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Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberization-associated highly anionic peroxidase

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Summary. A highly anionic peroxidase induced in suberizing cells was suggested to be the key enzyme involved in polymerization of phenolic monomers to generate the aromatic matrix of suberin. The enzyme encoded by a potato cDNA was found to be highly homologous to the anionic peroxidase induced in suberizing tomato fruit. A tomato genomic library was screened using the potato anionic peroxidase cDNA and one genomic clone was isolated that contained two tandemly oriented anionic peroxidase genes. These genes were sequenced and were 96% and 87% identical to the mRNA for potato anionic peroxidase. Both genes consist of three exons with the relative positions of their two introns being conserved between the two genes. Primer extension analysis showed that only one of the genes is expressed in the periderm of 3 day wound-healed tomato fruits. Southern blot analyses suggested that there are two copies each of the two highly homologous genes per haploid genome in both potato and tomato. Abscisic acid (ABA) induced the accumulation of the anionic peroxidase transcripts in potato and tomato callus tissues. Northern blots showed that peroxidase mRNA was detectable at 2 days and was maximal at 8 days after transfer of potato callus to solid agar media containing 10^{-4} M ABA. The transcripts induced by ABA in both potato and tomato callus were identical in size to those induced in wound-healing potato tuber and tomato fruit. The anionic peroxidase peptide was detected in extracts of potato callus grown on the ABA-containing media by western blot analysis. The results support the suggestion that stimulation of suberization by ABA involves the induction of the highly anionic peroxidase.

Key words: Abscisic acid – Peroxidase – Potato – Suberization – Tomato

Introduction

Plant cell walls are suberized to make them effective barriers to diffusion when developmental or stress factors make such barriers necessary (Kolattukudy 1980, 1987). For example, chemical studies have shown that periderms, endodermis of roots, and cell walls of the chalazal region of seed coats are suberized (Kolattukudy and Espelie 1989). According to the working hypothesis that is consistent with the available information, suberin is a polymeric material that consists of an aromatic matrix attached to the cell wall and aliphatic domains that are attached to the aromatic matrix. The aliphatic polyester domains are composed of very long chain fatty acids, fatty alcohols, ω-hydroxy fatty acids and dicarboxylic acids.

A highly anionic peroxidase is most probably involved in polymerizing phenolic monomers to generate the aromatic matrix of suberin (Borchert 1978; Cottle and Kolattukudy 1982; Kolattukudy 1980, 1987). Such a peroxidase has been purified from wound-healing potato tuber slices (Espelie and Kolattukudy 1985) and immunocytochemically shown to be localized exclusively at the site of suberization, in the cell wall of suberizing potato slices (Espelie et al. 1986). The potato anionic peroxidase cDNA has been cloned and sequenced (Roberts et al. 1988). Induction of potato anionic peroxidase mRNA was observed in suberizing potato slices, and a homologous anionic peroxidase mRNA was induced in suberizing tomato fruit (Roberts et al. 1988).

Resistance of a tomato isoline to the pathogen *Verticillium albo-atrum* has been correlated with its ability to coat its xylem vessels more rapidly after inoculation than a susceptible near-isoline (Robb et al. 1987; Street et al. 1986). Histochemically this coating appears to consist of suberin and lignin (Street et al. 1986) and gas chromatography/mass spectroscopy (GC/MS) analysis of the depolymerization products has shown aliphatic components characteristic of suberin (P.E. Kolattukudy, J. Robb, unpublished). Presumably, the deposition of suberin aids in the defense of the plant by forming a barrier that contains the pathogen as previously suggested (Kolattukudy 1980; Pearce and Rutherford 1981).

The anionic peroxidase appears to be a key component in the regulation of suberization. The level of this enzyme correlates with progression of suberization induced by different factors such as wounding and abscisic acid (ABA) treatment (Borchert 1978; Cottle and Kolattukudy 1982). Thus it is likely that timely elicitation of this enzyme could be an important defense mechanism against stress and pathogens. As a first step toward the elucidation of the regulation of expression of this gene, its structure and organization need to be established.

In this report we describe the cloning and sequencing of two tomato anionic peroxidase genes. One of these genes is expressed in wound-healing tomato fruits, as shown by primer extension analysis. There appear to be two copies

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of each gene in the haploid tomato genome. ABA induction of the anionic peroxidase is also reported.

Materials and methods

Plants. Potato plants (*Solanum tuberosum* cv Russett Burbank) and tomato plants (*Lycopersicon esculentum* cv Castlemart II) were grown from seed in the greenhouse.

Screening of tomato genomic library. A genomic library prepared from *Eco*RI partial digests of tomato DNA in lambda charon 4A was screened with ^{32}P-nick translated pAP_3 (Roberts et al. 1988). This plasmid contains the first 800 bp from the 5′ end of potato anionic peroxidase cDNA. The host strain of *Escherichia coli* used was KH802. Plaque replicas on nitrocellulose filters were hybridized with labeled probe at 42° C as described (Maniatis et al. 1982).

Sequencing of peroxidase genes. Restriction fragments of positive clones that hybridized to potato anionic peroxidase cDNA were subcloned into pUC19 or Bluescript plasmid (Stratagene). Deletion derivatives of these subclones were generated by exonuclease III digestion (Henikoff 1984) in order to obtain overlapping segments for sequencing. The dideoxy termination method (Sanger et al. 1977) was used to sequence double-stranded plasmid DNA. Synthetic primers (prepared with an Applied Biosystem 381A DNA synthesizer) were used to fill gaps in the sequence and for confirmation.

Southern blotting. DNA was isolated from leaves as described (Bendich et al. 1979). Restriction enzyme digests of the nucleic acids were electrophoresed in 0.5% agarose along with copy number standards. Inserts used as copy number standards were purified from subclones by restriction enzyme digestion (Maniatis et al. 1982). Nucleic acids were blotted onto nitrocellulose and probed with ^{32}P-nick translated potato anionic peroxidase cDNA.

Primer extension. Oligonucleotide primers were synthesized on an Applied Biosystems synthesizer and were end labeled with [^{32}P]ATP. Total RNA (50 µg), prepared from wounded tomato fruit as previously described (Roberts et al. 1988), was hybridized to 10 pmol labeled primer at 30° C overnight (Kingston 1987). The primers were extended with reverse transcriptase and dNTPs, precipitated, and loaded onto a sequencing gel along with size standards.

ABA treatment of tissue culture. Tomato callus (*L. esculentum* × *L. peruvanium*) was maintained on LS medium (Linsmaier and Skoog 1965). Potato callus (*S. tuberosum* cv Lemhi Russet) was maintained on modified LS medium in which 10^{-5} M picloram (Aldrich) was substituted for 2,4-dichlorophenoxyacetic acid (2,4-D). Modified LS medium containing 0.03 mg/l kinetin was used for ABA and control experiments. ABA was dissolved in ethanol (13.2 mg/400 µl for 1 l of medium) and added to the medium before autoclaving; to the control medium, 400 µl ethanol was added prior to autoclaving.

RNA blotting. RNA was purified from callus tissue or wound-healing tuber (Flurkey and Kolattukudy 1981), fractionated on agarose gels containing formaldehyde and blotted onto nitrocellulose (Maniatis et al. 1982). The blots were hybridized to ^{32}P-nick translated anionic peroxidase cDNA as described (Roberts et al. 1988), washed in 2 × SSPE at 65° C for 1 h, and subjected to autoradiography.

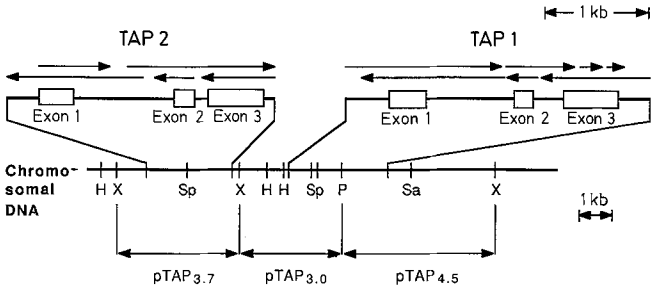


Fig. 1. Restriction map and sequencing strategy for tomato anionic peroxidase genes. *Arrows* represent total lengths of sequence obtained on a given strand. *Open boxes* represent exons. Abbreviations used: H, *Hin*dIII; X, *Xba*I; Sp, *Sph*I (TAP 1 has two *Sph*I sites in close proximity); P, *Pst*I; Sa, *Sal*I. The three major clones that span the two genes and flanking regions are indicated at the bottom

Western blotting. Callus tissue was homogenized in 0.01 M potassium phosphate, pH 7 at 0° C (4 ml buffer/g tissue) using an Omni mixer. Homogenates were centrifuged at 15000 *g* for 20 min and protein concentrations of the resulting supernatants were determined as described (Bradford 1976). Samples containing 5 µg protein were subjected to electrophoresis in 0.04 M potassium acetate buffer, pH 4.6 using an LKB 2117 Multiphore apparatus (Cottle and Kolattukudy 1982). The separated proteins were blotted onto nitrocellulose filters in the same buffer. Blots were treated with rabbit antiperoxidase antiserum and [^{125}I]protein A (Roberts et al. 1988) and were subjected to autoradiography. Purified anionic peroxidase was prepared as described (Espelie and Kolattukudy 1985).

Results

Characterization of genomic clone

A tomato genomic library in lambda charon 4A was screened with pAP_3, a plasmid containing the first 800 bp from the 5′ end of potato anionic peroxidase cDNA (Roberts et al. 1988). One clone was obtained that hybridized strongly after a second screen. DNA purified from the isolated positive phage was digested with restriction enzymes and subjected to Southern blot analysis with pAP_3 as probe. A restriction map of the genomic clone is shown in Fig. 1. Three segments were selected for subcloning into pUC19: the 3.7 kb *Xba*I fragment, the 4.5 kb *Xba*I-*Pst*I and the 3.0 kb *Pst*I-*Xba*I fragment. These clones were designated pTAP 3.7, pTAP 4.5, and pTAP 3.0, respectively. When pAP4, a plasmid containing the 3′ sequences of the potato anionic peroxidase cDNA (Roberts et al. 1988) was used to probe the Southern blots of digests of the three plasmids, only pTAP 3.7 and pTAP 4.5 hybridized (data not shown). This result suggested that the genomic clone consisted of two genes highly homologous to potato anionic peroxidase cDNA.

Structures of tomato anionic peroxidase genes

Portions of plasmids pTAP 3.7, pTAP 3.0 and pTAP 4.5 were sequenced according to the scheme shown in Fig. 1.

```
          1                                                                                                  100
TAP1      gcagggatcg ttaataatta tactcaagta ttaacgtcaa ttaagaacta atatctttgc accaaatgag aaaagaaaca taaatctatc ctcgaaatta
TAP2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          101                                                                                                200
TAP1      tctttccttc ttaaaatcga tattgtgact acaacatgaa agaaaattat tgacatttga cataattatc ggataaatat agctaagaaa acatcacacg
TAP2      .......... ......actt tttaataaaa ttgaatgtgt taacattagt agtaactata tatatcatca tatcatattg tctgaatatt ttatcacgat

          201                                                                                                300
TAP1      ttacacagct agaactttag aaaagaaaga aaagtcactt tcacatgaaa catcaattct cagtttgtaa ttacacgata attcaaag.. atgaattaat
TAP2      attctcgcaa aagaataaac aaatataaaa agtaattttt attttacaaa atataaattt gattttgtaa tcacacaatt atttaaagca gtaaattaat

          301                                                                                  **∇           400
TAP1      aataacagta atattgacaa gtaattataa gtcttcacct gcctatataa atagaggcat aaataaaagc aaa...atca tccctatcga aagcctttaa
TAP2      catataatac taattcatga ccaatagtaa gtcttcactt gcc..tataa atagaagtga aaatataggc aaacagacca tccctaaggc ctctgtttct

          401                                                                                                500
TAP1      tttgtttcct cgatagacat ttaaattaag tgATGGGTTT TCGTTTGAGT CATTTGAGTC TTGCATTGAG TTTTGTGGCA CTTGCACTTG CAGGTGTTGC
TAP2      tccaaataga catttaataa tttctttaag tgATGGGTTT TCGTTTGAGC CTTTTGAGCC TTGCGGTGAG CCTTGTGGCA CTTGCACTCG CAGGTGTTGC

          501                                                                                                600
TAP1      TATTTATAGA AATACTTATG AAGCCATCAT CATGAAAAAC GGTTCACTCC TCAAAAATGT TTCTCCAGAT TTCGATTCGT TGGAATCAGG CGTAGCCAGT
TAP2      CATTTATAGA AATACTTATG AGGCCATGAT CATGAATAAC GGATCACTCC TCCAAAATAT CTCTCCAGAT ATCGATTCGT TGGAATCAGG CGAAGTCAGT

          601                                                                                                700
TAP1      ATTTTAACCT TAAATAATAA TAAGAAGAGA AATTCAGACA AGTACTTGAG ACAGCAACTA ACTCCGGAGG CATGCGTTTT CTCAGCTGTA CGAGCAGTTG
TAP2      ATTTTAATTT TAAATGAT.. .AAGAAGAAA AATTCAGACA AATATTTGAG TCAGCAATTA ACTCAGGAGT CTTGCGTTTT CTCAGCTGTG AAAGGAGTTG

          701                                                                        →                       800
TAP1      TGGACAGTGC TATTGATGCA GAAACTCGCA TGGGAGCTTC TCTCATTCGT CTCCACTTTC ATGACTGCTT CGTTGATgta aatatatatt tccttcgctc
TAP2      TGGACAGTGC CATTGATAAT GAAACGCGCA TGGGAGCTTC TCTCATTCGT CTACATTTCC ATGACTGCTT TGTTGATgta aatattcttt cgtccttttt

          801                                                                                                900
TAP1      ttttttttatc gtatttgatt atgcacaaaa ttttatttga agtcacttaa atattttatg cttgttcaaa caaatttgaa aattctttca gtttttcctg
TAP2      ctcccgctca atattttata ataatgtaat tttaaggttg atataatcgt atgaataagt atttacttat tttttatcat aaattttaaa ttttttaatt

          901                                                                                               1000
TAP1      aactcacaat gcatgcagtc aattacttta ctagggaaaa tgcacaagta cattctcaac ctatgtccga aattacagag acacacttat attatactaa
TAP2      ttaactttta aaatctctta caactaaacg cttaatataa aaatagaaag atcggatttt ttattttatt tttgaggaca tattaaaaaa ataaaacagt

          1001                                                                                              1100
TAP1      ggttctatta ctcccctgaa cttattttat aagtaatatt ctacctagct tgaaaaaaaa gtcaacctgc gtaaggccaa caagatagtg tcacgtaggc
TAP2      atgttaggtg tagtatataa attgaatccg agtcctcctt tctcttatta aaattaaaag taaaaacctg gctccatgga ccatggtcat ataatattat

          1101                                                                                              1200
TAP1      cgaaaaaaag taaaaaatta ttaataaaat aagttaagta aaataatagg accttagtat agtataagcg tgtctctaaa atttcgggta taggttaaag
TAP2      ttaatgtaaa tatgtgtaag tggtccgaaa ggaaagtgat ccgtaaaatt atttgaaaaa tctgacggaa cgaatttcaa aacatgctta tgaaaactgt

          1201                                                                                              1300
TAP1      ggactactta aattgaaatg aaaagagtag agggatatca ttttttaaga caggctaaaa agagtacaat gttaggtgta gcatattaat tgaatccgag
TAP2      gtgggttttt attagtgtag tagttgtaaa gttgcatcat gaatggtacc atcttcttgt aatttgtgaa atgaacagtt tcttattagt tttttctgcc

          1301                                                                                              1400
TAP1      tccttttcct cttattaatt aaacctggcc gtatgatcat atttttcttt cataaataaa taaattgttg aaagatctga ggctacgaac tccaaattaa
TAP2      ttacaactct tttttatcag ccacttggct aattttgggc aactttatct cctttatctc actcatcact ttctcttttg ctcgattctc tttgcatatt

          1401                                                                                              1500
TAP1      aattataatg ccatacttgt tgttttaaat cgcaaatatt taaaaaaaaa atacttttttc ttttgaactc cccgtcaagt aaaatatctt cgcacacatt
TAP2      ttagatatat ataaatctag tcccattttt atatacctta aataaatagt ctgttttact cattttcatt ctatctagca tgctcttttt ctatattaat

          1501                                                                                              1600
TAP1      ttgcttctac atgaactcca aaaactttga aaagtctagt ctgctttcca gttttatttt attttagatc ttctgcagtt .......... ..........
TAP2      atattgataa aaaaaatata tgattttaca atatctaact taaaatctaa tataaaatta ttgattagta agacaatatt taatttgttt gaactataag

          1601                                                                                              1700
TAP1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
TAP2      gagaactagt aaatatagta ggattattta tctaattagg tttgtttttt ctttaaataa attcatgtta aattaaacca catccatata aattgaagca

          1701                                                                        ←                       1800
TAP1      .......... gatttatgta tttgactaat tatacctctc tcgttttctt ttttattacg aaaaata... .agGGTTGTG ATGGAGGTAT TCTGCTAGAT
TAP2      atagagtaac taattatgta tacatataat tgactaatta cagttatgtt tttggtttgg aaaaaaaaaa cagGGTTGTG ATGGAGGTAT TCTGCTAGAT
```

Fig. 2. Sequences of tomato anionic peroxidase genes. Untranslated sequences are in *lower case letters.* TATA and CAAT boxes are *double underlined.* The cap site is denoted by a *triangle.* Positions corresponding to minor primer extension elongation products of the TAP 1 primer are *starred.* 5′ sequences of TAP 1 and TAP 2 that are highly conserved are *overlined.* Sequences of TAP 1 and TAP 2 to which complimentary oligonucleotides were directed are *underlined.* Sequences of TAP 1 and TAP 2 introns that are highly conserved are *underlined with dotted lines.* The polyadenylation signal is *underlined* and the poly[A] addition site is denoted with a *vertical arrow* between the two A residues, either of which could be the site. The 5′ and 3′ ends of each intron are marked with *horizontal arrows.* The stop codon is underlined with *stars*

```
          1801                                                                                                1900
TAP1      GATATTAATG GAACATTTAC AGGGGAACAA AACTCACCAC CCAACGCCAA CTCAGCCAGA GGTTATGAAG TAATAGCACA AGCTAAACAA AGTGTCATAA
TAP2      GATATTAATG GAACATTCAC CGGGGAACAA AACTCACCAC CCAACAACAA CTCAGTCAGA GGCTTTGAAG TGATAGCACA AGCTAAACAA AGTGTTGTAG

          1901                                                                                    →           2000
TAP1      ACACATGCCC CAACGTATCT GTATCTTGTG CCGACATATT AGCTATTGCT GCTCGTGATT CTGTTGCTAA Agtaagtc.t atcattcatt ttacagtttg
TAP       ATTCATGCCC CAACATATCT GTATCTTGTG CTGATATTTT AGCTATTGCT GCTCGTGATT CCCTTGCTAA Agtaagtcct attgctcaat ttttagt...

          2001                                                                                                2100
TAP1      ataatttcta ccaaaattaa ttacgaatag taacaaatgg atatttaatc aaactctaaa gtcatcgtgg aagttgagaa atgttcaaag agatgaccaa
TAP2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

          2101                                                                                                2200
TAP1      attccacatc cacaatcaat gtggaaattg tgacacctcg cctgtgggcg tctaacattt aatagatgaa gcattgtatt tatttaggtt atgatgatat
TAP2      .......... .......... .......... .......... .......... ....caaagt tactattttt aatttagctt tacactacat gtttaattaa

          2201                                                  ←                                             2300
TAP1      gacaaagaaa ttgacttggg ctaactcaaa ctctgtttta ttggtatttg acagTTAGGA GGACAAACTT ATAGCGTTGC ATTAGGGAGA AGCGATGCTA
TAP2      cttatatcat atgatcaata gtaagttaaa ctctatttta ttggtatttg acagTTAGGA GGACAAACTT ATACCGTTGC ACTAGGGAGA AGCGATGCTA

          2301                                                                                                2400
TAP1      GAACTGCCAA CTTTAGTGGT GCTATAAATC AACTTCCAGC TCCATTCGAC AACCTAACAG TCCAAATACA GAAATTCAGC GACAAAAATT TCACCCTCCG
TAP2      CAACAGCCAA CTTTAGTGGT GCTATAAATC AACTTCCAGC TCCATCCGAC AACCTAACTG TTCAAATACA GAAATTCAGC GACAAAAATT TCACTGTCCG

          2401                                                                                                2500
TAP1      TGAAATGGTT GCACTAGCTG GTGCACACAC GGTAGGTTTC GCCAGGTGTT CCACAGTTTG CACCAGCGGT AACGTTAATC CTGCCGCACA ACTCCAATGC
TAP2      TGAAATGGTG GCACTAGCTG GCGCGCACAC GGTAGGTTTC GCCAGGTGTT CCACAGTTTG CACCAGCGGT AACGTTAATC CTGCCGCACA ACTCCAATGC

          2501                                                                                                2600
TAP1      AACTGCTCCG CCACGCTAAC TGATTCTGAT TTGCAACAAT TGGATACTAC TCCTACTATG TTCGACAAAG TTTACTACGA TAACTTAAAC AGCAACCAAG
TAP2      AACTGCTCCG CCACGCTAAC TGATTCCGAT TTGCAACAAT TGGATACAAC TCCTGCTGTG TTCGACAAAG TTTACTATGA TAACTTAAAC AACAACCAAG

          2601                                                                                                2700
TAP1      GCATAATGTT TTCAGATCAA GTTTTGACCG GGGATGCTAC AACTGCTGGT TTTGTAACGG ATTATAGCAA CGATGTGAAC GTTTTCCTTG GAGATTTTGC
TAP2      GGATAATGTT CTCGGATCAA GTTTTGACTG GGAATACTAC AACTGCTGGT TTTGTAACCA CTTATAGCAA CAATGTTACT GTTTTTCTTG AAGATTTTGC

          2701                                                                                                2800
TAP1      TGCTGCCATG ATCAAGATGG GAGACTTGCC CCCCTCCGCC GGCGCTCAAT TGGAAATTCG TGATGTTTGT AGCAGGGTTA ATCCCACTTC AGTGGCTTCT
TAP2      TGCTGCTATG ATCAAAATGG GGAATTTACC TCCCTCCGCT GGCGCTCAAT TGGAAATTCG CGATGTTTGC AGCAGGGTTA ATCCCACTTC TGTGGCTTCT

          2801                                                                                                2900
TAP1      ATGTGAaaaa tt.cggaaat tttaattaac ttatctcgcg actagttgct t...catgtt ttcttcaaaa taaaagaatc actggctcca gaatgtggtt
TAP2      ATGTGAaaaa tttcggaaat tttaagtagc ttttatc.tt actatttgtc gcatcatgtt ttcatctttt tcagtattga tgcaataata tatatgtccc
             ***
          2901                                                                  ↓                             3000
TAP1      tggttttggt atttgttttg taatccatcc tattttatat gaatattgtt ttccttgtgt gctaatgttg gagctgatca gaatagttca attcgttatt
TAP2      tatgttcgca gtgtttttaa tccaatttat tcttcttaat ccatatagta attaggaaat cggtagcaa. ..........  ..........  ..........

          3001                                                  3050
TAP1      aaaaatttaa taatctgcaa aataaaaata ggactcaaat agccttatgaa
TAP2      .......... .......... .......... .......... ..........
```

Fig. 2 (continued)

```
          1                                                                                                   60
AP        .......... ..SFVALALA GVAIYRNTYE AIIMNNGSLL QNASPHFDSL ESGVASILTL
TAP1      MGFRLSHLSL AL                      K       K V  D
TAP2      MGFRLSLLSL AV L                  M           I  DI        EV   I

          61                                                                                                  120
AP        NN.KKRNSDM YLRQQLTPEA CVFSAVRGVV DSAIDAETRM GASLIRLHFH DCFVDGCDGG
TAP1        N     K                  A
TAP2      ND.  K   K   S    Q S        K          N

          121                                                                                                 180
AP        ILLDDINGTF TGEQNSPPNA NSARGYEVIA QAKQSVIDTC PNISVSCADI LAIAARDSVA
TAP1                                                  N    V
TAP2                        N   V  F            V S                      L

          181                                                                                                 240
AP        KLGGQTYNVA LGRSDARTAN FTGALTQLPA PFDNLTVQIQ KFNDKNFTLR EMVALAGAHT
TAP1            S              S  IN                    S
TAP2            T          T   S  IN       S           S     V

          241                                                                                                 300
AP        VGFARCSTVC TSGNVNPAAQ LQCNCSATLT DSDLQQLDTT PTMFDKVYYD NLNNNQGIMF
TAP1                                                                      S
TAP2                                                  AV

          301                                                                                                 360
AP        SDQVLTGDAT TAGFVTDYSN DVSVFLGDFA AAMIKMGDLP PSAGAQLEIR DVCSRVNPTS
TAP1                          N
TAP2            NT        T    N T   E           N

          361
AP        VASM
TAP1
TAP2
```

Fig. 3. Amino acid sequences deduced from the nucleotide sequence of anionic peroxidase genes TAP 1 and TAP 2 and the amino acid sequence of potato anionic peroxidase (AP) deduced from cloned cDNA. For TAP 1 and TAP 2, only the amino acid differences are shown. The first 12 amino acids in potato anionic peroxidase are not known. Potential glycosylation sites are marked with *stars*. The putative N-terminus is marked with a *large black arrow*. Positions in the peptide that correspond to intron placement in the coding sequence are denoted by *vertical lines*

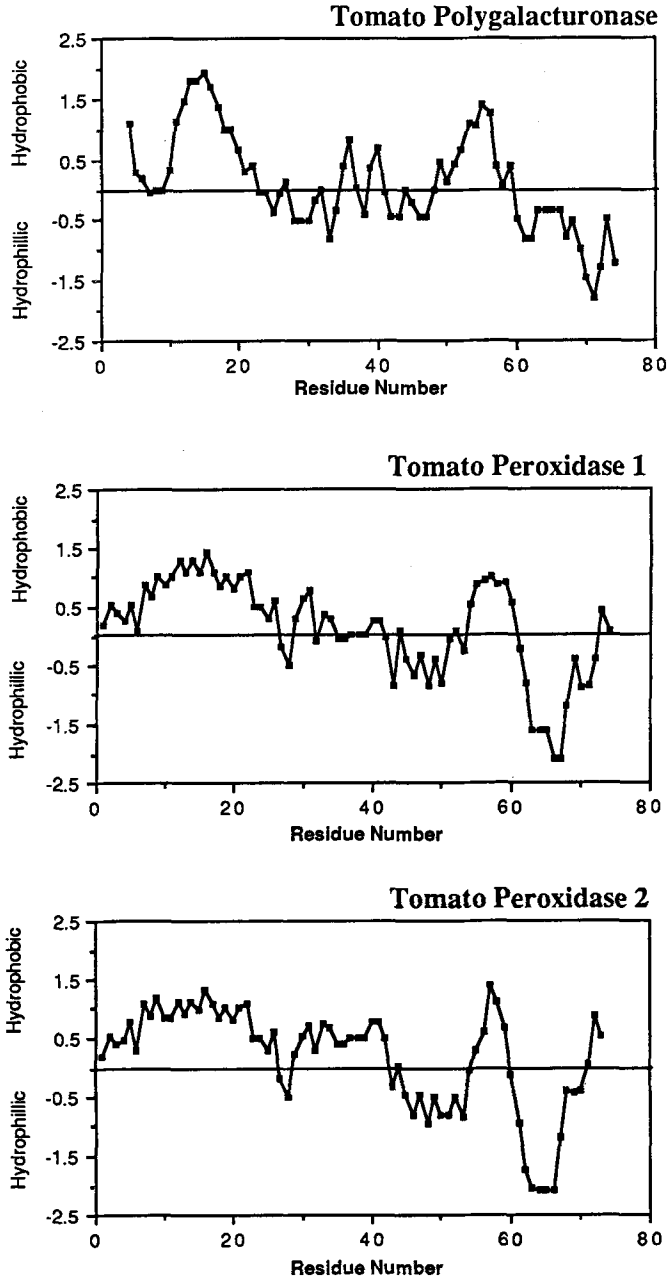


Fig. 4. Hydropathy plots of leader peptides of tomato polygalacturonase and tomato anionic peroxidase. Hydropathic indexes (Henikoff 1984) averaged over 6 residues, were plotted against residue number

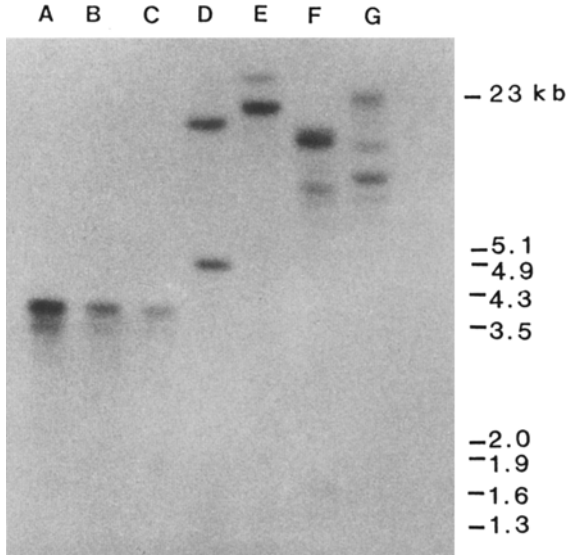


Fig. 5. Southern blot of potato and tomato genomic DNA. DNA from potato (lanes F and G) or tomato (lanes D and E) was cut with *Eco*RI (E, G) or *Hin*dIII (D, F) and hybridized with potato anionic peroxidase cDNA. For copy number standards, purified 3.7 kb *Xba*I fragment of the gene (Fig. 1) was loaded onto the gel in quantities of 80, 40, and 20 μg (lanes A, B, and C) and was hybridized to the anionic peroxidase cDNA

The sequences of the two tomato anionic peroxidases genes (designated TAP 1 and TAP 2) are shown in Fig. 2. TAP 1 has a *Pst*I site in the first intron so that its 5′ end is subcloned into pTAP 3.0 and its 3′ end is subcloned into pTAP 4.5. The entire TAP 2 gene is included in pTAP 3.7. The two genes are separated by approximately 1500 bp. TAP 1 consists of three exons interrupted by two introns of 761 and 271 bp. TAP 1 also has the same number of introns and exons; however, the introns are of different sizes, 996 and 123 bp in length. When gaps are introduced into the predicted potato anionic peroxidase mRNA and the predicted TAP 1 mRNA, so that maximal homology is achieved, the two are 96% identical to each other. Similar analysis of TAP 2 mRNA and potato anionic peroxidase mRNA indicates that the two have 87% identity. The codon usage of TAP 1 and TAP 2 was analyzed for similarity with that of potato anionic peroxidase cDNA using a correspondence test as defined (Grantham et al. 1981). The distance between the coding region of any mRNA and itself is 0, and as coding regions increase in dissimilarity, the value for the distance increases. The distances between coding regions of potato anionic peroxidase cDNA and TAP 1 or TAP 2 were calculated to be 0.489 and 0.700, respectively. Therefore, the codon usage of potato anionic peroxidase cDNA is more similar to that of TAP 1 than that of TAP 2.

The polypeptides predicted by TAP 1 and TAP 2 coding sequences are presented in Fig. 3. Both polypeptides are predicted to be anionic and have molecular masses of 39000 daltons. When gaps are introduced to optimize alignment of homologous regions, TAP 1 polypeptide is 96% identical to potato anionic peroxidase, and TAP 2 polypeptide is 89% identical to potato anionic peroxidase. Although the N-terminus of mature potato AP has not been identified, it is probably a cyclized glutamine (residue 75 in Fig. 3) on the basis of comparison with other plant peroxidase sequences (Roberts et al. 1988). Similarly, the mature tomato anionic peroxidases are assumed to begin with this residue so that the leader peptides for these proteins contain 74 (TAP 1) and 73 (TAP 2) amino acid residues. A comparison of the hydropathy plots of the tomato peroxidase leader sequences with a polygalacturonase leader is shown in Fig. 4. The net charges of mature TAP 1 and TAP 2 polypeptides are predicted to be -13 and -12 respectively at pH 7.4.

Estimation of gene copy number

Southern blots probed with ^{32}P-nick translated pAP3 revealed that there is more than one anionic peroxidase gene in the genomes of both potato and tomato (Fig. 5). As

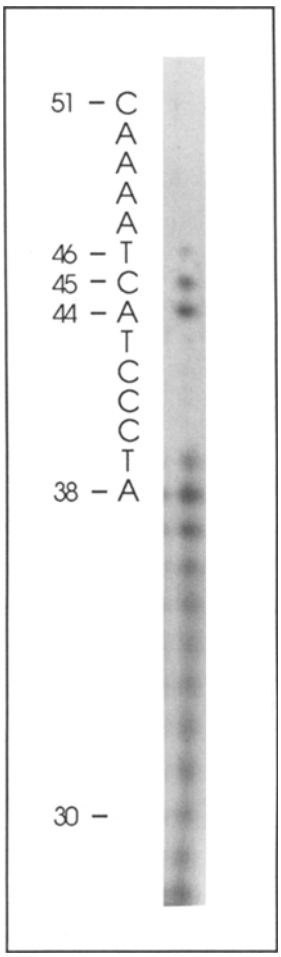


Fig. 6. Primer extension analysis of TAP 1. Radiolabeled oligonucleotide primer that specifically complements TAP 1 (shown in Fig. 2) was hybridized to total RNA prepared from suberizing tomato fruit aged for 3 days or to tRNA or to total RNA from fresh tissue for control that showed no bands

a copy number standard, purified 3.7 kb *Xba*I insert from pTAP 3.7 was loaded onto the gel alongside the genomic DNA digests, in quantities representing one, two, and four copies of the 3.7 kb segment. From comparison of the band intensities of genomic fragments to those of the standards, one can estimate that approximately two copies each of two highly homologous genes are present per haploid genome in tomato. In potato, presumably a similar set of copies of the two subfamilies of genes are present; however, since the potato genes have not been sequenced, the exact nature of these genes remains to be established. When Southern blots of tobacco DNA digests were probed with pAP3, no homologous fragments were detected (data not shown).

Primer extension analysis

The 5′ end of anionic peroxidase mRNA was determined using primer extension analysis. Two oligonucleotide primers that were specifically complimentary to 5′ regions of TAP 1 or TAP 2 (underlined in Fig. 2) were synthesized. The primers were labeled and were hybridized to either total RNA from wounded tomato fruit or tRNA. After elongation, the products were loaded on a sequencing gel alongside a sequencing ladder. The primer specific for TAP 1, when hybridized to RNA prepared from suberizing tomato fruit, yielded an elongation product (Fig. 6). This result suggests that wounded tomato fruits aged for 3 days accumulate the TAP 1 mRNA. The elongated products were 44, 45 and 46 bp in length with the most intense band occurring at 44 bp. The sequence of TAP 1 mRNA begins with ATCCC as shown in Fig. 2. The TAP 2 specific primer did not yield a discernible elongation product. Therefore, whether or not TAP 2 is expressed in wound-healing tomato fruits remains unclear.

ABA induction of peroxidase transcript

To investigate the apparent induction of the anionic peroxidase activity resulting from ABA treatment, potato callus was transferred to solid agar medium containing 10^{-4} M ABA or to medium without ABA. RNA, extracted at 1, 2, 3, 4, 6, or 8 days after transfer, were subjected to northern blot analysis, in which the probe used was pAP_3 (Roberts et al. 1988). Tissue grown on ABA-containing medium accumulated an mRNA species that cross hybridized with the cDNA for anionic peroxidase (Fig. 7). Callus tissue that had been transferred to medium without ABA accumulated no such mRNA species. The ABA-induced transcript was detectable at 2 days after transfer, and reached a maximum level at 8 days after transfer. Northern blot analysis showed that the ABA-induced message was identical in size (ca. 1.6 kb) to the anionic peroxidase mRNA that accumulated in suberizing potato tuber slices aged for 3 days.

Tomato callus grown on medium with ABA for 3 days also accumulated a transcript that hybridized with potato anionic peroxidase cDNA (Fig. 7). Northern blot analysis showed that this induced transcript was of the same size as the wound-induced transcripts found in potato and tomato. The peroxidase transcript was absent in tomato callus grown on medium without ABA.

ABA induction of peroxidase protein

Extracts of callus tissue grown on medium containing 10^{-4} M ABA or medium without ABA for up to 8 days after transfer were subjected to western blotting after the proteins were separated by native polyacrylamide gel electrophoresis at pH 4.6. An autoradiogram of the blot corresponding to the negatively charged proteins is shown in Fig. 8. At 4 days after transfer to ABA-containing medium the highly anionic peroxidase was barely detectable; by 6 days, this peroxidase was clearly detectable and it became very dominant by the 8th day. On the other hand, in tissue resting on medium containing no ABA the highly anionic peroxidase could not be detected even after 8 days. Although the antiserum used in this experiment was raised against purified highly anionic peroxidase, a second, less anionic protein cross reacted with the antiserum.

Discussion

A highly anionic peroxidase is most probably responsible for polymerizing the aromatic components to generate the aromatic matrix of suberin. The temporal and spatial occurrence of this peroxidase in wound-healing tissue (Borchert 1978; Espelie et al. 1986; Roberts et al. 1988) and the correlation of the level of the enzyme with inhibition or stimula-

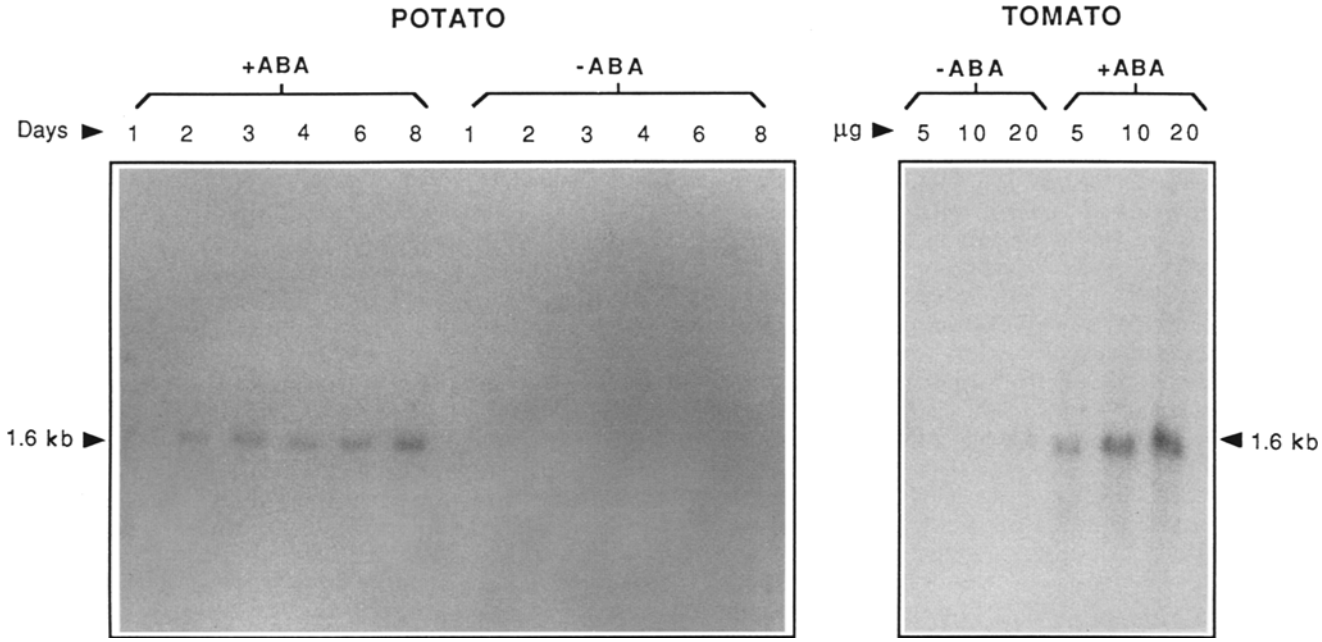


Fig. 7. Induction of anionic peroxidase mRNA in potato and tomato callus by abscisic acid (ABA). The callus tissue was on the ABA-containing agar for 3 days (tomato) or the number of days indicated on top (potato) before RNA was extracted. Electrophoresis was on agarose containing formaldehyde and pAP_3 was used as the ^{32}P-labeled probe. The amount of RNA used is indicated on top for tomato, and for potato 20 µg was used

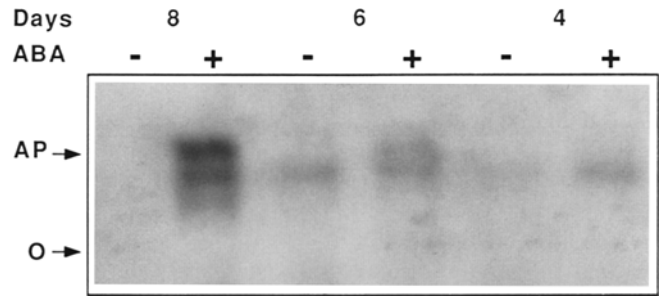


Fig. 8. Western blot analysis of potato callus proteins grown on medium with ABA. Proteins were extracted from potato callus at different periods after transfer to ABA-containing or control medium (number of days shown on top). The proteins were electrophoresed at pH 4.6 on a horizontal gel, the anionic proteins were blotted from the gel onto nitrocellulose, and the blots were treated with anti-anionic peroxidase followed by [^{125}I]protein A. The autoradiogram of the blot is shown. O, origin, AP, the most highly anionic peroxidase

tion of suberization (Cottle and Kolattukudy 1982; Roberts et al. 1988; Sijmons et al. 1985) strongly support this hypothesis. With cloned cDNA for the suberization-associated peroxidase from potato a highly homologous mRNA was detected in wound-healing tomato fruits (Roberts et al. 1988). Both in potato tuber and tomato fruit, the appearance of the message correlated with suberization. Using this potato cDNA as the probe we cloned and sequenced two genes for tomato anionic peroxidase. The 5′-flanking regions of the two anionic peroxidase genes include sequences (Fig. 2) similar to the TATA box typical of eukaryotic promoters. In TAP 1, the TATA box is positioned 25 bp upstream from the transcription start site, and 78 bp upstream from the first codon. Sequences similar to the CAAT box are located 84 bp and 64 bp upstream from the TATA boxes of TAP 1 and TAP 2, respectively. The polyadenylation site of TAP 1 is predicted to be at 2962 as determined by comparison to the potato anionic peroxidase cDNA. The polyadenylation signal AATATT is located 16 bp upstream from the site of poly[A] addition. A polyadenylation signal is located at 2985 in TAP 2.

The two tandemly oriented peroxidase genes most probably evolved from one ancestral gene by a duplication event. The exons are well conserved between the two genes, having 90% identity. The 5′-untranslated regions of the genes are generally divergent, with the exception of sequences at the transcription initiation site, at and preceding the TATA box, and at a segment 70 bp upstream from TATA as noted in Fig. 2. The introns have diverged, although their positions within the coding sequences are the same in both genes. A highly homologous segment of 50 bp is found in intron 1 of both genes (Fig. 2), although this segment occurs at different locations in the intron. Also, sequences immediately flanking the 5′ and 3′ ends of the third exon are conserved between the two genes as shown in Fig. 2. Recently, the genomic sequences of two peroxidase isozymes from horseradish have been reported (Fujiyama et al. 1988). Interestingly, these genes were also tandemly oriented. The intron/exon structures of the genes differed from the tomato genes only in that a third intron, at a location corresponding to a point within the third exon of the tomato peroxidase genes, was present. The first two introns of the peroxidase genes are in corresponding positions with respect to the peptide sequences. The coding sequences of the horseradish peroxidase genes indicate that a C-terminal peptide, 15 residues long, is removed during processing of the horseradish enzyme. The C-terminus of mature potato anionic peroxidase has not been determined, and therefore it is not known whether C-terminus processing occurs during its

maturation. It should be noted that tomato polygalacturonase is also processed at the C-terminus (Sheehy et al. 1987).

The coding sequences of the two tomato anionic peroxidase genes predict that unusual leader peptides precede the mature proteins. The first 25 amino acids of each of the leader peptides resemble a signal peptide typical of most extracellular proteins, where 18–20 relatively hydrophobic amino acids are flanked by charged residues. A second segment of 50 amino acids is located between the putative N-terminus and the typical signal peptide of each of the anionic peroxidase leader peptides. Two potential glycosylation sites are located within the second region of the putative leader peptide of each of the three anionic peroxidases. Other immature cell wall proteins whose primary structures are known (Chen and Varner 1985; Tucker et al. 1987) including another anionic peroxidase from tobacco (Lagrimini et al. 1987) have typical signal peptides at their N-termini rather than the unusual structure found in the present anionic peroxidase. At least one other cell wall protein, tomato polygalacturonase, has an unusually long leader peptide of length comparable to those of the anionic peroxidases (Sheeny et al. 1987). Comparisons of the hydropathy plots of the predicted leader peptide of tomato polygalacturonase to those of the products of the tomato peroxidase genes (Fig. 4) show some similarities. All three have a hydrophobic core at the N-terminus and a second hydrophobic region followed by a hydrophilic region near the C-terminus. Comparisons of the amino acid sequences of the three leader peptides show that there is no strong homology between the polygalacturonase leader and peroxidase leaders but they have a few residues in common which may be of importance. The significance of the existence of two classes of leader peptides for cell wall proteins is not obvious. The two classes of leaders may reflect different ultimate sublocations within the cell wall.

TAP 1 is most likely to be functional in wound-inducible suberization. This conclusion is based on the evidence that an oligonucleotide primer specific for TAP 1 was extended by reverse transcriptase after hybridization to RNA prepared from wounded tomato fruit tissue aged for 3 days. Therefore an mRNA species representing TAP 1 is actively transcribed in suberizing tomato fruit. It is not known if TAP 2 is expressed in suberizing tomato fruit, as the primer extension yielded no detectable elongation product. In TAP 2 there are no premature termination codons, and putative promoters such as TATA box and polyadenylation signal are present; the consensus bases GT and AG are at the 5′ and 3′ ends, respectively, of both introns. Thus there are no obvious alterations that would render this gene inactive or a pseudogene. The anionic nature of the putative product of TAP 2 and its high homology to the suberization-associated potato anionic peroxidase suggest that this gene may have a similar role. Thus TAP 2 may function in suberin deposition that is induced by stimuli other than wounding or ABA. For example, inoculation of tomato petioles with *V. albo-atrum* induced production of transcripts that cross hybridized with potato anionic peroxidase cDNA and this induction occurred 1 day earlier in the resistant tomato line when compared to a near isogenic susceptible line (P.E. Kolattukudy, R. Mohan, J. Robb, unpublished). It is possible that the pathogen induces expression of a different copy of the gene than that induced by wounding. Such possibilities have not been explored.

Several observations indicate that TAP 1 may have a role in suberization more analogous to that of the potato anionic peroxidase gene as opposed to TAP 2. TAP 1 is more highly homologous to potato anionic peroxidase cDNA than is TAP 2. Additionally, the 3′-untranslated sequences of transcripts are more conserved between TAP 1 and potato anionic peroxidase than between TAP 2 and potato anionic peroxidase. Sequences present in the 3′-untranslated regions of wound-induced transcripts have been proposed to have some significance (Sanchez-Serrano et al. 1986; Thornburg et al. 1987). Also, codon preferences are more highly conserved between TAP 1 and potato anionic peroxidase than between TAP 2 and potato peroxidase.

ABA induction of the highly anionic peroxidase in potato callus tissue correlated with suberization induction by this hormone (Cottle and Kolattukudy 1982; Espelie and Kolattukudy 1985; Roberts et al. 1988). The western blots presented here show that a highly anionic protein is induced by ABA treatment of potato callus. This protein has similar mobility to that of purified anionic peroxidase and therefore we suggest that this ABA-induced protein is the highly anionic peroxidase. The antiserum used to probe the western blots is apparently not specific for the most anionic isoperoxidase. Non-specificity of peroxidase polyclonal antiserum has been observed before (van Huystee 1987). However, it is clear that the most highly anionic peroxidase was induced by ABA treatment. The results presented here show that ABA induction of peroxidase is not limited to potato tuber but occurs also in aerial parts of plants such as suberizing tomato fruits. Northern blots presented here show that in both potato and tomato callus ABA induced the accumulation of transcripts that hybridized with potato anionic peroxidase cDNA. These transcripts were identical in size to the wound-induced anionic peroxidase mRNAs in potato tuber and tomato fruit. These observations suggest that wounding and ABA treatment induce the same anionic peroxidase as is involved in suberization. The maximum level of anionic peroxidase mRNA accumulation in ABA-treated callus was much lower than that found in periderm from potato slices aged for 3 days. One possible explanation for this observation is that the callus grown on medium with ABA was not uniformly suberized. The callus culture grown on ABA-containing agar included a suberized brownish outer layer of tissue and an inner white core of tissue. The entire tissue mass was used to prepare RNA. On the other hand, when RNA was prepared from aged tuber slices, only the developing periderm was used so as to enrich for suberized tissue.

The evidence presented here suggests that induction of anionic peroxidase by ABA involves transcriptional activation of the anionic peroxidase gene or an increase in stability of the transcript rather than a post-translational activation of a pre-existing protein. ABA induction of suberization in wound-healing tuber slices is probably mediated not directly but through intermediate factors (Soliday et al. 1978). The results presented in this report are consistent with this conclusion, as 2 days of growth on medium with ABA were required for a detectable level of the anionic peroxidase transcript in potato callus. The concentration of ABA required to induce suberization and the anionic peroxidase is most likely very much lower than the 10^{-4} M originally added in the agar medium on which the tissue mass is resting. ABA level in the tissue was not determined.

In addition to the outer layer of underground organs and wound periderm, suberin is found in other barrier

layers generated during development such as the Casparian band of endodermis (Bonnert 1968; Espelie and Kolattukudy 1979) and in the chalazal region of seed coats (Espelie et al. 1980). Clearly, suberization must be regulated by developmental triggers as well as by wound-induced triggers. ABA may be important in developmental regulation of suberization. For example, ABA is known to prevent precocious germination of immature embryos (Norstog 1979) perhaps through resisting water uptake prior to the desiccation phase of maturation (Finkelstein et al. 1987). The suberized regions of the inner seed coat are thought to be the major moisture diffusion barriers influencing inhibition and germination of the seed (Espelie et al. 1980). Possibly, ABA prevents precocious germination in part through the induction of an anionic peroxidase involved in suberization of the chalazal region of the seed coat. In potato tubers, water stress of the vines before harvest is thought to be beneficial in preventing crop loss during transportation and storage due to decay (Braue et al. 1984). The elevated ABA levels that result from the water stress might help to stimulate suberization that provides the beneficial effects. Tubers stored for more than 6–8 months deteriorate if transported and stored elsewhere. We have observed that upon such long storage the tubers loose their ability to suberize and thus cannot repair injury caused by handling (Braue et al. 1983). This loss in wound-healing capability correlates with the observation that ABA content of tubers decreases upon such long term storage.

Developmental factors, stress factors including mineral stress, wounding and infection by pathogens induce suberization that requires expression of the gene for the highly anionic peroxidase. The availability of tomato anionic peroxidase clones of known sequence should help in the elucidation of the mechanisms of regulation of expression of this gene by such factors.

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