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Hydrogen Peroxide Is Required for Poly(phenolic) Domain Formation during Wound-Induced Suberization

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The requirement for hydrogen peroxide (H_2O_2) during suberization was demonstrated in wound-induced potato tubers by monitoring the extent of phenolic polymerization after the inhibition of H_2O_2 production using diphenyleneiodonium (DPI). In DPI-treated tissues the extent of phenolic polymerization in suberized tissues, measured using DFRC (Derivatization Followed by Reductive Cleavage) and thioglycolic acid analyses, was greatly reduced relative to untreated controls. Concomitantly, a large quantity of new soluble phenolics accumulated in the DPI-treated tissue some of which were not present in the controls. We suggest that the inhibition of H_2O_2 production prevented these phenolics from being oxidized by cell wall peroxidases. As a result, these phenolics were left unpolymerized and accumulated in the tissue. Several of the soluble phenolics were identified as hydroxycinnamic acid derivatives. From the data presented, it was concluded that H_2O_2 is required for the polymerization step in the formation of the poly(phenolic) domain of suberized potato tubers.

KEYWORDS: ***Solanum tuberosum*; Derivatization Followed by Reductive Cleavage (DFRC); diphenyleneiodonium; hydrogen peroxide; hydroxycinnamic acids; NADPH-dependent oxidase; poly(phenolic) domain; soluble phenolics; thioglycolic acid; wall-bound phenolics; wound-induced suberization**

INTRODUCTION

Over the course of evolution, plants have developed effective defense mechanisms to overcome environmental challenges, including stresses caused by injuries, pathogens, and a desiccating environment. Plant defenses tend to be site specific such that the affected cells or tissues are healed or isolated from healthy, unaffected ones. In the case of injury, this is usually accomplished by producing high quantities of toxic chemicals (e.g., H_2O_2, secondary metabolites) to kill pathogens, followed by the formation of a polymeric barrier next to the infected site (e.g., a suberized layer). For example, in potato tubers challenged with pathogens, large amounts of free radicals are produced (*1*) and rapid suberization is necessary to provide an effective resistance against dehydration and pathogen penetration (*2*). Apart from the direct toxic effect of H_2O_2 on pathogens, other roles for this reactive oxygen species (ROS) have also been proposed, including the oxidative cross-linking of cell wall components, (reviewed in *3*). This latter role is potentially important during the wound-healing process, since it would allow the formation of a physical barrier to prevent water loss and further pathogen penetration, (reviewed in *4*, *5*). Furthermore, in wound healing potato tubers, suberizing cells not only accumulate H_2O_2, but also actively produce it via the disproportionation of O_2^- (*1*, *6*). This production and accumulation of H_2O_2 is an indication of the potential involvement of H_2O_2 in suberization.

Suberization is the name given to the deposition of a specific cell wall modification in periderm, wound periderm, and endo- and exodermal cells, that is characterized by both a poly(phenolic) domain and wax-embedded poly(aliphatic) domain. The term *suberin*, however, is often associated with the aliphatic component of suberized tissues, while the poly(phenolic) domain has been labeled a lignin. And while there is an element of truth to this description, two recent major breakthroughs have reshaped our understanding of suberization: (i) direct evidence that the poly(phenolic) domain contains a significant amount of covalently cross-linked hydroxycinnamic acids (*7*, *8*), and (ii) that glycerol is a significant component of the poly(aliphatic) domain (*9*, *10*). Based on these seminal results, and many supporting papers, a new conceptual picture of suberized cells has emerged (*10*, *11*). In this model, the poly(phenolic) domain (herein referred to as the SPPD) contains a significant amount of nonlignin precursors (principally hydroxycinnamic acids and their derivatives) that are covalently linked to one another and embedded in the primary cell wall. Suberin, which is covalently linked to the SPPD at the cell wall surface, is depicted as a linear, 3-dimensional, glycerol-bridged polyester network.

The polymerization of phenolics into the SPPD of potato has been hypothesized to occur via a peroxidase/H_2O_2 mediated free radical coupling process (*5*) and a specific anionic peroxidase has been implicated (*12*, *13*). In order for this hypothesis to be correct, there has to be a supply of H_2O_2 at the site of polymerization. It should be noted, however, that H_2O_2 involvement in suberization has not yet been demonstrated and no H_2O_2 generating system has been linked to it. Furthermore, the

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molecular machinery responsible for H_2O_2 generation in situ remains controversial (*6*, *14*). Several enzymatic systems have been demonstrated to be able to generate H_2O_2, including cell wall peroxidases (*15*, *16*), polyamine oxidases (*17*), and plasma membrane NADPH-dependent oxidases (*4*, *18*−*21*). A tentative model for H_2O_2 production during SPPD formation was recently presented (*6*). In this model, H_2O_2 produced by a plasma membrane-bound NAD(P)H oxidase is used by a cell wall peroxidase to polymerize phenolics. In support of this model, we recently reported that the treatment of wound healing tissue with the flavoprotein inhibitor diphenyleneiodonium (DPI) inhibited the production of H_2O_2 (based on histochemical detection with tetramethylbenzidine) in a dose dependent manner, at low μM levels (*6*) and resulted in the accumulation of new soluble compounds. In the present study we extended these earlier observations by demonstrating the effect of DPI treatment directly on SPPD formation, and report the identity of several of the compounds that accumulate. We show for the first time that H_2O_2 is *required* for the polymerization of the SPPD of potato.

MATERIALS AND METHODS

Tissue Wounding, DPI Treatment, and Phenolic Extraction. Potato (*Solanum tuberosum* L. cv. Russet Burbank) tubers were cut (i.e wounded) transversely into approximately 1 cm slices, under sterile conditions as described earlier (*22*). Slices were either soaked in 25 μM DPI (Sigma) for 15 min (DPI-treated) or dH_2O (control) for the same time and incubated at 25 °C for up to 7 days as described earlier (*22*). The suberizing layers were collected as described earlier (*22*), immediately frozen in liquid nitrogen, ground to a fine powder using a pestle and a mortar, and stored at −20 °C until extracted. Soluble compounds were extracted overnight with 80% aqueous MeOH in a Soxhlet extractor. Extracts were concentrated to aqueous in vacuo at < 40 °C, adjusted to 3 mL g^{-1} dried tissue and analyzed by HPLC. The cell wall residue was re-extracted with acetone and air-dried prior to cell wall analysis (see below). Three independent time course studies were conducted, with triplicate samples taken at each time point within each study. For cell wall analysis, three sub-samples were taken from each triplicate after solvent extraction.

HPLC Apparatus and Measurements. Liquid chromatographic analyses were performed on a Beckman analytical HPLC consisting of a model 126 binary pump, a model 168 diode-array detector, and a model 508 autosampler (Beckman Instruments, Inc., Fullerton, CA). For separation of potato phenolics, a Nucleosil C-18 reversed phase analytical column (4.6 × 150 mm, 5 μm; Macherey-Nagel, Düren, Germany) was used. Solvent A: 1.5% (v/v) aqueous phosphoric acid; solvent B: MeOH:CH_3CN:H_2O (1:1:1). Gradient elution was performed as follows: 0−10 min, isocratic at 5% B in A; 10−35 min, 5 to100% B in A. The flow rate was 1 mL min^{-1} and the injection volume 10 μL. The eluent was monitored over the range of 200 to 350 nm, with 280 nm used for calibration and quantification.

Isolation of Phenolic Compounds. For compound isolation, a large-scale harvest of "suberized" layers from DPI-treated tissue, 4 days post wounding was used (approximately 500 g). The tissue was Soxhlet extracted overnight in 80% aqueous MeOH, working in small batches of 50 g each. Extracts were concentrated to aqueous in vacuo at < 40 °C and filtered through Whatman No.1 filter paper to remove debris. The filtered crude extracts (approximately 100 mL each) were partitioned (three times) with an equal volume of hexane. The aqueous phase was acidified to pH 2−3 using HCl and partitioned (three times) with an equal volume of ethyl acetate (EtoAc). Both organic and aqueous phases were concentrated in vacuo, reconstituted in dH_2O and loaded separately onto an LH-20 column (1.5 × 64 cm) preequilibrated with dH_2O. After an initial 60 min isocratic elution with dH_2O, an 80 min linear gradient to 100% MeOH followed by an isocratic elution for 60 min with MeOH, was used to elute compounds. The eluent was monitored at 280 nm and analytical HPLC used to guage fraction purity. Some fractions were reloaded onto LH-20, and eluted with longer linear gradients of MeOH in dH_2O for further purification.

Soluble Phenolics Identification. Phenolics isolated by LH-20 column chromatography were identified on the basis of their UV spectra (diode-array detection between 200 and 350 nm), co-chromatography with authentic standards, and their mass spectra (measured by GCMS). For GCMS, residues from LH-20 fractions were derivatized using bis-(trimethylsilyl)trifluoroacetamide in pyridine at room temperature for 40 min. The TMS derivatives were immediately analyzed by GCMS using a Varian CX3400 gas chromatograph (equipped with a model 8200 autosampler) linked with a Saturn 2000 mass detector. Samples (1 μL) were injected in splitless mode onto a CP−Si15−CB low bleed/MS capillary column (30 m × 0.25 mm ID) and eluted with a linear temperature gradient as follows: initial column conditions 70° for 2 min, followed by a linear gradient (10° min^{-1}) to 310°. The injector temperature was 250°. Helium was used as carrier gas at 1 mL min^{-1} on column (12 psi head pressure). Electron impact ionization spectra (10−650 amu range) were recorded every second using an emission current of 40 μAmp and electron multiplier voltage of 1450 V.

Determination of Wall-Bound Phenolics. Cell wall analysis was carried out using DFRC as described (*23*). Briefly, 20 mg samples of extractive-free cell wall residue were treated with acetylbromide derivitazing agent and subsequently treated with zinc dust (reductive cleavage). After acetylation (acetic anhydride in pyridine), samples were analyzed by gas chromatography as described (*23*), except detection was via a Saturn 2000 mass detector as above. Thioglycolic acid analysis was carried out as described by Bruce and West (*24*) with minor changes to accommodate smaller amounts of tissue (*25*).

RESULTS

Cell Wall Phenolic Analysis. Analysis of suberized tissues using the DFRC method (*23*) showed that the amount of polymerized monolignols (represented by monolignol diacetates, **Figure 1a**) were significantly higher in control tissue than DPI-treated tissue (**Figure 1b**). The syringyl:guaiacyl ratio was approximately 1:1 in both control and DPI-treated tissues. Thioglycolic acid derivatization yielded results consistent with DFRC analysis: that is a greater amount of thioglycolic acid derivatives were released from control tissues compared with DPI-treated tissue (**Figure 1c**). Both DFRC and thioglycolic acid analyses showed the incorporation of significant amounts of phenolics into the cell walls of control tissues within 3 days post-wounding. By this time, it was possible to peel off the suberized layer intact from the control tissue. In contrast, the surface layer of the DPI-treated tissues collapsed into an unstructured mass and had to be scraped off during collection. Interestingly, the surface layer of control and DPI-treated suberized tuber slices appeared very similar prior to removal.

Analysis of Soluble Phenolics. At the time of wounding (day 0), two predominant soluble phenolic compounds appeared in potato tubers (**Figure 2a**). One compound was identified as *p*-coumaroylputrescine **3** (**Figure 3**). Within 4 days of wound

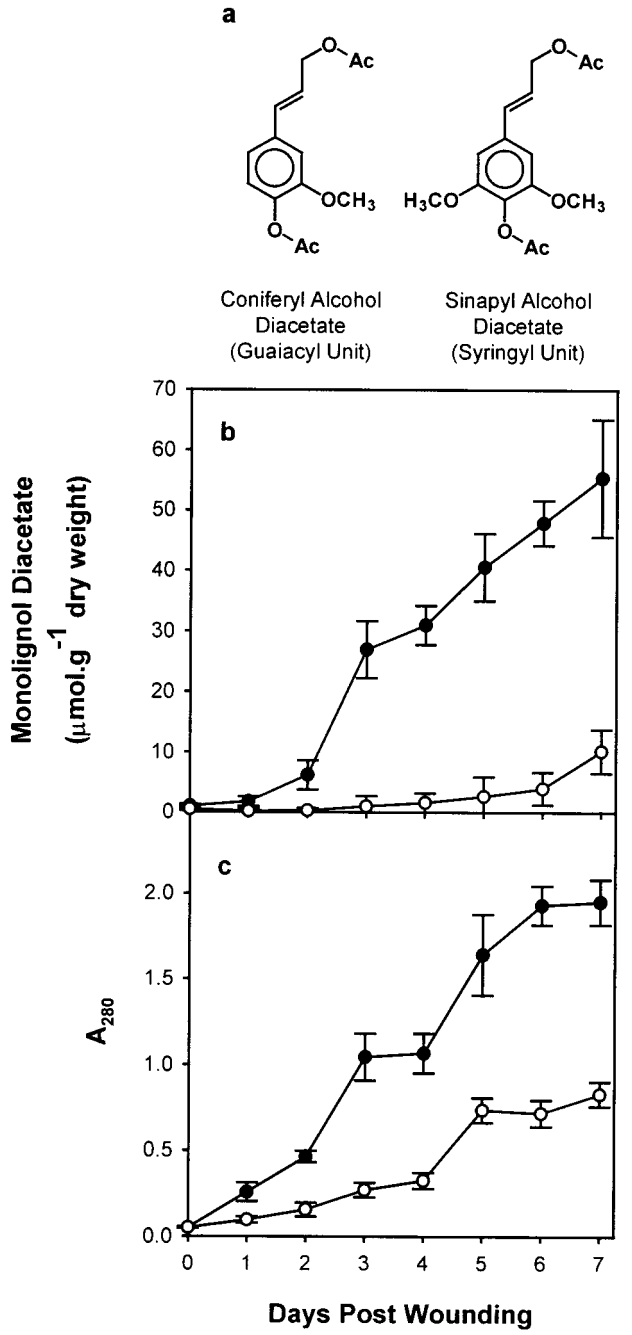

Figure 1. Cell wall phenolic analysis of suberized potato tubers. (a) Structures of the monolignol diacetates released by DFRC treatment. (b) Time course of monolignol diacetate recovery from the extractive-free cell wall residues of suberized layers of control (closed symbols) and DPI-treated (open symbols) tuber tissue by DFRC. (c) Time course of phenolic release from the extractive-free cell wall residues of suberized layers of control (closed symbols) and DPI-treated (open symbols) tuber tissue after thioglycolic acid derivatization. Suberized layers were collected daily (0–7 days post-wounding), extracted with 80% MeOH and acetone and the residues subjected to DFRC analysis or thioglycolic acid derivatization (see Material and Methods). Each data point represents the average of three independent time course experiments, with triplicate measurements made within each time course. Error bars represent one standard deviation. Data for guaiacyl and syringyl units are combined into a single data point for each time point in (b).

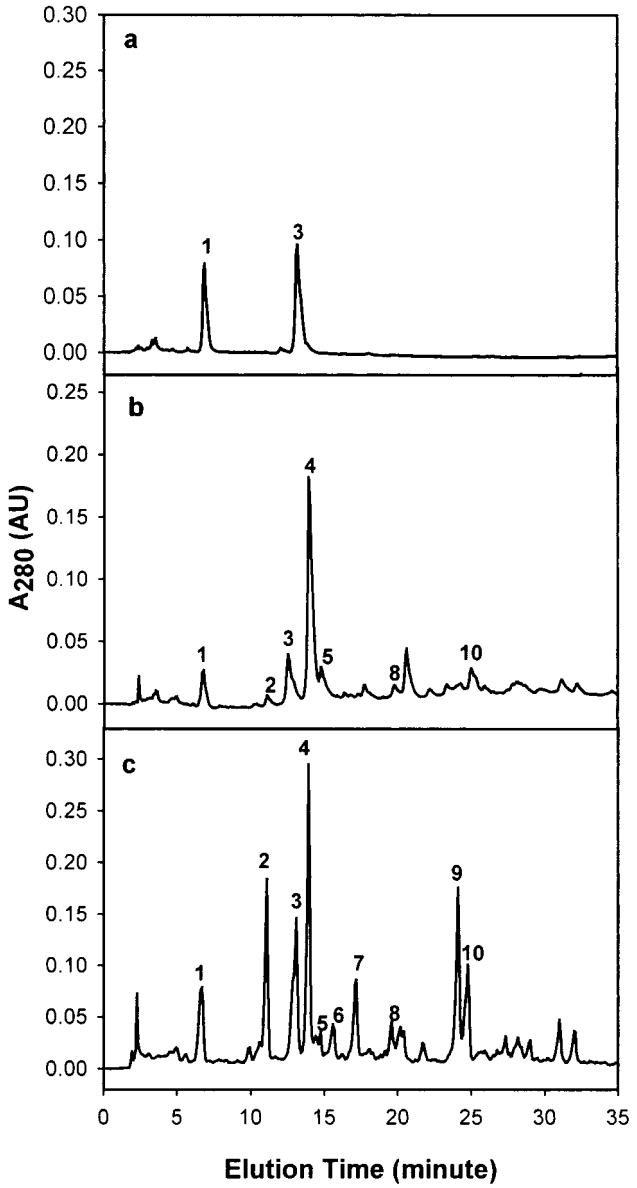

Figure 2. HPLC analysis of soluble potato tuber extracts. (a) Un-suberized tissue (day 0), (b) control tissue (day 4 post wounding) and (c) tissue treated with 25 μM diphenyleneiodonium (DPI) (day 4 post wounding), were Soxhlet extracted with 80% MeOH and the extracts analyzed by HPLC (see Materials and Methods). Labeled peaks are: (**1** = unknown; **2** = unknown; **3** = *p*-coumaroylputrescine; **4** = feruloylputrescine; **5** = chlorogenic acid; **6** = syringic acid; **7** = unknown; **8** = ferulic acid; **9** = *p*-coumaroyltyramine; **10** = feruloyltyramine. Numbers correspond to structures in Figure 3.

healing, many additional compounds appeared in both control and DPI-treated tissues (**Figure 2b,c**). Several of these compounds have been identified including feruloylputrescine **4**, chlorogenic acid **5**, syringic acid **6**, ferulic acid **8**, *p*-coumaroyltyramine **9** and feruloyltyramine **10** (**Figure 3**). [Compounds **1**, **2** and **7** remain unidentified.] While all compounds but **6** and **7** are present in control tissues, they are less abundant (**Figures 4** and **5**) with compound **9** not apparent until day 6 post wounding (**Figure 5**). Feruloylputrescine **4** was the major compound accumulating in both control and DPI tissues in response to the wound treatment (**Figures 2** and **4**). There was no evidence of soluble monolignols at any time post-wounding in either DPI or control tissues.

DISCUSSION

The initiation of suberization by wounding triggers the formation of poly(phenolic) (SPPD) and poly(aliphatic) (i.e.,

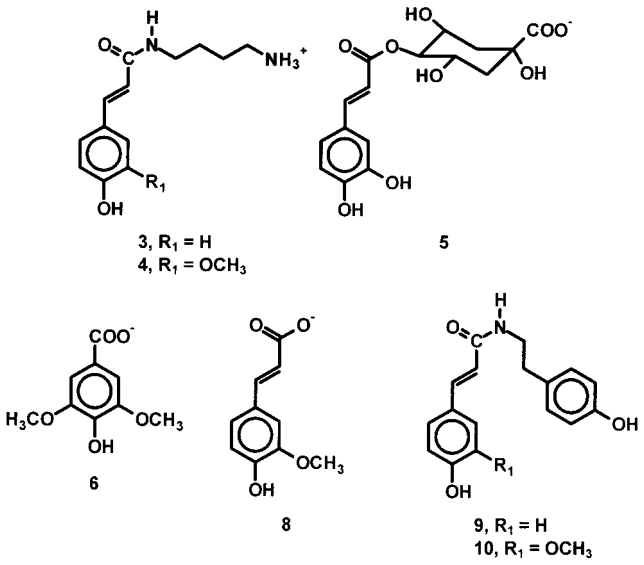

Figure 3. Structures of phenolic compounds identified in suberizing potato tubers. Numbers correspond to peak labels in **Figure 2**.

suberin) domains within the same tissue. The SPPD contributes 50−75% of the suberized wall modification, while the suberin components constitute 25−50% (*26*). The SPPD of potato tubers contains, in addition to small amount of monolignols, a considerable amount of hydroxycinnamic acids, especially ferulic acid derivatives (*7*, *8*). The suberin domain is a glycerol-linked polyester (*9*, *10*) covalently attached to the SPPD. Thus, while the SPPD and suberin domains are distinct, they are covalently linked when they coexist in the same cell (*11*).

The question of how the SPPD is assembled in vivo remains unresolved. It has been argued that the phenolic polymerization step is analogous to that described for lignification; that is via a peroxidase/H_2O_2 mediated free radical coupling process (for a recent review of the evidence supporting this view for lignification, see ref. *14*). A key component in this process is the availability of H_2O_2, and while peroxidases have been associated with the suberization process (*12*, *13*), to date there is no convincing evidence that H_2O_2 is a requirement for the SPPD formation. To approach this, we asked the question: what would happen if H_2O_2 was not available during suberization? Two consequences were predicted: First, fewer phenolics would be incorporated into the SPPD, and second, a greater amount of soluble (i.e., unpolymerized phenolics) would accumulate. Our data support both predictions and clearly point to the requirement of H_2O_2 for the formation of the SPPD during suberization.

Diphenyleneiodonium (DPI) Inhibition of Oxidases in Suberizing Potatoes. We have used DPI to inhibit an oxidase that we believe is involved in suberization (at least in Solanaceae) (*6*). However, DPI has been reported to also inhibit the H_2O_2-generating properties of peroxidases, albeit at much higher concentrations (i.e., > 1 mM) (*20*) than used here. While H_2O_2 generation has been suggested to be mediated by cell wall peroxidases in other plant species, e.g. French bean (*Phaseolus vulgaris*) (*20*) and lettuce (*Lactuca sativa* L.) (*16*, *27*), it should be noted that in the Solanaceae, NADPH-dependent oxidases have been more generally implicated. For example, an NADPH oxidase similar to the mammalian phagocyte plasma membrane oxidase has been suggested as a mediator in O_2^- production in wounded potato tubers inoculated with *Phytophthora infestans* (*1*). An oxidase showing high homology with human *gp91^{phox}* has also been cloned from tomato (*Lycopersicon esculentum*)

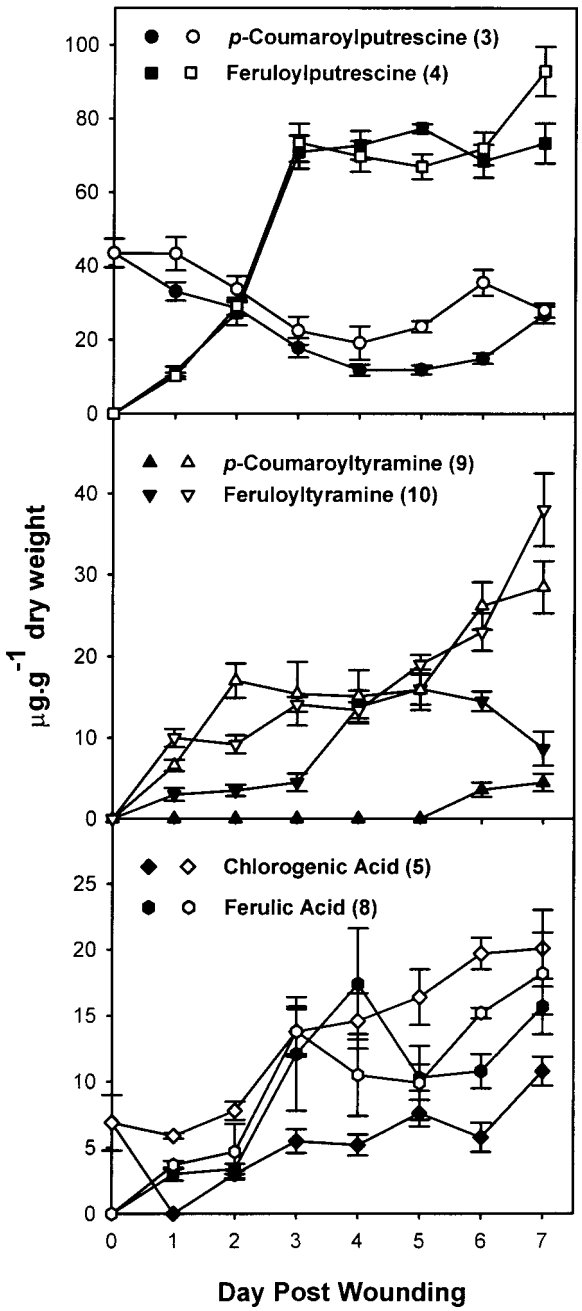

Figure 4. Time course of soluble phenolic accumulation in suberized potato tissue. The amounts of phenolic compounds (μg g^{-1} dry weight) released by Soxhlet extraction overnight in 80% MeOH from control (closed symbols) and DPI-treated (open symbols) tissues are shown. Three analyses were performed for each time point except for days 4 and 7, where at least five analyses were performed. Error bars represent one standard deviation.

(*28*). Similar oxidases have also been described in rice (*Oryza sativa*) (*29*) and *Arabidopsis thaliana* (*30*).

The level of ROS remains under homeostatic control during normal growth and development. This equilibrium is overridden by the production of large amounts of ROS under stress conditions (*15*). The superoxide anion is produced either by the reduction of dioxygen in reactions catalyzed by oxidases, particularly NADPH oxidase, or by the autoxidation of electron carriers (*31*). Superoxide then disproportionates either spontaneously or in a reaction catalyzed by superoxide dismutase, to form H_2O_2. In our hands, the level of DPI used (25 μM) is not sufficient to completely inhibit H_2O_2 production by peroxidases (*20*), and has no effect on normal per-oxidative activity (data

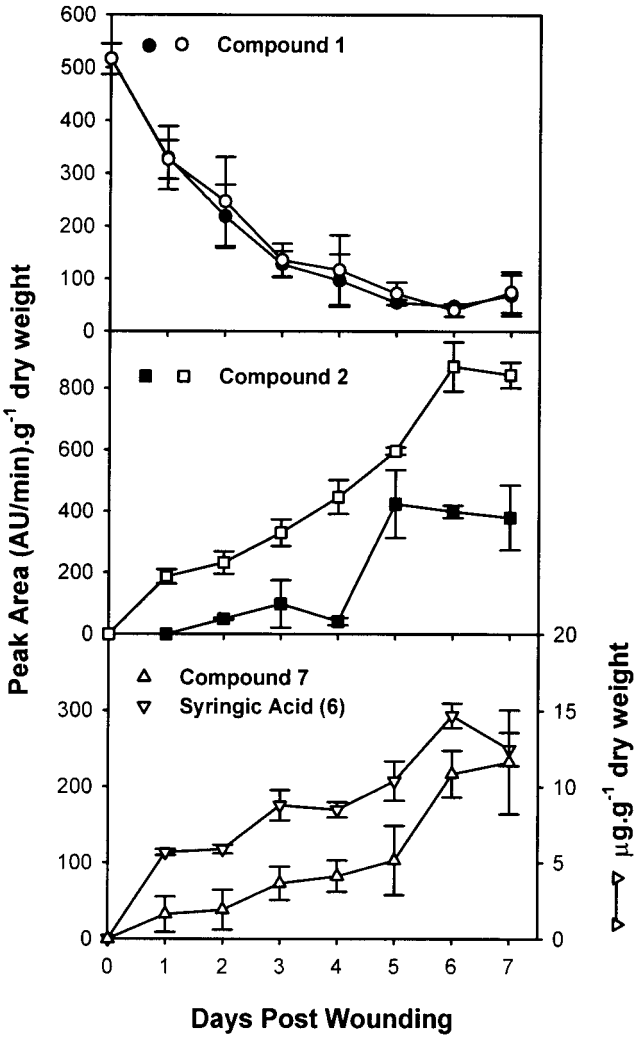

Figure 5. Time course of soluble phenolic accumulation in suberized potato tissue. The amounts of syringic acid **6** (μg g^{-1} dry weight) and the peak areas of unidentified phenolic compounds, **1**, **2** and **7** (AU/min μg mg^{-1} dry weight) released by Soxhlet extraction overnight in 80% MeOH from control (closed symbols) and DPI-treated (open symbols) tissues are shown. Note that syringic acid **6** and compound **7** were below the level of detection in control tissues, throughout the time course study. Three analyses were performed for each time point except for days 4 and 7, where at least five analyses were performed. Error bars represent one standard deviation.

not shown). We believe, therefore, that the DPI treatment used in this study was sufficient to prevent H_2O_2 production by putative oxidases without interfering with the peroxidase(s) involved in PPD formation (*6*).

Phenolic Incorporation into the SPPD. During the process of suberization a SPPD is laid down within the primary cell wall of induced cells, typically in advance of the poly(aliphatic) domain (reviewed in ref *11*). While it is becoming clearer that the SPPD is unique and distinct from that of lignified cells, no SPPD-specific analytical techniques have been developed. To date, alkaline nitrobenzene oxidation, cupric oxide oxidation, and thioacidolysis (all techniques developed for the analysis of lignified cell walls) have been employed to study the SPPD of various plants, but the relatively new DFRC technique (*23*) has not. However, its relative ease of use and promise of higher yields than for thioacidolysis prompted us to try it. From **Figure 1** it is clear that DPI-treated tissues contain significantly less DFRC-extractable material than controls, which we take as evidence for a greatly reduced amount of covalently cross-linked cell wall monolignols. The amount of coniferyl alcohol diacetate and syringyl alcohol diacetate released from control tissues 7 days post wounding is greater (50.4 $\pm$ 9.8 μmol g^{-1}) than that reported for the products of thioacidolysis (approximately 13 μmol g^{-1}) (*32*), consistent with the higher yield expected from this technique. It remains significantly lower, however, than that released from lignified cells (e.g., 640 and 1440 μmol g^{-1} for pine and basswood xylem, respectively) (*23*), consistent with the nonlignin nature of the SPPD.

Since it is well established that hydroxycinnamates make up a significant proportion of the potato SPPD, we also used the less specific thioglycolic acid derivatization method to characterize the extent of wall bound phenolics after DPI treatment. Although thioglycolic acid derivatization is less specific than DFRC (i.e., the latter cleaves specific interunit linkages), it will release soluble derivatives from phenolic-encrusted cell walls, yielding a relative measurement of total cell wall phenolics. It is therefore a useful tool to determine the time post wounding when the suberizing cells incorporate phenolics into their cell walls regardless of their metabolic origin. Thioglycolic acid analysis indicated the incorporation of wall-bound phenolics within 60 h post-wounding in both DPI-treated and control tissues consistent with the appearance of cell wall autofluorescence well before this time (*2*). As with the DFRC analysis, however, significantly fewer phenolics were released from DPI-treated tissues. Incidently, 60 h post-wounding is also the time when suberized layers attain enough structural integrity that they can be peeled off intact from control tissues (unpublished observation).

Soluble Phenolics. Treatment of suberizing potato tubers with DPI should inhibit H_2O_2 formation by flavoprotein-oxidases, but it should not interfere directly with phenolic metabolism. This raises the question of what happens to the phenolics that are synthesized in response to wounding, but not incorporated into the SPPD when DPI is used? In our study, wound-induced tissue incubated with DPI, accumulated more soluble phenolics, particularly **6**, **7**, **9,** and **10**, that were either not present or present in significantly lower amounts in the controls (**Figures 4** and **5**). It is tempting to speculate that this may be due to the (partial) inhibition of polymerization following DPI treatment and that these phenolics would otherwise have been incorporated into the SPPD. This is particularly true for **10** since this hydroxycinnamic acid derivative has been reported to be incorporated into the cell walls of suberized potato tubers (*8*), presumably via oxidative cross linking. In this study, we could detect only very small amounts of **10** until 3 days post wounding in the control suberized tissue, while in the DPI-treated tissue, relatively large amounts were detected within 1 day (**Figure 4**). This may indicate that in control tissues **10** is immediately polymerized into the cell walls, while in DPI-treated tissue it is not. It should be noted, however, that using DFRC analysis we were not able to confirm whether **10** was incorporated into the cell walls of control tissues (i.e., the analysis is tailored to the detection of monolignols diacetates). With the exception of **6**, **8**, and **10**, which have been previously isolated from suberized cell walls (*8, 32*), none of the other phenolics that accumulated in DPI-treated tissues have been hitherto associated with the SPPD. For example, the build up of **9** after DPI-treatment was obvious within 3 days post-wounding (**Figure 4**), while it was not found in controls until 6 days after wounding (and then only in very low amounts). Similarly, the unidentified compound **7** was below the level of detection in controls, but appeared in significant amount in the DPI-treated tissues (**Figure 5**). The

question remains as to what role the phenolics that accumulate in DPI-treated tissues would normally play in SPPD formation. Is **9** a metabolic precursor to **10** that accumulates because the latter is not efficiently incorporated into the SPPD under the conditions employed? What about **6**, which is undetectable in controls but accumulates to a significant level after DPI treatment? This compound has been isolated from potato periderm after extensive alkaline hydrolysis (*32*), but its structural role in the SPPD (if any) remains undefined. Of the remaining compounds, **1**, **3,** and **4** are assumed to be unrelated to SPPD formation since their accumulation patterns are essentially identical in control and DPI-treated tissues. Chlorogenic acid (**5**), on the other hand, accumulates to a greater extent after DPI-treatment, and as with the accumulation of **9**, may reflect its role as an intermediate in phenolic metabolism in the Solanaceae. The other two unidentified compounds (i.e., **2** and **7**) either accumulate to a greater extent after DPI treatment (**2**), or are undetectable (**7**) in controls at any time after wounding. Elucidating their involvement in the SPPD formation awaits their identification (underway in our lab). Curiously, the syringyl composition of potato SPPD is not represented in the compounds that accumulate after DPI treatment. None of the unidentified peaks in the DPI-treatment chromatograms represent syringin, syringyl alcohol, sinapic acid, sinapoyltyramine, or sinapoyl glucose, all of which are potential syringyl precursors for the potato SPPD.

In conclusion, our results clearly point to the requirement for H_2O_2 during suberization for SPPD formation. The inhibition of H_2O_2 production with DPI resulted in significantly lower amounts of covalently linked wall-bound phenolics and larger amounts of soluble phenolics. These two pieces of information are consistent and provide strong evidence of at least the partial inhibition of phenolic polymerization during the macromolecular assembly of the potato SPPD when H_2O_2 production is inhibited.

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