Plant Physiol. (1993) 101: 201–208

Abolition of an Inducible Highly Anionic Peroxidase Activity in Transgenic Tomato[1]

Bruce A. Sherf[2], Aslam M. Bajar, and Pappachan E. Kolattukudy*

The Ohio State Biotechnology Center, 1060 Carmack Road, Columbus, Ohio 43210

Locally induced expression of a highly anionic peroxidase has previously been correlated temporally and spatially with suberization of tissues responding to pathogen assault, wounding, or exogenously applied abscisic acid or fungal elicitors. DNA sequences corresponding to the 5′ regions of two tomato (*Lycopersicon esculentum*) genes encoding homologous anionic peroxidases were fused, inserted into a pTi-based plasmid designed to express a composite antisense transcript, and introduced into tomato via *Agrobacterium*-mediated transformation. RNA gel-blot analyses showed high expression of the antisense transcript in most transgenic plants and no detectable induction of native anionic peroxidase transcripts in wounded or abscisic acid- or pathogen-treated tissues. Plants and fruits expressing the antisense transcript appeared normal in all respects. Electrophoretic analysis of anionic proteins from selected transgenic plants showed no detectable anionic peroxidase protein or activity. Depolymerization of polymeric material from the wound periderm of transgenic tomato fruits and analysis of the aliphatic products by gas-liquid chromatography/mass spectrometry showed that the content and composition of C_{16}/C_{18} ω-hydroxy and dicarboxylic acids, characteristic of suberin, were not affected by the absence of the anionic peroxidase. Autofluorescence generated from cell wall phenolics at the wound lesion was also not affected by the absence of the highly anionic peroxidase.

Peroxidases constitute a diverse family of heme-containing enzymes believed to function in a variety of normal and stress-related physiological processes of plants. Peroxidases have been implicated in numerous biochemical processes such as lignification (Lagrimini et al., 1987), suberization (Espelie and Kolattukudy, 1985; Espelie et al., 1986), cross-linking of hydroxyproline-rich wall proteins and feruloylated polysaccharides (Smith and O'Brian, 1979; Fry, 1986), both oxidation and polymerization of soluble phenolics (Strivastava and van Huystee, 1977), the formation of hydrogen peroxide (Mader et al., 1980), Chl degradation and senescence (Yamauchi and Watada, 1991), and auxin degradation (Jinnman and Lang, 1965).

The highly anionic peroxidases of tomato (*Lycopersicon esculentum*) appear to be related to pathogenesis. Although the basal levels of transcripts of these genes are very low in the root, stem, leaf, and red fruit tissues of healthy tomato plants, expression of these genes is induced to high levels in tissues responding to challenge by fungal pathogens, wounding, or exposure to either ABA or fungal elicitor preparations (Roberts and Kolattukudy, 1989; Mohan and Kolattukudy, 1990; Robb et al., 1991).

We have previously described the cloning and characterization of a cDNA encoding a highly anionic (isoelectric point = 3.15) peroxidase from potato (Roberts et al., 1988). An 800-bp segment of this cDNA was later used to probe a tomato genomic DNA library from which two genes encoding homologous isozymes of TAP were isolated. These genes, designated *tap1* and *tap2*, display a high degree of sequence identity with each other as well as with potato anionic peroxidase (Roberts and Kolattukudy, 1989). A correlation has previously been established between the temporal and spatial expression of wound-, fungal elicitor-, and ABA-inducible anionic peroxidase activities with the onset of suberization in potato and tomato tissues (Espelie and Kolattukudy, 1985; Espelie et al., 1986; Roberts et al., 1988; Robb et al., 1991). Deposition of suberin occurs on the cell wall of tissues responding to pathogen ingress or wound trauma (Espelie et al., 1986; Robb et al., 1991), thus fortifying the wall structure of affected cells. Suberin, like lignin, comprises a polyphenolic matrix composed primarily of hydroxy and methoxy derivatives of cinnamic acid monomers. Suberin is distinguished from lignin, however, in that it also contains covalently attached aliphatics, most often consisting of esterified ω-hydroxy C_{16} saturated and C_{18} monounsaturated fatty acids (Kolattukudy, 1984). The aromatic and aliphatic domains of the suberin polymer are interspersed with waxes that provide a barrier against moisture diffusion and desiccation. The timely induction of suberin biosynthesis is therefore important once the plant's primary defense barrier, the cuticle, has been breached. The observed correlation between the wound- and pathogen-induced expression of TAP and the deposition of suberin have led us to propose that the activity of this particular peroxidase is involved in catalyzing the cross-linking of suberin monomers.

The in vivo expression of antisense RNA in tomato has recently been used to study several developmentally expressed genes (ethylene-forming enzyme, Hamilton et al., 1990; polygalacturonase, Smith et al., 1990; ACC synthase, Oeller et al., 1991; pTOM5, Bird et al., 1991; ACC oxidase, Peñarrubia et al., 1992; pectin methylesterase, Tieman et al., 1992), providing important information about the physiolog-

[1] This work was supported by grants from The National Science Foundation (DCB8819008) and The Midwest Biotechnology Consortium (MPBC#593–0009–13).

[2] Present address: Promega Corp., 2800 Woods Hollow Road, Madison, WI 53711.

* Corresponding author; fax 1–614–292–5379.

Abbreviations: 35S CaMV, 35S enhancer region of the cauliflower mosaic virus; TAP, tomato anionic peroxidase.

ical function(s) of the proteins they encode. To test whether an antisense approach can selectively abolish the expression of the highly anionic peroxidase and to determine the biological consequences of the absence of this gene expression, we generated transgenic tomato plants producing a chimeric antisense transcript corresponding to the 5′ region of both *tap*1 and *tap*2. In this paper, we report that the constitutive, high-level expression of this antisense-(*tap*1/*tap*2) transcript in transgenic tomato plants prevented the wound-, ABA-, and pathogen-induced accumulation of TAP transcripts and, hence, synthesis of the highly anionic peroxidase protein. Abolishing this inducible TAP activity does not appear to significantly inhibit suberization of the wound periderm of the tomato fruit.

MATERIALS AND METHODS

Plasmid Construction

All restriction endonuclease, T4 DNA polymerase fill-in, and ligase reactions were performed using the buffer conditions recommended by their respective manufacturers.

The antisense-(*tap*1/*tap*2) construct used throughout this work, designated pBIN:α(T1/T2), is shown schematically in Figure 1. All intermediary plasmid constructions were made in pBluescript II KS$^+$ (Stratagene, La Jolla, CA) and propagated in *Escherichia coli*. The antisense plasmid was engineered by first excising the extreme 5′ end of *tap*1 from pTAP3.0 (Roberts and Kolattukudy, 1989) as a 1.1-kb *Sph*I (filled-in)/*Cla*I DNA fragment and cloning it into the *Eco*RV/*Cla*I sites of pBluescript. This plasmid was subsequently linearized with *Cla*I, blunted by fill-in with T4 DNA polymerase, and ligated with a 0.32-kb DNA fragment generated by *Dra*I digestion of pTAP3.7 (Roberts and Kolattukudy, 1989). The resulting plasmid contained a portion of the 5′ ends of both *tap*2 and *tap*1 fused in a tail-to-head fashion, the DNA sequences of which correspond to a portion of both untranslated leader and coding regions from both genes. This plasmid was linearized by digestion with *Eco*RI (filled-in)/*Pst*I and was ligated with a 0.60-kb DNA generated by *Xba*I (filled-in)/*Pst*I digestion of pCa2 (Kay et al., 1987). The resulting construct contained the fused *tap*1/*tap*2 5′ regions downstream from, and in an antisense orientation to, twin 35S CaMV enhancer regions. A 0.43-kb DNA containing the nopaline synthase (*nos*) transcription terminator was cloned into vector *Hin*cII and *Kpn*I sites immediately 3′ to the antisense-(*tap*1/*tap*2) DNA sequence. The complete [2 × 35S CaMV + anti-(*tap*1/*tap*2) + *nos*] construct was excised from pBluescript via *Sac*I/*Kpn*I digestion and cloned into the complementary sites of the disarmed pTi-based vector pBIN19 (Bevan, 1984). The resulting plasmid, designated pBin:α(T1/T2), was introduced into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) and transformants were selected on YEB medium containing 50 μg/mL of kanamycin.

Plant Materials and Transformation

Tomato (*Lycopersicon esculentum* L. cv Better Boy) was used as the transformation recipient in this work. Conditions for *Agrobacterium*-mediated transformation of cotyledon explants and the media formulations used to propagate putative transformed calli, shoots, and whole plants were as previously described by Fillatti et al. (1987). Individual plants from S_1 progeny of primary transformants were identified as being homozygous by scoring for 100% seed germination and viability on MSSV medium containing Murashige and Skoog salts, 3% sucrose Nitsch vitamins, and 0.8% Bactoagar (pH 6.0) (Fillatti et al., 1987) and kanamycin at 50 mg/L. Tomato plants were grown to maturity in Terra Lite Metro Mix 360 and maintained in an environmental chamber at 23 ± 2°C under cool white fluorescent lighting with a 16 h/d photoperiod.

Pathogen Cultures and Preparation

A tomato isolate of *Verticillium albo-atrum*, the causative agent of vascular wilt, was cultured at 22°C on Bacto potato dextrose agar medium in 100-mm Petri dishes for a minimum of 3 weeks prior to use. Conidia were harvested for the purpose of challenging tomato petioles by adding 10 to 15 mL of sterile distilled water to each of three plates and scrubbing the mycelium with a gloved finger. Conidia suspensions were diluted with sterile distilled water to give a final concentration of 10^7 conidia/mL.

Treatment of Plant Materials

Cut tomato petioles and fruits were treated to induce expression of the plants' native anionic peroxidase. Petioles ranging in size from 8 to 12 cm were excised from 4- to 6-week-old plants, and the cut ends were placed in vials containing either a suspension of *V. albo-atrum* conidia (10^7/mL), ABA (1 μM), or water alone. All petioles were exposed for 1 h to a stream of warm air to enhance transpiration and, hence, to promote vascular uptake of the spores or ABA solution. Petioles challenged with the conidia suspensions were subsequently rinsed and transferred to vials containing water alone, whereas petiole sets treated with ABA or water

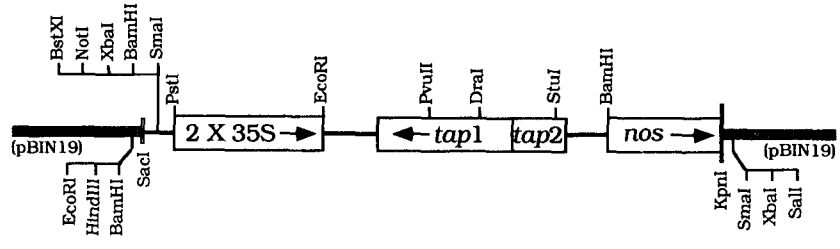

Figure 1. pBIN:α(T1/T2). DNA sequences corresponding to the mRNA leader and part of the coding regions of *tap*1 and *tap*2 were fused and cloned in an antisense orientation downstream from tandem 35S CaMV promoter sequences (2 × 35S). The anti-(*tap*1/*tap*2) transcript is terminated by a bacterial nopaline synthase (*nos*) termination sequence. The disarmed *Agrobacterium* vector pBin19 was used to mobilize the antisense construct into the genome of tomato.

alone remained in their respective solutions for the duration of the assay. All petioles were placed in a high-humidity chamber and incubated at room temperature (22–25°C) under incidental fluorescent lighting for an additional 72-h period. The bottom 3 mm of each petiole was removed and discarded, and 6 cm of the remaining petiole was harvested and processed for the extraction of either total RNA or protein.

Fruits from nontransformed and anti-(*tap1*/*tap2*)-expressing plants were examined for the induced synthesis of both TAP and suberin in response to mechanical wounding. Uniform wounding of large green fruits was accomplished by slicing away the cuticle and underlying periderm tissue to a depth of approximately 1 mm, encompassing a surface area of several cm^2 (Dean and Kolattukudy, 1976). Wounded fruits of both nontransformed and transgenic plants were allowed to heal on the vine for a period of 4 d prior to harvesting of tissue for RNA and protein extractions, or for 7 d prior to excising and processing of wound-periderms for suberin analysis.

Nucleic Acid Extraction and Gel-Blot Analyses

To isolate total DNAs, young tomato leaves (1.5 g) were frozen and ground to a fine powder with a mortar and pestle precooled in liquid nitrogen. The powder was homogenized in 6 parts (w/v) DNA extraction buffer (50 mM Tris, pH 8.0, 12% sucrose, 70 mM each EDTA and EGTA, 50 mM 2-mercaptoethanol, 35 μg/mL RNase A) for 30 s with a motorized homogenizer (Brinkmann Instruments, Westbury, NY). The mixture was extracted once with both phenol:chloroform (50:50, v/v) and chloroform, and DNA was isolated from CsCl density gradients using established procedures (Sambrook et al., 1989). Total DNAs (8 μg) from nontransformed and transgenic plants were digested with various restriction endonucleases using conditions recommended by the manufacturers. Restriction enzyme-digested DNAs were resolved on 1.2% agarose gels and subsequently transferred to nylon membranes via alkaline capillary blotting (Sambrook et al., 1989). Gel-blot analyses of nucleic acids were performed using a ^{32}P-labeled 490-bp DNA corresponding to exon-1 of *tap1*, which was derived from *DraI*/*NsiI* digestion of plasmid pTAP4.5 (Roberts and Kolattukudy, 1989). DNA-DNA hybridizations were carried out in formamide buffer (50% formamide, 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 0.1 mg/mL of sheared salmon sperm DNA) at 42°C for 16 h. Membranes were subsequently washed in 2× SSC/0.1% SDS for 30 min at 25°C and for 10 min each at 50 and 55°C prior to autoradiography.

Total RNA was isolated from finely ground fresh tissues (200–400 mg) of tomato root, stem, leaf, and green and red fruits by LiCl precipitation from cleared homogenates, as previously described (Sambrook et al., 1989). RNA samples were pretreated with ethidium bromide, resolved in 1.5% agarose gels containing 1.9% formaldehyde, then transferred to a nylon membrane via capillary blotting with 10× SSC. RNA gel blots were probed using the same hybridization conditions and ^{32}P-labeled 490-bp *tap*-specific DNA as described above for DNA-DNA hybridizations.

Protein Extraction and Acid-Gel Analysis

Proteins were extracted by grinding plant tissues to a fine powder in a small mortar and pestle precooled in liquid nitrogen. A portion of the powder (typically 200–400 mg) was transferred to a precooled 1.5-mL microcentrifuge tube and weighed, and an equal (w/v) portion of 2× protein extraction buffer (25 mM KH_2PO_4, pH 6.0, 200 mM sucrose, 2.5 mM $CaCl_2$, 0.25 mM leupeptin) was added. Each sample was thoroughly homogenized using a motorized microcentrifuge pestle (Kontes Corp., Vineland, NJ). Homogenates were clarified twice by spinning them in a microcentrifuge for 10 min at 14,000 rpm at 4°C. Protein concentrations of clarified extracts were determined using a Bradford dye reagent kit (Bio-Rad).

Electrophoretic resolution of highly anionic plant proteins was accomplished using a vertical acid-PAGE set-up consisting of a 6% acrylamide/290 mM acetate (pH 4.3) separating gel and 175 mM β-alanine (pH 4.6) running buffer, as described by Borchert (1978). Typically, 20 μg of total protein was loaded per lane of a miniature (7 cm) vertical slab gel unit and gels were run in a 4°C room at 100 V for 6 h. Peroxidase-positive bands were visualized by first soaking the gel in phosphate-buffered saline (30 mM KH_2PO_4, pH 6.0, 150 mM NaCl) containing 0.06% (w/v) 4-chloro-1-naphthol and 0.18% H_2O_2. The peroxidase staining reaction was terminated, typically after 2 to 3 min, by exchanging buffers with protein-fixative solution (40% methanol and 7% acetic acid).

Suberin Analysis

Several green fruits from both nontransformed and anti-(*tap1*/*tap2*)-expressing plants were wounded by removing regions of the fruit's exocarp (Dean and Kolattukudy, 1976), then allowing them to heal on the vine for a period of 7 d. The resulting wound-periderms were excised and carefully cleared of residual underlying pericarp by scraping with a razor blade. The total surface area of the periderm materials was determined for both experimental samples. Wound-periderms were lyophilized for 48 h and finely powdered in a Wig-L-Bug amalgamator (Crescent Dental Manufacturing Co., Chicago, IL) for 2 min. The reductive depolymerization with $LiAlD_4$, extraction of aliphatic products, TLC purification, chemical modification, and identification and quantification of the suberin aliphatics by capillary GLC/MS analyses were repeated twice for each sample and have been described in detail elsewhere (Kolattukudy and Agrawal, 1974; Robb et al., 1991). The silylated diols were analyzed by capillary GLC/MS on a 15-m SPB-5 (Supelco) column in a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 59888A mass spectrometer equipped with a Chemstation computer data system. Mass spectra were recorded at 70 eV.

Autofluorescence Analysis of Cell Wall-Associated Polyphenolics

Wound-healing periderms of immature green fruits were excised, fixed overnight in 100 mM sodium phosphate buffer (pH 7.4) containing 4% freshly depolymerized *para*-formal-

dehyde, washed three times for 15 min in phosphate buffer alone, placed in phosphate buffer containing 500 mM sucrose for 30 min, then transferred to phosphate buffer containing 1 M sucrose for a period of 24 h. The periderm samples were embedded in O.C.T. compound (Miles, Inc., Elkhart, IN) at −20°C and cut into 40-μm sections using a cryostat microtome (Reichert Histostat, Cambridge Instruments, Buffalo, NY). Samples were UV irradiated (330–380 nm filter) and autofluorescence of cell wall-associated polyphenolics was observed at 400× magnification using a Nikon photomicroscope.

RESULTS

We have previously demonstrated that the genome of *L. esculentum* cv Castlemart contains two tandem genes encoding highly anionic peroxidases that share approximately 95% sequence identity (Roberts and Kolattukudy, 1989). Therefore, plasmid pBin19:α(T1/T2) (Fig. 1) was engineered to produce high-level, constitutive expression of a composite antisense transcript complementary to the 5′ regions of both *tap*1 and *tap*2 mRNAs so that synthesis of peroxidases encoded by both genes can be suppressed. pBin19:α(T1/T2) was mobilized into the cells of cultured tomato cotyledons via *Agrobacterium*-mediated transformation. Twenty-three tomato plants were regenerated from individual calli cultured axenically on media containing kanamycin as a selective marker for positive transformation. Total RNAs isolated from young leaf tissues of regenerated plants were gel-blotted and analyzed using a 490-bp ^{32}P-labeled DNA probe corresponding to the extreme 5′ end of *tap*1. Nineteen of the 23 putative transgenic tomato plants revealed varying levels of expression of the desired 1.4-kb anti-(*tap*1/*tap*2) transcript (results not shown).

Several tomato plants displaying high expression of the introduced anti-(*tap*1/*tap*2) gene were further examined to determine their ability to block synthesis of both the native TAP transcript and protein under inducing conditions that, in control plants, result in exceedingly high expression of the *tap* gene products. Tomato petioles were excised from both nontransformed and selected transgenic plants and treated for induction of endogenous TAP by placing the cut end of each petiole in a vial containing either a suspension of conidia from *V. albo-atrum*, ABA, or water alone. After 72 h of incubation in a humid environment, total RNA was isolated from each petiole and gel-blot analysis (Fig. 2) demonstrated that most of the primary transformants that expressed the anti-(*tap*1/*tap*2) transcript failed to accumulate the anionic peroxidase transcript. Despite the high-level of expression of the anti-(*tap*1/*tap*2) transcript in transformant 1 (Fig. 2), the induced expression of *tap* transcript is seemingly unaffected, suggesting that this may be a chimeric plant.

Based on the high expression of the antisense transcript and its ability to block efficiently the induced transcription of tomato's endogenous *tap* gene(s), one transgenic plant, designated BB:α(T1/T2)-3, was selected for subsequent analyses. Heritable transmission of the anti-(*tap*1/*tap*2) gene was confirmed by nucleic acid analyses (Fig. 3) of an S_1-generation plant selected for its phenotype of 100% germination and seedling viability on Murashige and Skoog agar containing kanamycin. Total DNAs extracted from young leaf tissue of control and BB:α(T1/T2)-3 plants were digested with restriction endonucleases selected to cut outside of the targeted hybridization region for both the introduced anti-(*tap*1/*tap*2) and endogenous *tap* genes. Hybridization analysis of DNA restriction enzyme digested separately with *Ban*I and *Sau*96I revealed that the genome of BB:α(T1/T2)-3 contains two unlinked copies of the introduced [2 × 35S + anti(*tap*1/*tap*2) + *nos*] construct (Fig. 3A). It is interesting that Southern blot analysis suggested that *L. esculentum* cv Better Boy contains only one genomic copy of *tap*, unlike the genome of cv Castlemart from which two genes, *tap*1 and *tap*2, were previously cloned and characterized (Roberts and Kolattukudy, 1989). Northern-blot analysis of total RNA extracted from the root, stem, leaf, and green and red tomato fruits confirmed that the anti-(*tap*1/*tap*2) transcript is constitutively expressed, albeit at varying levels, in all tissues of BB:α(T1/T2)-3 (Fig. 3B). *tap* mRNA was not detected in any tissues of this transgenic plant, including the exocarps of green fruit, which is the only tissue where constitutive expression of *tap* is found. Abrogating expression of the highly anionic peroxidase seemingly does not affect the normal growth or development of transgenic plants or fruits, which display phenotypes that are indistinguishable from their nontransformed counterparts.

ABA- and wound-induced synthesis of the native TAP protein was investigated by treating young petioles harvested from BB:α(T1/T2)-3 and nontransformed plants with ABA and by wounding large green fruits of each plant. Proteins from treated petioles and wounded fruit sections were resolved electrophoretically in nondenaturing 6% polyacrylamide gels under acidic (pH 4.3) conditions, thereby excluding all but very highly anionic proteins. Proteins with peroxidase activity were visualized as intense purple bands against a clear background when gels were soaked briefly in aqueous 4-chloro-1-naphthol and H_2O_2. Acid gel analysis

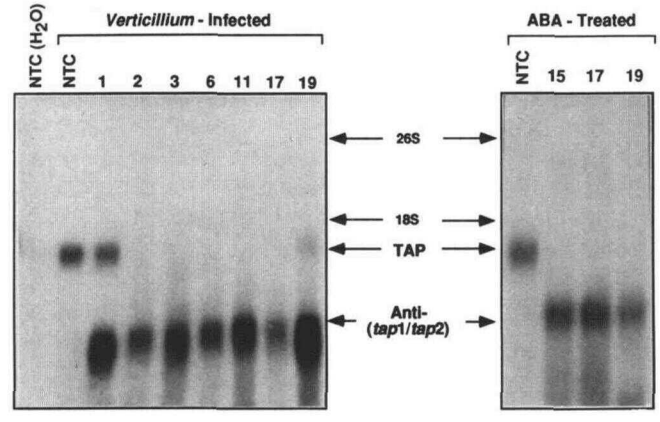

Figure 2. RNA gel-blot analysis of induced TAP transcripts in petioles of nontransformed control (NTC) and several transgenic tomato plants (designated 1, 2, 3, 6, 11, 15, 17, and 19) that constitutively express the anti-(*tap*1/*tap*2) transcript. Petioles were treated with either a suspension of *V. albo-atrum* conidia (10^7/mL), ABA (1 μM), or water. The 18S and 26S rRNAs are indicated as reference markers.

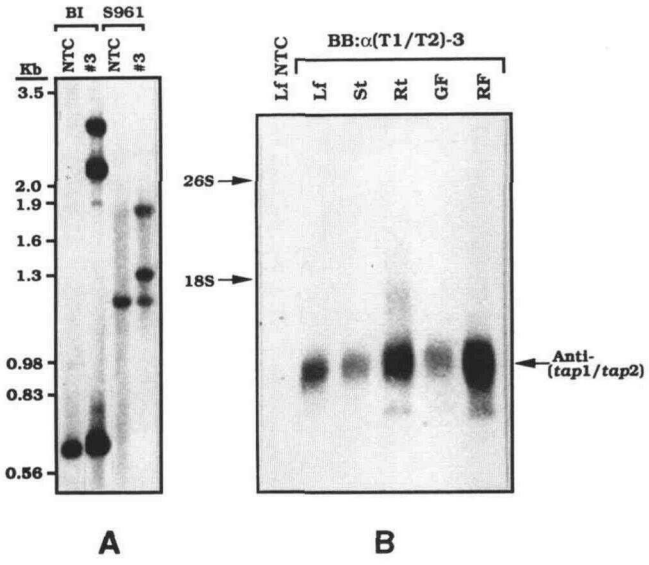

Figure 3. Nucleic acid analyses of the nontransformed parental tomato plant (cv Better Boy) and a selected S_1 transgenic plant, BB:α(T1/T2)-3. A, Southern blot of total DNAs harvested from nontransformed (NTC) and transformed (#3) plants. Eight micrograms of each DNA was digested with *Ban*I (BI) or *Sau*96I (S961). B, Northern blot of total RNAs isolated from uninduced BB:α(T1/T2)-3 leaf (Lf), stem (St), root (Rt), and green and red fruit (GF and RF) tissues, as well as from leaf tissue of uninduced Better Boy (Lf NTC). A ^{32}P-labeled 490-bp DNA corresponding to exon 1 of *tap*1 was used to probe both the blotted DNA and RNA preparations.

demonstrated the presence of high levels of a single highly anionic peroxidase in both the ABA- and wound-induced tissues of control tomato plants (cv Better Boy), whereas this peroxidase was not detectable in BB:α(T1/T2)-3 plants, which express the antisense transcript (Fig. 4).

Green tomato fruits of both control and BB:α(T1/T2)-3 plants were wounded and allowed to heal on the plant for a period of 7 d prior to excising the wound-periderm for analysis of suberin aliphatics. Depolymerized aliphatic monomers were isolated, silylated, and analyzed by capillary GLC/MS. Wound-healed periderms from nontransformed fruits expressing high levels of the anionic peroxidase and transgenic fruits devoid of the anionic peroxidase both yielded nearly equal amounts of C_{16} and C_{18} α,ω-diols (Fig. 5). Because both diols originate from the corresponding ω-hydroxy acid and dicarboxylic acid, we used the number of deuterium atoms incorporated during $LiAlD_4$ reduction to assess the content of the ω-hydroxy acids and dicarboxylic acids (Dean and Kolattukudy, 1976). The C_{18} diol produced an ion at m/e = 201 representing the doubly charged ion at (M-30)/2 generated from the tetradeuterated diol derived from octadec-9-ene-1,18-dioic acid. This ion had twice the intensity of the ion at m/e = 200 that was similarly generated from the diol derived from 18-hydroxy-octadec-9-enoic acid. Similarly, in the mass spectrum of C_{16} diol derived from 16-hydroxy-hexadecanoic acid and the corresponding dicarboxylic acid, the ratio of the intensity of the ion (m/e = 188) representing tetradeuterated diol derived from the dicarboxylic acid to that of the ion (m/e = 187) from dideuterated diol from the ω-hydroxy acid was near 2:1. These results show that the relative amounts of dicarboxylic acids and ω-hydroxy acids in the polymer was 2:1, a value characteristic of suberin (Dean and Kolattukudy, 1976). Thus, the level of suberin aliphatics deposited in the wound-healing periderm of the transgenic tomato fruits was unaffected by the absence of the inducible highly anionic peroxidase.

The deposition of phenolics in the wound lesions of both control and anti-(*tap*1/*tap*2)-expressing green fruits was assessed by observing autofluorescence (Smith and O'Brian, 1979). Numerous 40-μm cryostat sections of wound-healed periderms were evaluated microscopically under UV irradiation to determine the location and relative intensities of polyphenolic autofluorescence. The wound periderms of fruits from control and transgenic tomato plants were observed to display similar fluorescence patterns and intensities in the walls of cells at the wound surface, as depicted by the typical appearance shown in Figure 6. In all cases, this layer spanned no more than two cell layers and formed a boundary between an outer region of nonfluorescent broken cells and the cells of intact internal tissue displaying characteristic faint green and red fluorescence localized to the cell wall matrices and enclosed chloroplasts, respectively.

DISCUSSION

We have previously reported the cloning and sequencing of two linked genes, designated *tap*1 and *tap*2, encoding highly homologous anionic peroxidases from *L. esculentum* cv Castlemart (Roberts and Kolattukudy, 1989). Plasmid pBIN:α(T1/T2) was constructed for high-level expression of a transcript complementary to regions of exon 1 of both *tap*1

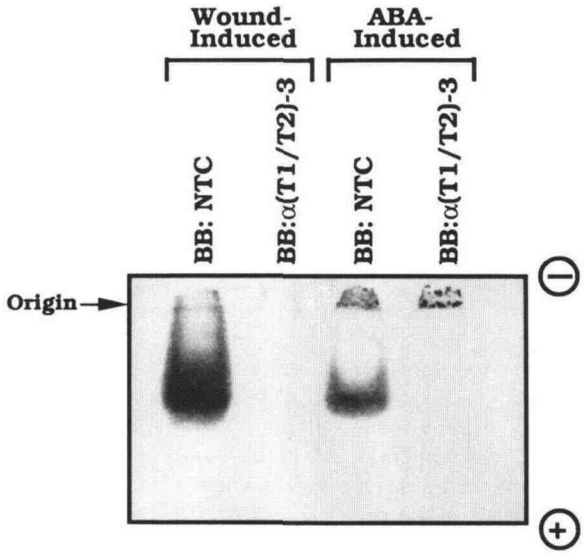

Figure 4. Peroxidase activity staining of highly anionic proteins resolved in nondenaturing acidic (pH 4.3) 6% polyacrylamide gels. Twenty micrograms of total proteins extracted from the wound-healing periderms of green fruits and ABA-treated petioles of nontransformed (BB:NTC) and transformed (BB:α(T1/T2)-3) plants were loaded per lane. Peroxidase-positive proteins were visualized via staining with 4-chloro-1-naphthol and H_2O_2.

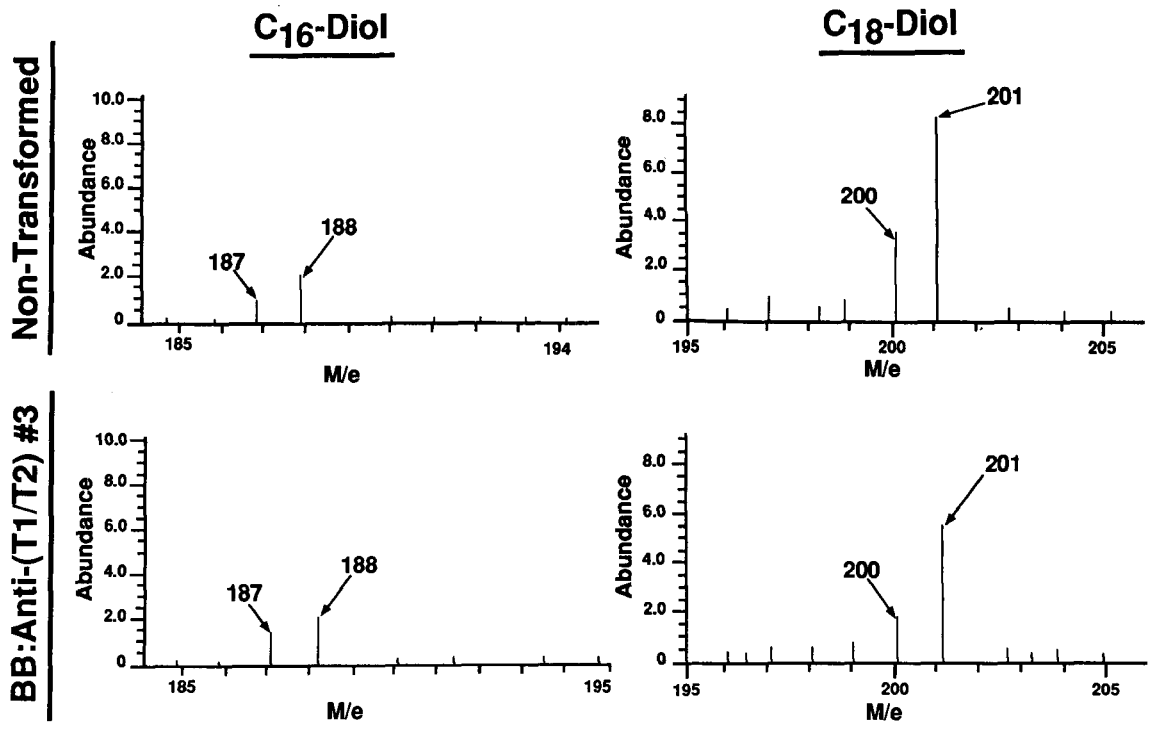

Figure 5. Mass spectral analysis of suberin aliphatics isolated from the wound periderms of transgenic and control tomato fruits. Wound periderms from 7-d wound-healed green fruits were processed and depolymerized with $LiAlD_4$, and the aliphatic monomers were subjected to capillary GLC/MS. The portion of the spectra showing the doubly charged ion resulting from the loss of a methyl group each from the trimethylsilyl groups of the two diols is shown. The ion at m/e = 188 is from tetradeuterated C_{16}-diol and the ion at m/e = 201 is from tetradeuterated monounsaturated C_{18}-diol.

and *tap*2. The constitutive, systemic expression of the anti-(*tap*1/*tap*2) transcript in tomato BB:α(T1/T2)-3 has been demonstrated to negate effectively the induced expression of the native *tap* gene products in wound- or pathogen-challenged and ABA-treated tissues of this transgenic plant. Thus, a means is now available to evaluate the specific effects of abrogating the inducible expression of this anionic peroxidase.

In addition to the wound- and pathogen-induced expression of *tap*, a pattern of developmental regulation has recently been observed for this gene in unripe tomato fruits (Sherf and Kolattukudy, 1993). Constitutive expression of *tap* mRNA occurs during the normal developmental progression from immature to mature green fruits. However, at the climacteric stage the expression of *tap* transcripts plummets to the same, nearly undetectable levels observed in the other tissues of healthy tomato plants. The otherwise constitutive expression of *tap* in maturing green fruits has been effectively negated in the transgenic fruits without any obvious changes in the development and timing of fruit and seed maturation, color progression, fruit softening, or overall seed viability. Indeed, the phenotype and developmental progression of transgenic plants experiencing constitutive expression of the antisense-(*tap*1/*tap*2) transcript are indistinguishable from those of nontransformed control plants. Thus, the constitutive expression of *tap* in maturing green fruits may not play a role in the normal development of fruits per se, but rather may reflect an evolutionary adaptation to ensure an elevated level of pest resistance in the reproductive tissues of plants. This protective strategy has also been proposed by Wingate and Ryan (1991) for the expression of proteinase inhibitor I in wild tomato (*L. peruvianum*). They report the expression of this gene to be both wound-induced in leaves and developmentally expressed in unripe fruits. It is also possible that whatever developmental role TAP plays in the fruits of nontransformed plants is compensated for by other peroxidases in the transgenic plants, which are no longer able to express this anionic peroxidase.

We performed repeated analyses of suberin aliphatics and the qualitative assessment of total phenolic polymers in wound-healed periderms of normal and TAP-deficient tomato fruits. The composition of the deuterium-labeled aliphatic components of suberin showed a 1:2 ratio of ω-hydroxy acids and dicarboxylic acids in both the C_{16} and C_{18} groups of suberin monomers. Such a ratio is what is expected from tomato periderm suberin, as previously described (Dean and Kolattukudy, 1976). Absence of the highly anionic peroxidase in wounded transgenic fruits did not affect the amount or composition of suberin aliphatics. This finding raises the possibility that another peroxidase might substitute for the abolished anionic peroxidase during the synthesis of suberin in the wounded tissues of transgenic plants. If so, deposition of suberin's phenolic matrix should also not be affected in the antisense plants. Rigorous quantitation of

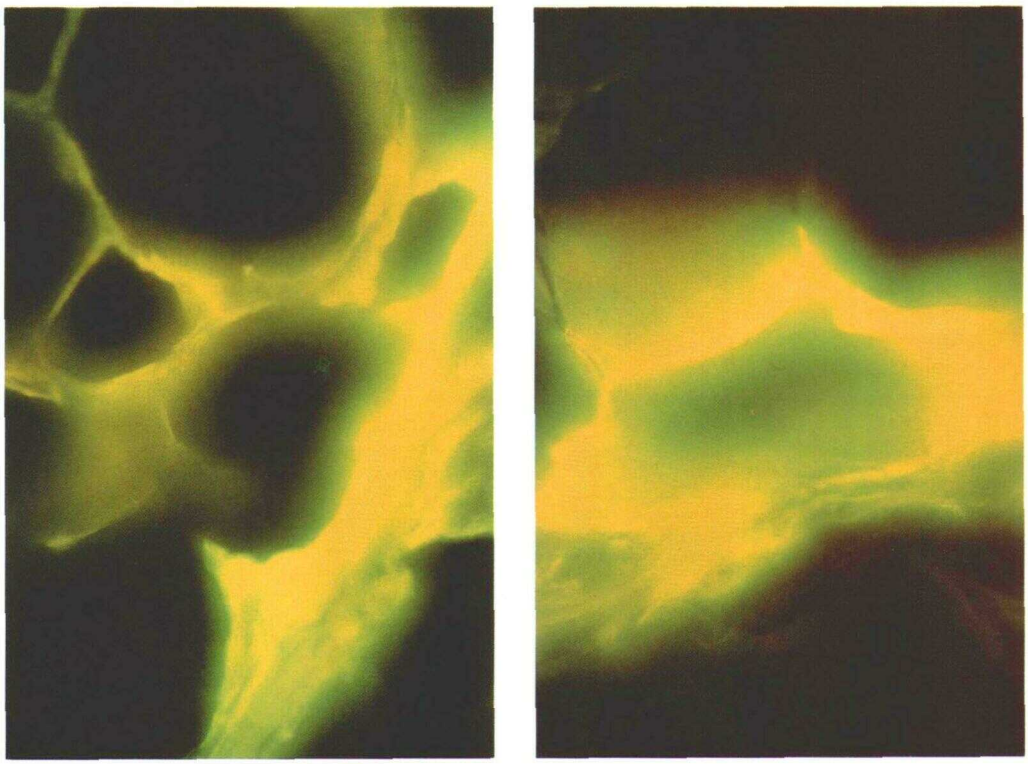

Figure 6. Autofluorescence of polyphenolic compounds in the cell walls of wound-healed periderm in green tomato fruits from control and BB:α(T1/T2)-3 plants. Tissue samples were cut into 40-μm sections and viewed (400× magnification) under UV (330–380 nm filter) irradiation.

wall-bound phenolics has not yet been performed; however, microscopic analyses showed autofluorescence originating from cell wall polyphenolics in the wound-healed periderm of transgenic green fruits that is equally intense as that from similarly treated tissues of nontransformed fruits. This result provides preliminary evidence that total phenolic deposition was not significantly affected by the loss of TAP activity in transgenic plants.

Because the homozygous plant used for the detailed analysis was phenotypically identical to the nontransformed plants, and because the peroxidase transcript was undetectable in this plant, the results obtained show the direct consequence of the absence of expression of this highly anionic peroxidase. Thus, the results suggest that the expression of this specific anionic peroxidase gene is not essential for suberization. On the other hand, many lines of indirect evidence strongly suggest that the highly anionic peroxidase may be involved in suberization. Time-course and location of anionic peroxidase appearance and suberization matched exactly when they were induced by wounding in potato tuber and tomato fruit, by ABA in potato and tomato tissue cultures, by pathogens in tomato petioles (Roberts and Kolattukudy, 1989; Robb et al., 1991), and by fungal elicitors in tomato cell-suspension cultures (Mohan and Kolattukudy, 1990). Additionally, enhanced suberization of corn roots by Mg^{2+} deficiency, inhibition of suberization by iron deficiency in bean roots and reversal of this inhibition by the addition of iron (Sijmons, 1985), and inhibition of suberization in potato tuber slices by 10% CO_2 in the air and suberization induced by the replacement of CO_2 by normal air (R. Mohan, P.E. Kolattukudy, R.A. Boyd, and G.G. Laties, unpublished observation) correlated with corresponding changes in the anionic peroxidase level or its transcript level. Despite such evidence suggesting that the anionic peroxidase is involved in the polymerization of suberin monomers, the possibility that all such results constitute coincidental correlation cannot be ruled out. However, in view of the multiplicity of peroxidases known to exist in plants, it seems possible that in the antisense transgenic plants another peroxidase has substituted for the abolished activity of the highly anionic peroxidase isozyme. Indeed, electrophoretic analysis demonstrates that at least one other nonhomologous peroxidase isozyme is induced to high levels in the wound-periderm of both nontransformed and anti-(*tap1*/*tap2*)-expressing tomato fruits (B.A. Sherf and P.E. Kolattukudy, unpublished observation). This peroxidase was detected by enzyme activity staining and did not cross-react with antibodies or DNA probes specific

for the highly anionic peroxidase. Thus, the nature of this peroxidase remains to be elucidated.

ACKNOWLEDGMENTS

We thank Dr. Joan McPherson, University of British Columbia, for her gift of plasmid pCa2 containing the tandem 35S CaMV enhancer cassette. We gratefully acknowledge Dr. Barry Stein, The Ohio State Biotechnology Center, for his expert assistance in processing wound-periderm sections for microscopic analysis of autofluorescence.

Received July 6, 1992; accepted September 8, 1992.

LITERATURE CITED

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acid Res **12:** 8711–8721

Bird CR, Ray JA, Fletcher JD, Boniwell JM, Bird AS, Teulieres C, Blain I, Bramley PM, Schuch W (1991) Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. Bio/Technology **9:** 635–639

Borchet R (1978) Simultaneous separation of acidic and basic isoperoxidases in wounded potato tissue by acrylamide gel electrophoresis. Plant Physiol **62:** 794–797

Dean BD, Kolattukudy PE (1976) Synthesis of suberin during wound-healing in jade leaves, tomato fruit and bean pods. Plant Physiol **58:** 411–416

Espelie KE, Franceschi VR, Kolattukudy PE (1986) Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. Plant Physiol **81:** 487–492

Espelie K, Kolattukudy PE (1985) Purification and characterization of an abscisic acid-inducible anionic peroxidase associated with suberization in potato (*Solanum tuberosum*). Arch Biochem Biophys **240:** 539–545

Fillatti JJ, Kiser J, Rose R, Comai L (1987) Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technology **5:** 726–730

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol **37:** 165–186

Hamilton AJ, Lycett GW, Grierson D (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature **346:** 284–287

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303:** 179–180

Jinnman RL, Lang J (1965) Peroxidase catalyzed oxidation of indole-3-acetic acid. Biochemistry **4:** 144–158

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **236:** 1299–1302

Kolattukudy PE (1984) Biochemistry and function of cutin and suberin. Can J Bot **62:** 2918–2933

Kolattukudy PE, Agrawal VP (1974) Structure and composition of aliphatic constituents of potato tuber skin (suberin). Lipids **9:** 682–691

Lagrimini LM, Burkhart W, Moyer M, Rothstein S (1987) Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. Proc Natl Acad Sci USA **84:** 7542–7546

Mader M, Ungemach J, Schloss P (1980) The role of peroxidase isozyme groups of *Nicotiana tabacum* in hydrogen peroxide formation. Planta **147:** 467–470

Mohan R, Kolattukudy PE (1990) Differential activation of expression of a suberization-associated anionic peroxidase gene in near-isogenic resistant and susceptible tomato lines by elicitors of *Verticillium albo-atrum*. Plant Physiol **92:** 276–280

Oeller PW, Min-Wong L, Taylor LP, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254:** 437–439

Peñarrubia L, Aguilar M, Margossian L, Fischer RL (1992) An antisense gene stimulates ethylene hormone production during tomato fruit ripening. Plant Cell **4:** 681–687

Robb J, Lee SW, Nazar RN, Mohan R, Kolattukudy PE (1991) Chemical characterization of stress-induced vascular coating. Plant Physiol **97:** 528–536

Roberts E, Kolattukudy PE (1989) Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberization-associated highly anionic peroxidase. Mol Gen Genet **217:** 223–232

Roberts E, Kutchan T, Kolattukudy PE (1988) Cloning and sequencing of a cDNA for a highly anionic peroxidase from potato and the induction of its mRNA in suberizing potato tubers and tomato fruits. Plant Mol Biol **11:** 15–26

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning, A Laboratory Manual, Vols 1–3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sherf B, Kolattukudy PE (1993) Developmentally regulated expression of the wound- and pathogen-responsive tomato anionic peroxidase in green fruits. Plant J (in press)

Sijmons PC, Kolattukudy PE, Bienfait HF (1985) Iron deficiency decreases suberization in bean roots through a decrease in suberin-specific peroxidase activity. Plant Physiol **78:** 115–120

Smith MM, O'Brian TP (1979) Distribution of autofluorescence and esterase and peroxidase activities in the epidermis of wheat roots. Aust J Plant Physiol **6:** 201–209

Smith CJS, Watson CF, Morris PC, Bird CR, Seymour GB, Gray JE, Arnold C, Tucker GA, Schuch W, Harding S, Grierson D (1990) Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Mol Biol **14:** 369–379

Strivastava OP, van Huystee RB (1977) An inter-relationship among peroxidase, IAA oxidase, and polyphenoloxidase from peanut cells. Can J Bot **55:** 2630–2635

Tieman DM, Harriman RW, Ramamohan G, Handa AK (1992) An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. Plant Cell **4:** 667–679

Wingate VPM, Ryan CA (1991) Uniquely regulated proteinase inhibitor I gene in a wild tomato species. Plant Physiol **97:** 496–501

Yamauchi N, Watada AE (1991) Regulated chlorophyll degradation in spinach leaves during storage. J Am Soc Hortic Sci **116:** 58–62