tissue and cultivar specific. When leaves were wounded, more pch313-related RNA was detected in Bailey and the rate of ethylene evolved was also higher in Bailey. When fruits were wounded, the levels of ethylene evolved were nearly identical but Suncrest accumulated more pch313-related RNA. Southern analysis of the DNA indicated a small number of related genes.

Softening is one of the major problems in harvesting peach (*Prunus persica* L.) fruit at optimum quality. The fruit soften too fast to be able to market, hence they are picked at a time earlier than optimum for quality. We are taking an approach similar to that being done in tomatoes to attempt to extend this softening period so that fruit may be harvested at a time closer to optimum quality. That is, we want to decrease the amount of softening-associated enzymes present by insertion of antisense genes for those enzymes or processes affecting those enzymes (9, 19, 20). Initially, we isolated three cDNAs that represented RNAs that accumulated primarily during the softening phase of peach fruit (3, 5). Pch201, from the "Loring" soft fruit cDNA library, was isolated by differential hybridizations with cDNA made from two different stages of fruit firmness. Pch307, from the B612615 ripe fruit cDNA library, was isolated by sequence homology to tomato polygalacturonase, and pch313 was isolated from the same ripe fruit library because it had no homology to tomato polygalacturonase (3, 5). Pch313 had the most dramatic accumulation of RNA, a 30-fold increase, at the end of softening. For this reason, we chose to further characterize this gene's relationship to softening in peach fruit.

One way of understanding the role of a particular gene is to compare wild-type phenotypes with those of mutants. In perennial fruit trees, it is difficult to generate specific mutants. To this end, we have used two existing cultivars that have different phenotypes, but also different genotypes. "Bailey" is a rootstock cultivar not used for fruit production. The fruit soften very rapidly. "Suncrest" is a successful commercial cultivar that remains firm enough to ship to market with adequate shelf-life. Therefore, we have examined the accumulation of pch313-related RNA in Bailey and Suncrest cultivars.

To further characterize pch313, we sequenced the insert and compared it to the GenBank sequences. It was found to be related to pTOM13, a cDNA clone isolated from tomato fruit (11). pTOM13 was involved in ripening and also induced by wounding. Holdsworth et al. (11–13) hypothesized that it was involved in ethylene biosynthesis because its accumulation paralleled the evolution of ethylene during fruit ripening and wounding. Hamilton et al. (9) have decreased ethylene evolution and EFE[2] activity by the insertion of an antisense construct of pTOM13. This supported the idea of the role of pTOM13 in ethylene biosynthesis. Spanu et al. (21) have expressed an RNA related to pTOM13 in oocytes and have obtained EFE activity. Hamilton et al. (8) have also expressed a pTOM13-like clone in yeast and have obtained EFE activity. In this report, we have investigated the effect of wounding on pch313 RNA accumulation and the differences between the two cultivars with regard to the relationship of ethylene evolution and pch313 RNA accumulation. We also verified that pch313 RNA accumulates during fruit softening, fruit wounding, and leaf wounding.

MATERIALS AND METHODS

Plant Tissue

Two different peach (*Prunus persica* L. Batsch) cultivars were used in this study: cv Suncrest, a commercial-quality

[1] Co-senior author.

[2] Abbreviations: EFE, ethylene-forming enzyme; N, newton; ACC, 1-aminocyclopropane-1-carboxylate.

fruit cultivar that bears large, yellow, freestone/melting flesh fruit (2), and cv Bailey, a small, poor-quality, white, freestone/melting flesh fruit (16). The softening period of Bailey is very short, with the fruit taking as little as 5 d to change from 100 N to 4 N in firmness, whereas that of Suncrest is more prolonged, taking as long as 2 weeks.

Fruit from mature bearing trees were collected at multiple times ranging from 15 to 135 d after bloom and frozen with liquid nitrogen in the field. Leaf tissue was collected in the early spring and frozen with liquid nitrogen in the field. Peach fruit are not uniform in the time it takes for them to soften. Softening was first detected at 104 DAF but it varied in individual fruits. A second series of fruit was collected to look more carefully at softening. This was based on firmness as measured by a Magness-Taylor penetrometer and was collected when the fruit had reached full size and had developed color. The fruit were sorted into the following classes as had been done previously (3): first class (61–80 N), second class (41–60 N), third class (21–40 N), and final class (4–20 N). The fruit and leaf material for RNA extractions were lyophilized, batched by cultivar and date, and stored at −20°C until use. The leaf material for DNA extraction was kept frozen at −80°C until use.

Isolation of Full-Length cDNA Clones

The construction of a B612615 ripe fruit cDNA library in λZAP and the isolation of λ313 from that library has been described (3). Sixty random clones from the same cDNA library were screened with the λ313 insert using standard techniques (17). Two clones were isolated, λ308 and λ336. A 5-mL culture of each was grown and the DNA was extracted (17). Polymerase chain reactions were performed on this DNA using a GenAmp kit and a thermal cycler according to the manufacturer's directions (Perkin Elmer/Cetus)[3] using the vector-encoded primers of SK and KS (Stratagene, La Jolla, CA) to verify appropriate-sized cDNA inserts.

Wounding

Tissue was cut into 1-cm squares and pooled. Aliquots were placed in air-tight tubes with a stopper and stored for various times at room temperature. At the appropriate time, gas samples were removed and analyzed by GC for the amount of ethylene present. Three independent samples were taken for each time point. The values of the three samples were averaged. Tissue was then frozen in liquid nitrogen, lyophilized, batched, and RNA was extracted.

Sequencing

The λ clones were converted into pBluescribe clones according to the manufacturer's directions (Stratagene). Plasmid DNA was isolated according to the alkaline extraction procedure of Maniatis et al. (17) and further purified by elution through an elutip-D column (Schleicher and Schuell, Keene, NH). Sequencing was performed with Sequenase (U.S. Biochemical, Cleveland, OH) according to the manufacturer's directions. Commercial sequencing primers, SK and KS (Stratagene), were used initially and additional primers (Research Genetics, Huntsville, AL) were synthesized based on a newly generated sequence to extend the sequence. Sequences were analyzed using PC/GENE (IntelliGenetics, Inc. Mountain View, CA).

DNA and RNA Extractions

Genomic DNA was isolated as described by Doyle and Doyle (7) using 3% mixed alkyltrimethyl-ammonium bromide (w/v) and 1% PVP-40 (w/v) in the extraction buffer. Purified DNA was evaluated by UV light for integrity and amount after resolution on 0.7% agarose gels and ethidium bromide staining.

RNA was extracted by the following modification of a method of Callahan et al. (4). The tissue was extracted with preheated 65°C proteinase K buffer to which ascorbic acid was added (1 mM final concentration). The supernatant was extracted twice with phenol that had been heated to 65°C and then with room-temperature phenol. The protocol was continued as before (4) using phenol-chloroform extractions, chloroform extractions, LiCl precipitation, and ethanol precipitations. The integrity of the RNA was visualized following electrophoresis through a 1.4% agarose gel containing 5 mM methyl mercuric hydroxide (1).

Nucleic Acid Labeling

Inserts used as probes for northern and Southern analyses were isolated from plasmid DNA (17) and labeled by random primers following the directions provided by the manufacturer (Life Technologies, Gaithersburg, MD).

Northern and Southern Analysis

RNA samples, 5 μg/lane, were fractionated on 1.4% agarose gels containing 5 mM methyl mercuric hydroxide (1) and transferred to Nytran membrane according to the manufacturer (Schleicher and Schuell). The membranes were stained with methylene blue (10) to verify equal amounts of RNA in each lane following the transfer. Prehybridization and hybridization were performed using the buffer from an Oncor Sureblot kit (50% formamide [v/v], 6× SSC, 10% dextran sulfate [w/v]) (Oncor, Gaithersburg, MD), at 45°C, and the final wash was at 0.1× SSC at 52°C.

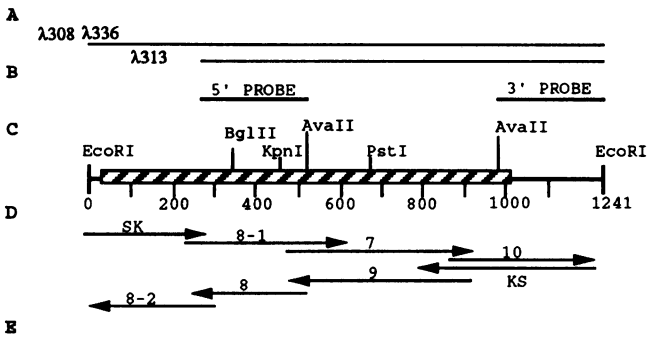
DNA samples, 6 μg/lane, were fractionated on 0.7% agarose gels using a Probe Tech I machine and reagents (Oncor). Prehybridization and hybridization were performed as in the northern analyses.

RESULTS

Sequencing

The cDNA inserts of three clones, λ313, λ308, and λ336, were sequenced by the Sanger dideoxy method using Sequenase (Fig. 1). The insert of the original clone λ313 was completely sequenced and found to be 978 bases, which is

[3] The mention of specific instruments, trade names, or manufacturers is for the purpose of identification and does not imply any endorsement by the United States government.

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                                                AAAGAAACACACACACAAAGAGAGAGAGAGAG  31
  M  E  N  F  P  I  I  N  L  E  G  L  N  G  E  G  R
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  K  A  T  M  E  K  I  K  D  A  C  E  N  W  G  F  F
AAAGCAACAATGGAAAAAATCAAAGATGCCTGTGAGAACTGGGGCTTCTTT 133
  E  L  V  S  H  G  I  P  T  E  F  L  D  T  V  E  R
GAGCTTGTGAGTCATGGGATACCAACTGAGTTTTTGGACACAGTGGAGAGG 184
  L  T  K  E  H  Y  R  Q  C  L  E  Q  R  F  K  E  L
TTGACAAAAGAACACTACAGGCAGTGCTTGGAGCAGAGGTTCAAGGAGCTG 235
  V  A  S  K  G  L  E  A  V  K  T  E  V  N  D  M  D
GTGGCCAGCAAGGGCCTCGAGGCTGTCAAGACAGAGGTCAATGATATGGAC 286
  W  E  S  T  F  Y  L  R  H  L  P  K  S  N  I  S  E
TGGGAAAGCACCTTCTACTTGCGCCATCTTCCAAAATCTAACATATCTGAA 337
  V  P  D  L  E  D  Q  Y  R  N  V  M  K  E  F  A  L
GTTCCAGATCTTGAGGATCAGTACAGGAATGTGATGAAGGAATTTGCATTG 388
  K  L  E  K  L  A  E  Q  L  L  D  L  L  C  E  N  L
AAGTTGGAGAAATTAGCAGAGCAGCTCCTAGACTTGCTCTGTGAGAATCTT 439
  G  L  E  Q  G  Y  L  K  K  A  F  Y  G  T  N  G  P
GGACTTGAACAAGGGTACCTCAAGAAGGCCTTCTATGGAACAAATGGACCA 490
  T  F  G  T  K  V  S  N  Y  P  P  C  P  K  P  E  L
ACTTTTGGCACCAAGGTTAGCAACTACCCTCCTTGTCCCAAACCTGAGCTG 541
  I  K  G  L  R  A  H  T  D  A  G  G  L  I  L  L  F
ATCAAGGGTCTCCGGGCTCACACCGATGCCGGCGGCCTCATCCTGCTCTTC 592
  Q  D  D  K  V  S  G  L  Q  L  L  K  D  G  Q  W  I
CAGGATGACAAGGTCAGTGGTCTGCAGCTCCTCAAAGATGGCCAATGGATT 643
  D  V  P  P  M  R  H  S  I  V  I  N  L  G  D  Q  L
GATGTGCCCCCCATGCGCCACTCCATTGTTATCAACCTTGGTGACCAACTT 694
  E  V  I  T  N  G  K  Y  K  S  V  E  H  R  V  I  A
GAGGTAATCACTAATGGGAAGTACAAGAGTGTGGAGCACAGAGTGATTGCC 745
  Q  T  D  G  T  R  M  S  I  A  S  F  Y  N  P  G  S
CAAACTGATGGCACCAGAATGTCAATAGCTTCCTTTTACAACCCTGGCAGT 796
  D  A  V  I  Y  P  A  P  T  L  V  E  K  E  A  E  E
GATGCTGTCATCTATCCTGCACCAACACTGGTGGAGAAAGAAGCAGNGGAG 847
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AAGAATCAAGTGTACCCGAAATTCGTGTTCGAAGACTACATGAAGCTCTAT 898
  A  G  L  K  F  Q  P  K  E  P  R  F  E  A  M  K  A
GCTGGCCTCAAGTTCCAGCCCAAGGAGCCAAGATTTGAAGCCATGAAAGCA 949
  V  E  T  N  I  S  L  G  P  I  A  T  A
GTGGAAACCAATATCAGTTTGGGTCCAATTGCAACAGCTTAAGAGAATTTA 1000
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GGCCAAAGTTTTTTATTGTCTCTTTAAAAAATTCAGTTAAAATTACTATTG 1102
AGGGTATCAAATATCAATCTGTTGTTTGTAGAAACTAAATTTATAGTAAAT 1153
AATGTGACCTGGGAATTTGTTAGTTTGCACTATTCCTGTACCGTGTACCAG 1204
TTTGTTAAATAAACTGTTAGTTACCAATTCTCAAAAA                1241
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Figure 1. Physical map and sequence of pch313. A, Schematic representing the insert of the three phage cDNA clones isolated that encompass pch313. λ308 was 1237 bp long, λ336 was 1242 bp long, and λ313 was 978 bp long. B, The region of the sequence used to construct the 5′ end (bp 264–574) and the 3′ end (bp 1001–1241) specific probes. C, The restriction map of the pch313 cDNA with the hatched area representing coding sequence. D, Schematic of the amount of sequence derived from each primer. Arrows pointed in one direction represent sequence from one strand and arrows in the other direction represent sequence from the other strand. Primers SK and KS were from Stratagene, and primers 7, 8, 9, 10, 8-1, and 8-2 were synthesized by Research Genetics. E, The nucleic acid sequence of pch313 and the predicted amino acid sequence. An open reading frame that starts with an ATG begins at base 32 and ends at base 988. The stop codon, TAA, is underlined, as is a potential polyadenylation sequence starting at base 1211.

smaller than either the 1.4- or 1.7-kb RNA transcripts detected by northern analysis in ripe fruit (3). For this reason, the other two clones were sequenced at both the 3′ and 5′ ends. All three clones had identical 3′ ends (except for different numbers of A residues at the end), although λ308 had an additional 260 bases at the 5′ end and λ336 had an additional 264 bases at the 5′ end. The complete sequence is presented in Figure 1. The largest open reading frame of 319 amino acids beginning with an ATG starts at base 32 and ends at base 988. Analysis of the predicted protein indicates that its unmodified molecular mass would be 36 kD, with a predicted isoelectric point of 5.0. There is no membrane-associated region. There are two potential N-glycosylation sites. A potential polyadenylation site is found at base 1211 to 1217.

The sequence was compared with sequences in the GenBank files and found to be homologous to a cDNA clone pTOM13 (11). It has 78% sequence identity at the nucleic acid level in the coding sequence and 78% at the predicted amino acid level. At the predicted amino acid level, pch313 also has sequence identity of 83% to tomato gene *eth1* (15), 83% to pRC13, a clone representing a mRNA similar to pTOM13 but with a two-base insertion (8), 81% to the wound-induced gene from tomato GTOMA (12), 77% to a ripening avocado cDNA clone pAVOe3 (18), and 74% to a cDNA from senescing carnation flowers (22). A comparison of the sequences is shown in Figure 2. There are three consensus cysteine residues that may indicate a potential disulfide bond and six consensus histidine residues that may indicate sites for ligand binding such as heme groups. There is one consensus glycosylation site and two others found in a subset of the sequences. Overall, the sequences have homology throughout the predicted amino acid sequence. A lesser degree of sequence identity exists with the tomato ripening-related gene E8 of Deikman and Fisher (6).

RNA Accumulation and Ethylene Evolution

Pch313-related RNAs did not accumulate in fruit tissue until the fruit was at a mature stage (3, 5). The two cultivars were chosen to see if pch313 RNA were more abundant in the faster softening Bailey than in Suncrest. Two sets of developmental and softening RNAs for both cultivars were resolved on the same gel and transferred to one piece of Nytran. Figure 3 shows the results of probing those RNAs with fragments of pch313 insert. Using the 5′ coding sequence probe (Fig. 1), pch313-related RNAs are detected in Suncrest when fruit begin to soften at 104 DAF, and accumulation peaks when the fruit is softest (4–20 N). The accumulation of pch313 RNA is detected in Bailey when fruit reach 61 to 80 N and it peaks when the fruit is 21 to 40 N (Fig. 3B). Both cultivars have two detectable transcripts, one being 1.4 kb long and the second, weaker transcript being 1.7 kb long. To determine the relationship of the two transcripts to our pch313 clone, we hybridized a duplicate north-

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                                                                    60
PCH     MENFPIINLEGLNGEGRKATMEKIKDACENWGFFELVSHGIPTEFLDTVERLTKEHYRQC
TOM(F)  ..........K...DE.AN...M.............N....H.VM....KM..G..KK.
TOM(L)  ..........K...AE.V......N.............N....H.VM....K...G..KK.
AVO     .DS..V..M.K.E.QE.A...KL.N.............N.S..V.LM.E.........KK.
CAR     IV.....D-------------.....H.....QV.N.SLSH.LM.K...M.....KKF
        MAN                  LNNYNGVERSLVLDQ
                                                                   120
PCH     LEQRFKELVASKGLEAVKTEVNDMDWESTFYLRHLPKSNISEVPDLEDQYRNVMKEFALK
TOM(F)  M................QA..T.L......F.....T....Q....DEE..E..RD..KR
TOM(L)  M.........K....G.EV..T.......F.....S....QL...D.V..E..RD.RKR
AVO     M.......M...VEG..VDA-.........FI....V..L..I...T.EH.K......E.
CAR     R..K..DM.QT...VSAESQ...I..........R.T.........D....KL.....AQ
                                                                   180
PCH     LEKLAEQLLDLLCENLGLEQGYLKKAFYG--TNGPTFGTKVSNYPPCPKPELIKGLRAHT
TOM(F)  ......E............K....N....--SK..N.............D.........
TOM(L)  ......K............KS...NT...--SK..N.............D.........
AVO     .......V...........K....M..A.TT.GL..............R...F.......
CAR     I.R.S..............KA...N....--A..................D.........
                                                                   240
PCH     DAGGLILLFQDDKVSGLQLLKDGQWIDVPPMRHSIVINLGDQLEVITNGKYKSVEHRVIA
TOM(F)  ....I.................E.............V.................I.....
TOM(L)  ....I..................R............V.................M.....
AVO     ............R.A........E.V.....N..........V...........M...V.
CAR     ....I..................H.V.....K....V.................M.....
                                                                   300
PCH     QTDGTRMSIASFYNPGSDAVIYPAPTLVEKEAEEKN-QVYPKFVFEDYMKLYAGLKFQPK
TOM(F)  ........L..............K.........ST-.........D............A.
TOM(L)  .K......L.......N..L.....A..D.....HNK......M.D.......N....A.
AVO     ....N...L............F...A.........-KE......-....N........A.
CAR     ....N.............................-...CRA-..........N..LK....E.
                                  324
PCH     EPRFEA--MKAVETNISLGPIATA
TOM(F)  ......--...M.----SD...S.
TOM(L)  ......--...M.----SD...I.
AVO     .....VMK......-AN.S..T.-
CAR     ......--...M..---T...P..
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Figure 2. A comparison of the predicted amino acid sequences between pch313 and related clones. PCH is the sequence derived from pch313. TOM(F) represents the tomato fruit cDNA sequence of pRC13 (8) that is based on a mRNA sequence homologous to pTOM13, a tomato ripe fruit cDNA clone from Holdsworth et al. (11). TOM(L) is a genomic tomato sequence representing an RNA induced during leaf wounding (12). AVO is an avocado clone, pAVOe3, isolated from a ripe fruit avocado cDNA library (17). CAR represents pSR120, a senescing carnation flower cDNA clone from Wang and Woodson (22). All the sequences begin at the first ATG of the open reading frame. Dashes represent gaps in the sequence and dots represent sequence identity with the pch313 sequence. Potential N-glycosylation sites, consensus C residues, and consensus H residues are underlined.

ern blot of fruit RNAs with the 3′ probe (Fig. 1). Figure 3B is the result of that hybridization where only the 1.4-kb transcript is detected. This indicates that the 1.4 kb-transcript is homologous to our pch313 clone at both the 5′ and the 3′ end, whereas the 1.7-kb transcript is related at the 5′ end only.

Previous work had shown that a pch313-related RNA accumulated following wounding (3). To better understand the relationship between ethylene evolution and pch313-related RNA accumulation, leaf tissue from Bailey and Suncrest were wounded and sampled at 0, 0.5, 1, 2, 4, 8, 24, and 48 h for ethylene and pch313-related RNA. Both the 5′ and 3′ pch313 probes detected only the 1.4-kb transcript. Figure 4A is an autoradiograph of a northern blot of those RNAs probed with the 3′ fragment. The transcript accumulated within the first 8 h and then decreased to background level. The corresponding rates of ethylene evolved are shown in Figure 4B. Bailey accumulates about 2-fold more RNA than Suncrest, and evolves about twice as much ethylene over the same time period. It appears that Suncrest RNA accumulation is maximum at the 1-h point, whereas Bailey RNA accumulation drops at the 1-h time point and then peaks by 4 h. This drop in accumulation is not understood. The Bailey 1-h

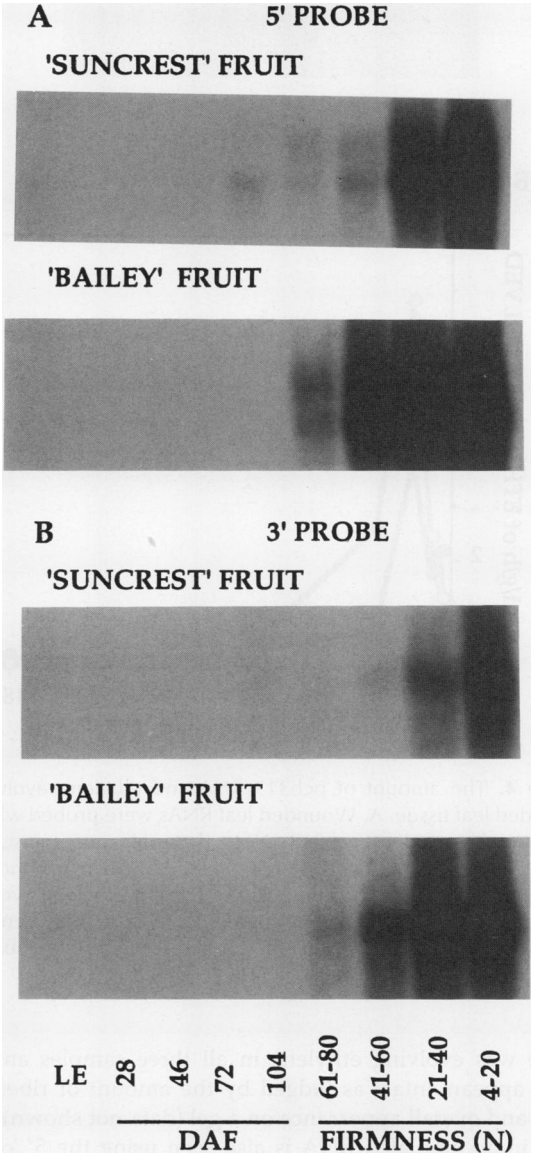

Figure 3. The accumulation of pch313 mRNA during fruit ripening of two cultivars. A, Northern analysis of fruit RNA probed with the 5′ probe of pch313. A major transcript of 1.4 kb and a minor transcript of 1.7 kb are detected. B, A northern analysis of fruit-ripening RNAs probed with the 3′ noncoding end of pch313. Only the 1.4 kb transcript is detected. LE represents leaf tissue, 28, 46, 72, and 104 represent DAF, when the fruit were sampled for RNA extractions. Fruit from both cultivars are not quite mature at 104 d after bloom. The fruit that were used for the firmness RNAs were all mature and sorted only by their resistance in N to a penetrometer.

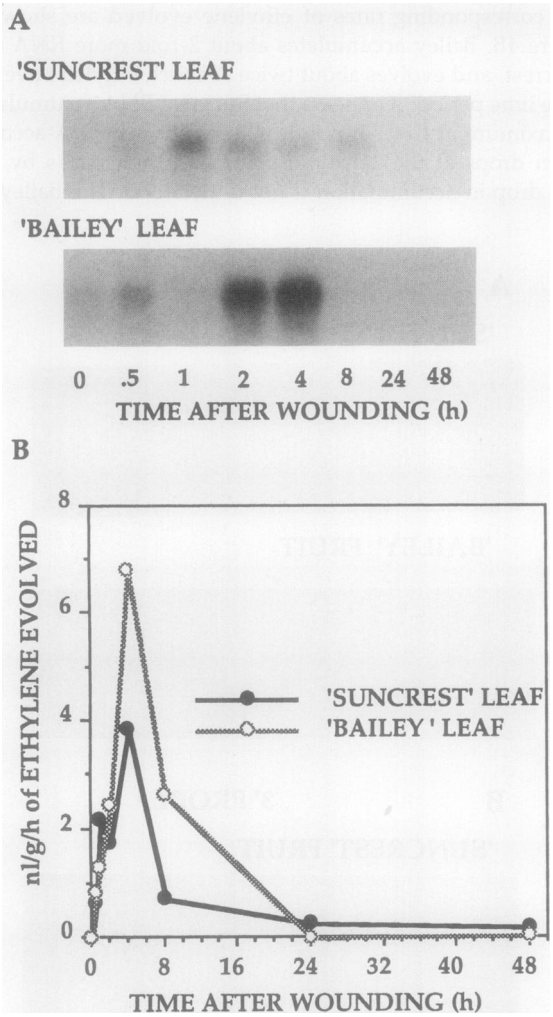

Figure 4. The amount of pch313 RNA and ethylene evolved in wounded leaf tissue. A, Wounded leaf RNAs were probed with the 3′ noncoding end of pch313. Note that only one transcript is detected, implying that pch313 is expressed following wounding of leaves along with fruit ripening. B, The rates of ethylene evolution by the wounded leaves are compared. Note that Bailey generates about two times more ethylene than Suncrest at 4 h after wounding.

tissue was evolving ethylene in all three samples and the RNA appears intact as judged by the amount of ribosomal RNA and overall appearance on a gel (data not shown). This drop in accumulated RNA is also seen using the 5′ coding sequence probe.

Preclimacteric fruit from both cultivars were wounded to see if the wound-induced pch313 RNA were leaf specific. Bailey and Suncrest exhibited an accumulation of the 1.4-kb transcript following wounding (Fig. 5A). The increase in amount was similar to that in leaf tissue. Peak accumulation was detected at 4 h for both cultivars and the rate of ethylene was maximum at 4 h (Fig. 5B). The amount of RNA hybridized was quantitated and indicated that Suncrest had 3-fold more RNA than Bailey, whereas the rate of ethylene evolved was identical.

Southern Analysis

DNA from both cultivars was probed with pch313 insert to determine the number of genes. The DNA was digested with five different restriction enzymes (Fig. 6). All digestions indicate that there are several genes with homology to the pch313 insert. There are slight differences between the two cultivars. The two large fragments detected in the *Eco*RI digest have inverse intensities potentially indicating polymorphisms resulting in different numbers of those fragments. The largest fragment in the *Eco*RV digest is more abundant in Suncrest than in Bailey, potentially indicating the same phenomena. There also may be some slight size differences with the Bailey fragments migrating faster in the *Hin*dIII and *Eco*RV digests.

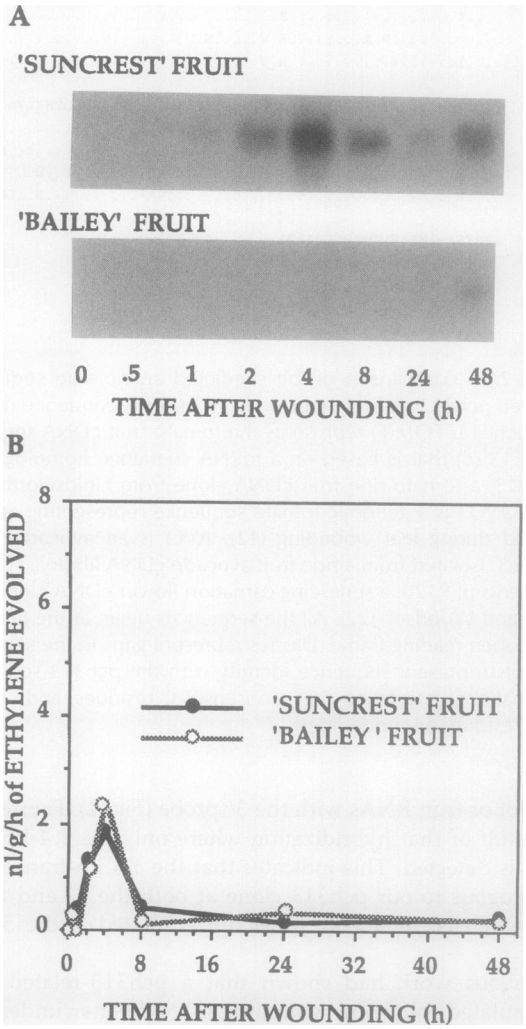

Figure 5. The amount of pch313 RNA accumulated and ethylene evolved in wounded fruit tissue. A, Wounded fruit RNAs were probed with the 3′ noncoding end of pch313. Note again that only one transcript is detected, implying that pch313 is expressed following wounding of fruit, leaves, and fruit ripening. B, The rates of ethylene evolution by the wounded fruit are compared. Note that they are similar even though the amount of pch313 RNA detected is different.

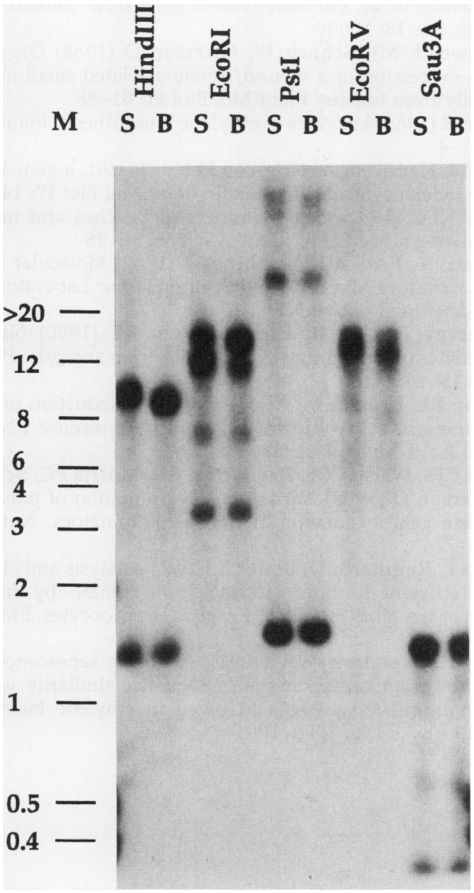

Figure 6. Southern analysis of DNA from Bailey and Suncrest leaves using pch313 insert as the probe. (M = size marker DNA; sizes are indicated in kb. S = Suncrest DNA digests; B = Bailey DNA digests.)

DISCUSSION

Pch313 detects several mRNAs that accumulate during the softening stage of fruit ripening and in response to wounding. Both of these phenomena are associated with increases in the amount of ethylene synthesized by plants. Pch313 is highly homologous to the pTOM13 family of genes, which has been shown to have EFE activity. Ethylene is synthesized from *S*-adenosylmethionine in two steps (14). The first step is catalyzed by the enzyme ACC synthase and the second step by EFE. Understanding how ACC synthase and EFE are regulated and their effect on the levels of ethylene will help to understand the regulation of the many functions that ethylene affects.

Experiments were carried out in peach to show that pch313 RNA accumulation does mirror ethylene evolution. The two wounding experiments demonstrate that the accumulation occurs prior to or at the same time as the peak ethylene evolution. During fruit softening, both cultivars have an increase in the amount of ethylene evolved when the fruit are 50 N in firmness, and this level peaks when the fruit are the softest (data not shown). The accumulation of the pch313-related RNA again precedes or parallels the increase in the amount of ethylene.

The first dilemma was to determine what gene is represented by pch313. There are at least two genes whose RNAs accumulate during fruit softening as determined by the two transcripts detected. Pch313 is related to both in the coding region and only to the 1.4-kb transcript in the 3′ region. Tissue wounding only induces the increase in accumulation of the 1.4-kb transcript, which is detected again by both the 5′ and 3′ probes of pch313. As indicated in the Southern analyses, there is a small family of related genes. Some of these genes may have similar transcript sizes and may even be related at the 3′ noncoding end. This might suggest that multiple transcripts of 1.4 kb are detected with the pch313 3′ probe and that possibly only a subset of these are accumulated during wounding and during fruit softening.

To test this hypothesis, more specific probes would need to be constructed and the northerns and Southerns probed with these specific probes to determine the relatedness of the genes. In addition, multiple clones related to pch313 could be isolated and sequenced to determine differences. In searching for full-length cDNAs, we isolated two clones that had sequences identical to the 978-base insert of λ313, implying that this represented the major transcript or at least the most clonable transcript. The simplest conclusion is that, like pTOM13 (13), pch313 represents a gene whose RNA accumulates during fruit softening and fruit and leaf wounding.

How this gene is regulated will be the subject of future research. The data presented here suggest that there are several levels of control. The level of ethylene evolved is not limited by the amount of pch313-related RNA. Suncrest leaf tissue evolves about two times as much ethylene as Suncrest fruit, yet the fruit has about three times as much RNA homologous to pch313. The wounding and ripening data suggest that there are several elements that regulate the accumulation of pch313 RNA. These include cultivar-specific differences, a fruit-ripening aspect, a wound-induced aspect, and a tissue-specific aspect. These factors may be acting on a single gene or they may each be regulating a subset of pch313-related genes. There may be other forms of regulation involved at the protein and transcription level that we would not be able to detect here.

We are currently attempting to isolate the second fruit gene in the form of a cDNA clone as well as the genomic sequences. The regulatory elements can then be analyzed and contrasted because the larger transcript is not detected following tissue wounding. We are also in the process of inserting an antisense construction of pch313 into our plum and peach transformation systems. We hope to test the wounding response on the seedlings and in the future test the effects on softening and fruit development.

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