Plant Physiol. (1991) 97, 1445–1455
0032-0889/91/97/1445/11/$01.00/0

Received for publication April 16, 1991
Accepted July 25, 1991

Rapid Accumulation of Anionic Peroxidases and Phenolic Polymers in Soybean Cotyledon Tissues following Treatment with *Phytophthora megasperma* f. sp. *Glycinea* Wall Glucan[1]

Madge Y. Graham and Terrence L. Graham*

Department of Plant Pathology, The Ohio State University and the Ohio Agricultural Research and Development Center, Columbus, Ohio 43210

ABSTRACT

***Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin (PMG) cell wall glucan has been extensively characterized as an elicitor of the pterocarpan phytoalexins, the glyceollins in soybean (*Glycine max* L.). Just recently, this glucan was shown to be a potent elicitor of conjugates of the isoflavones, daidzein and genistein as well. Here we report that PMG wall glucan also induces a rapid and massive accumulation of phenolic polymers in soybean cotyledon cells proximal to the point of elicitor application. Deposition of phenolic polymers is over ten times that in wounded controls within just 4 hours of elicitor treatment and reaches a maximum by 24 hours. In the same tissues, isoflavone conjugates begin to accumulate at 8 hours and glyceollin at 12 hours. By 24 hours, the total deposition of wall bound phenolics in elicitor-treated tissues is several times greater than the peak glyceollin and isoflavone responses combined. Histochemical stains and quantitation of phenolic residues released after saponification and nitrobenzene or copper oxide oxidation suggest that the covalently linked phenolics include both lignin- and suberin-like polymers as well as simple esterified coumaric and ferulic acid monomers. Accumulations of phenolic polymers are accompanied by equally rapid and massive increases in activity of a specific group of anionic peroxidases. Although increases in peroxidase activity are not strictly limited to cells immediately adjacent to the area of elicitor treatment, the deposition of phenolic polymers is significantly less extensive in distal cells.**

The soybean-PMG[2] association provides an excellent system to study molecular aspects of host-pathogen interactions. Multiple races of the pathogen have been defined based on their reactions to specific *Rps* resistance genes in various host differentials (35). Soybean tissues resistant to infection respond with the production of the pterocarpan phytoalexins, the glyceollins, which are synthesized from phenylalanine through well characterized enzymatic pathways (8). The accumulation of the glyceollins generally correlates very well with race-specific resistance as conditioned by the *Rps* genes (7, 8, 25).

In the laboratory, race-specific resistance to PMG is observed in all soybean seedling organs (*e.g.* 2, 3, 18, 20, 31, 40). Of these various organs, however, cotyledons are particularly suited for investigations of the spatial and temporal coordination of molecular defense responses (17). This is due to their distinctively simple cellular architecture; other than the epidermis and a few major vascular elements, they consist mainly of tightly aligned columns of highly uniform mesophyll parenchmya cells. To ensure that studies with cotyledons are physiologically meaningful, we have confirmed and extended earlier reports (31) of race specific infection in this organ. We demonstrated that all aspects of the response of the organ to infection by PMG, including disease progression, symptoms, and the timing, magnitude and spatial accumulations of glyceollin, resemble the race-specific responses of other organs (18).

Employing a sensitive HPLC profiling procedure (14) to study soluble phenylpropanoid-derived metabolites, we have disclosed the presence of large constitutive pools of conjugates of two closely related isoflavones, daidzein and genistein, in all soybean seedling organs (15, 18). Daidzein is a direct precursor of glyceollin and genistein itself is toxic to PMG (13) (LI Rivera-Vargas, AF Schmitthenner, TL Graham, unpublished results). In cotyledon tissues infected with an incompatible race of PMG, the isoflavone conjugates are hydrolyzed at the infection front leading to a rapid and sequential buildup of inhibitory levels of free genistein and glyceollin and rapid containment of the pathogen in a limited hypersensitive lesion (18). In tissues beyond the infection front, rather than being hydrolyzed, the conjugates often show a net increase in concentration.

These responses prompted us to reexamine the activities of the cell wall glucan from PMG, which was previously characterized as an elicitor of glyceollin (7, 8). Thin sectioning and analysis of discrete cell populations of soybean cotyledon tissues proximal and distal to the point of application of elicitor demonstrated that proximal cells accumulate both glyceollin and the isoflavone conjugates, while distal cells

[1] Partial salary and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center (OARDC). This is OARDC manuscript No. 101-91. Partial research support was also provided by the U.S. Department of Agriculture under Cooperative State Research Service grant No. 89-37231-4493 to T.L.G. and by a grant from the Midwest Plant Biotechnology Consortium.

[2] Abbreviation: PMG, *Phytophthora megasperma* Drechs. f. sp. *glycinea* (Hildeb.) Kuan & Erwin.

respond with an even more massive buildup of isoflavone conjugate reserves without generation of the phytoalexin (16). Elicitor treatment thus differs from incompatible infection in that the local hydrolysis of the isoflavone conjugates observed at the interface between the fungal and host tissues is not seen. Instead, isoflavone conjugates accumulate in both local and distal cell populations. Thus, the PMG wall glucan elicitor appears to stimulate some, but not all, of the events occurring in incompatible infections. We believe that the distal buildup of isoflavone conjugates, which is more explicitly demonstrated by elicitor treatment, may function as a third level of defense to strengthen the defense potential of underlying tissues.

Phenolic polymers are often important and major components of the secondary plant cell wall. In addition to the predominant aromatic polymers, lignin and suberin, simple phenolics such as hydroxycinnamic acids and isodityrosine are often covalently linked to wall polymers (11, 39) and appear as components in wall-bound conjugates of diamines and polyamines (9). Although wall bound phenolics have been proposed to play a potential role as barriers to infection, these molecules have traditionally been very difficult to extract and quantitate. Only recently have molecular studies with the wall-associated phenolics begun to provide convincing evidence for a role in host-pathogen interactions (19, 28, 36).

Although soybean-PMG interactions have been very thoroughly studied in relation to the soluble products of the phenylpropanoid pathways (8) (see discussion above), remarkably little work has been done to characterize the role of wall-bound phenolics in this system, even though they may account for some major diversions from the phenylpropanoid pathway. To address this issue, we have analyzed the deposition of wall-bound phenolics in parallel with soluble phenylpropanoids in various cotyledon cell layers proximal and distal to the point of elicitor treatment. Since peroxidase is proposed to be responsible for the last steps in the oxidative coupling of phenolic monomers for the formation of wall polymers, we also examined peroxidase isozyme patterns in these tissues. We have found that the deposition of phenolic polymers in the cell wall is a very early and major response to treatment with the PMG wall glucan. Their accumulation begins within 4 h of elicitor treatment, several hours before the accumulation of appreciable amounts of the isoflavones or of glyceollin, and represents a major commitment of phenylpropanoid intermediates. Correspondingly, a group of anionic, wall-bound peroxidases are induced over a very similar time frame. The possibility of the deposition of wall-bound phenolics as a protection mechanism is discussed in the context of its coordination with other phenylpropanoid-derived defense responses.

MATERIALS AND METHODS

Chemicals

All chemicals were reagent grade and obtained from Sigma or Aldrich.

Plant Growth and Treatment

Soybean (*Glycine max* L.) cultivars Williams and Williams 79 were grown as described previously (18). Wall glucan elicitor was prepared from race 1 of *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin (PMG) according to Ayers *et al.* (1). Its composition, as determined by elemental, protein, lipid and sugar analyses, was essentially the same as reported by Ayers *et al.* (1). The cotyledon elicitor assay, including thin sectioning of discrete cell populations proximal and distal to the site of elicitor treatment, was carried out as described previously (16). Thirty replicate cotyledons were used per treatment. The cut cotyledon surface was treated with either 30 μL of sterile water (as control) or 30 μL of elicitor at 50 μg/mL. This concentration of elicitor is that which causes half-maximal accumulation of both glyceollin and the phenolic polymers (data not shown). Incubations were performed in the light (100 $\mu E/m^2/s$) or in the dark at 25°C. The centers of the cotyledons were harvested at various times with a #1 cork borer (4 mm i.d.). The resulting cylinder of cells was cut into two cross-sections. The upper section consisted of a 0.6-mm slice, including the treated surface and approximately three cell layers from the wounded surface. The lower section (about 2.5 mm) contained an average of 16 cell layers. In some experiments, this lower section of cells was further sliced into two equal layers, providing three sections for analysis.

HPLC of Soluble Aromatics and Phenolic Polymer Measurement

Inasmuch as phenolic polymer quantitation can be carried out on the insoluble residue from alcohol extracted tissues, HPLC analysis and phenolic polymer quantitation were conveniently carried out on the same samples. Sections for 10 replicate cotyledons for a given treatment were pooled, weighed, and kept frozen in microfuge tubes at −80°C until extracted. Except where noted otherwise, all operations were carried out directly in microfuge tubes as previously described (14).

Phenolic polymer deposition was quantified by modifications of established protocols for the thioglycolic acid assay (21, 38). This procedure is the method of choice for the measurement of herbaceous phenolic polymers, since it involves minimal chemical alterations of individual phenolic residues and results in a thioglycolic acid derivative which is readily purified away from potential contaminants, including esterified phenolic monomers and noncovalently linked proteins (29, 34).

Samples for phenolic polymer analysis were first extracted in 80% ethanol (400 μL/50 mg fresh weight tissue) for HPLC profiling of soluble aromatics (14). The insoluble residue was collected by centrifugation and reextracted with 1 mL of 80% ethanol and twice with 1 mL of methanol. This procedure removed any residual alcohol-soluble phenolics and, through the removal of remaining moisture, made the residue highly friable. The precipitate was then suspended in 500 μL of 10% thioglycolic acid in 2 N HCl (v/v) and transferred to a glass HPLC autoinjection vial (2 mL, National Scientific Co., Lawrenceville, GA) with a fresh Teflon seal. The suspension

was heated at 100°C for 4 h, cooled, transferred to a fresh microfuge tube, and centrifuged at 18,000g for 6 min. The resultant pellet was washed with 500 μL of deionized, distilled water and recentrifuged. The washed pellet was then suspended in 500 μL of 1N NaOH and vortexed several times over a period of at least 2 h to solubilize the thioglycolic acid derivatives. The suspension was again centrifuged and the supernatant transferred to a fresh microfuge tube. The polymeric thioglycolic acid derivatives were then reprecipitated through the addition of 200 μL of concentrated (10 N) HCl and cooling the suspension on an ice bath for 1 h. After centrifugation of the flocculent reddish-brown precipitate, the pellet was redissolved in 1 mL of 0.5 N NaOH. The absorbance of the resultant orange solution was taken at 335 nm as a measure of phenolic polymer deposition. This preparation is essentially free of esterified phenolic acid monomers as shown by saponification and HPLC as described below. Although this protocol was generally applied to samples weighing 50 mg, it is highly sensitive and can be readily applied to samples as small as 1 mg without any changes in the derivatization, extraction, or precipitation volumes.

Purified phenolic polymer-thioglycolic acid derivatives were prepared from elicitor-treated cotyledon tissues for initial characterization and to obtain a standard curve for quantitation of the analytical samples. This preparation was made in a very similar manner as above, except that the derivatives were alternatively solubilized and reprecipitated four times in 0.5 N NaOH and 2 N HCl, respectively. The final precipitate was then dissolved in methanol, diluted in 0.5 N NaOH, and subjected to one more acid precipitation. This precipitate was again dissolved in methanol and dried to a fine powder. A standard curve was established with this preparation (Fig. 1).

Spectra of the analytical samples, as prepared above, were superimposable with that of the purified conjugates (Fig. 1); thus, the shorter protocol, as outlined above, effectively removes all soluble and insoluble interfering substances. Because the ratio of the 257 to 335 nm peaks also did not vary over relatively wide ranges of phenolic polymers, as analyzed in a variety of samples, we routinely used the broader absorbance at 335 nm as a measure of thioglycolic acid derivative concentration. Because the peak at 257 nm is very sharp, reliable quantitation of this peak required a scan rather than measurement at a single wavelength.

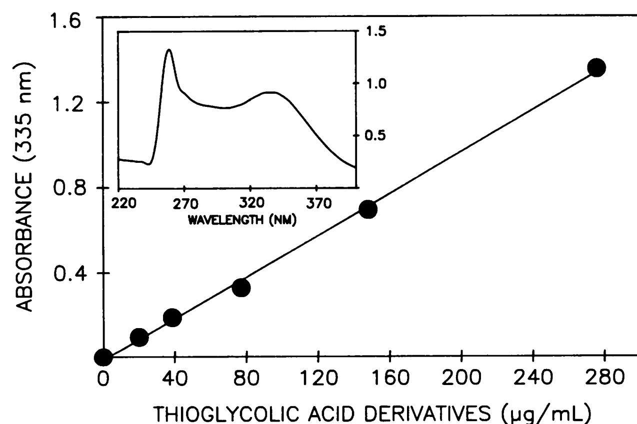

Figure 1. Standard curve for thioglycolic acid estimation of phenolic polymers. A preparative sample of purified thioglycolic acid derivatives from elicitor treated cotyledon tissues was diluted at the concentrations indicated in 0.5 M NaOH and the absorbance taken at 335 nm. The insert is the ultraviolet spectrum of a 180 μg/mL solution of these same thioglycolic acid derivatives.

Estimation of Wall-Bound Esterified Phenolic Acid Monomers and Nitrobenzene and Copper Oxide Oxidations of Phenolic Polymers

The various thin sections of soybean cotyledon tissue were extracted twice with 1 mL of 100 mM phosphate buffer (pH 6.8) for each 50 mg fresh weight tissue, followed by two extractions each with 1 mL of 80% ethanol and 100% methanol. In all cases, extractions were carried out directly in microfuge tubes by grinding with a modified polypropylene pestle (Kontes Glass Co., Vineland NJ) and centrifugation at 18,000 g for 4 min as described previously (14). The final, finely divided cell wall residue was suspended in 1 mL of 1 M NaOH and shaken for 24 h at 25°C (22). Under these mild saponification conditions, wall-esterified hydroxycinnamic acid derivatives were selectively released. The supernatant was acidified to pH 2 with concentrated phosphoric acid and extracted with four volumes of ethyl acetate. The ethyl acetate was evaporated under a stream of nitrogen and the residue taken up in 500 μL of 80% ethanol. The resultant solution, containing the majority of the wall-esterified ferulic and coumaric acids, was subjected to HPLC as described previously (14). The pellet from the above saponification reaction was subdivided into two equal aliquots, which were suspended and oxidized in alkaline nitrobenzene or copper oxide essentially by the methods of Hartley (22) and Rhodes and Wooltorton (32), respectively. Oxidations were carried out in sealed glass vessels in an autoclave at 130°C. The cooled reaction mixtures were acidified to pH 2 with concentrated phosphoric acid and extracted with four volumes of ethyl acetate. The ethyl acetate extracts were evaporated under nitrogen to provide a residue which was taken up in 200 μL of 80% ethanol for HPLC analysis of the constituent aldehydes.

HPLC of both the saponification and oxidation mixtures resulted in simple and well-resolved profiles of the constituent aromatic components. Standards of cinnamic, caffeic, coumaric, and ferulic acids and of the aldehydes p-hydroxybenzaldehyde, vanillin, and syringaldehyde were used to quantitate the released phenolic acids or aldehydes. The identity of each of these components was confirmed by its coelution with and by the superimposability of its ultraviolet spectrum with the appropriate authentic standard.

Electrophoretic Analysis of Peroxidases

Samples for electrophoresis of peroxidases were harvested from a concurrent but separate set of replicates from those used for HPLC and phenolic polymer quantitation. Sections from 20 replicate cotyledons were pooled for each treatment. Peroxidases were extracted by modifications of the methods of Bruce and West (4). Initial extractions of wall-bound (apoplastic) peroxidases from wound- or elicitor-induced cotyledon tissues by several rounds of vacuum infiltration with

0.05 M $CaCl_2$ yielded the same major peroxidase isozymes as grinding in this extraction solution. Because the latter method provided higher and more reproducible yields, it was used in all subsequent analyses. Samples were ground to a fine suspension in the extraction solution (100 μL/50 mg tissue) in microfuge tubes with a polypropylene pestle (Kontes Glass Co., Vineland, NJ) at 650 rpm. The samples were kept ice cold during all operations.

After incubation on ice for 20 min, the extracted samples were centrifuged at 18,000*g* for 5 min. Electrophoresis sample buffer was immediately added to the supernatant, and electrophoresis was accomplished essentially according to Bruce and West (4), except that it was carried out at 4°C in gels 1.5 mm thick and 17 cm long. Cooling of the electrophoresis gels resulted in more reproducible staining of activity, particularly for cationic species.

In addition to 3-amino-9-ethylcarbazole, we also tested 4-chloro-1-naphthol and 3,3′-diaminobenzidine for visualization of the peroxidase isozymes. Even though 4-chloro-1-naphthol gave the clearest background, 3-amino-9-ethylcarbazole gave higher overall sensitivity and better resolution of the banding pattern.

Histochemical Stains

A variety of stains were examined for their histochemical reaction with the phenolic polymers in hand-cut cross-sections through the treated cotyledons at various times after treatment. The stains employed included Sudan III-IV, Sudan Black, phloroglucinol, Nile Blue, and chlorine-sulfite (24), and 12 N KOH and crystal violet (23). Of these stains, the most useful for routine staining of thin sections of soybean cotyledon tissue were phloroglucinol (which reacts with free aldehyde groups in phenolic polymers) and Nile Blue (which reacts with the lipid or aliphatic side chains of suberin).

RESULTS

Phenolic Polymer Deposition in Wounded and Elicitor-Treated Soybean Cotyledons

In intact cotyledons, the internal parenchyma cells (which make up the surface layers in the cut cotyledon assay) contained very small amounts of phenolic polymers (<0.10 μg thioglycolic acid derivatives/g fresh weight tissue). Upon wounding of light-incubated cotyledons, however, the phenolic polymer content of cells immediately adjacent to the wounded surface increased with time (Fig. 2A). This was a relatively slow response, becoming apparent only after 16 h and continuing gradually over the period 24 to 72 h.

In contrast, treatment of cotyledons with PMG wall glucan elicitor in the light resulted in significant phenolic polymer deposition in the uppermost cell layers as early as 4 h (Fig. 2A). Although only a fraction of the final response, the phenolic polymer content of elicitor-treated tissues at 4 h was readily measurable and was consistently over 10 times that of wounded control tissues. Phenolic polymer deposition continued rapidly under this condition and leveled off by 24 h.

Because incubation of cotyledons in the light, but not in the dark, greatly enhanced the accumulation of the isoflavone conjugates in elicitor-treated tissues (16), we examined the

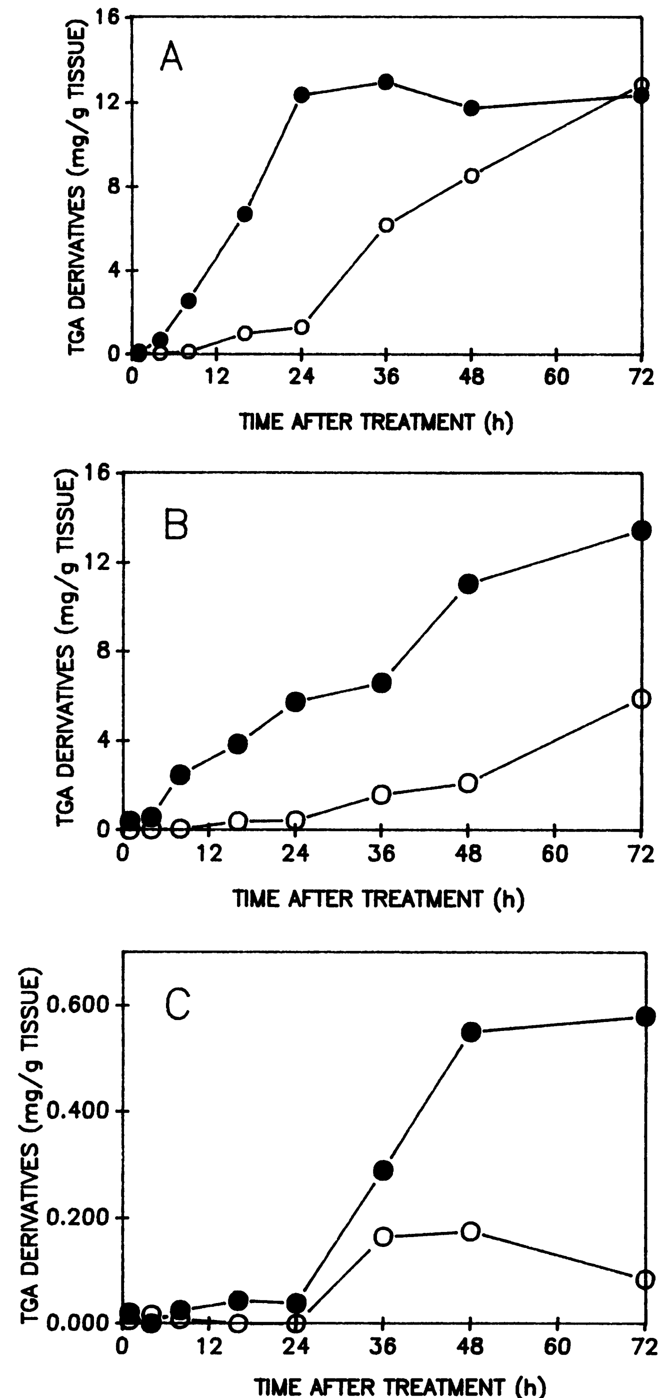

Figure 2. Phenolic polymer deposition in the upper and lower cell layers of wounded and elicitor-treated cotyledons. Cotyledon tissues were treated with 30 μL of water (○) or PMG wall glucan (50 μg/mL) (●) and incubated in the light (A, C) or the dark (B). The upper four cell layers (A, B) or the lower 16 cell layers (C) from a cylindrical plug of tissue was harvested at the indicated times and subjected to the thioglycolic acid (TGA) assay for phenolic polymers. Similar results were obtained in three separate experiments.

effects of dark incubation on the deposition of phenolic polymers as well. When incubated in the dark, cotyledons showed more gradual accumulations of phenolic polymers in response to both wounding and PMG wall glucan elicitor (Fig. 2B). As with light-incubated cotyledons, however, treatment with the elicitor resulted in a much more rapid deposition of phenolic polymers.

Although phenolic polymer deposition was detectable in lower cell layers in light-incubated cotyledons as well (Fig. 2C), the response was substantially delayed and the polymers accumulated to levels less than 5% of those in the upper cell layers (note the difference in scale). Phenolic polymer depositions in the lower cell layers of dark incubated cotyledons again showed considerably diminished and more gradual responses compared to those in the light (data not shown).

Peroxidase Isozyme Patterns in Wounded and Elicitor-Treated Cotyledons

Few anionic or cationic peroxidases were detectable, and only at low activities, in freshly excised, unwounded soybean cotyledon tissues. Wounding led to the induction or enhancement of several major anionic and cationic peroxidase bands. However, since the induction of the cationic peroxidases did not correlate well with phenolic polymer deposition in any of our experiments, and because elicitor treatment did not markedly influence their induction, we chose to focus on the anionic species in the present paper.

The anionic peroxidase isozyme patterns seen in response to wounding and elicitor treatment are shown in Figure 3 for upper cell layers and Figure 4 for lower cell layers. These activities can be divided into three groups according to their electrophoretic mobilities. The fastest moving doublet (group 3) was constitutive, weaker staining, and somewhat labile under the conditions employed. The doublet was present only in freshly extracted tissues and disappeared upon storage or repeated freeze-thawing. It did not appear in response to wounding or elicitor treatment. Another group of anionic isozymes (group 1) ran near the top of the gel. Although as many as four bands have been resolved in this group, the bands were generally weakly staining and somewhat more variable from experiment to experiment. The activity of two of these bands was enhanced by the PMG wall glucan in upper cell layers (Fig. 3). However, none of this group of bands clearly corresponded to phenolic polymer deposition as shown in Figure 2.

In contrast, the central group of anionic peroxidases (group 2) was always very strongly and reproducibly induced by wounding and PMG wall glucan. Although they are somewhat difficult to visualize in the gels presented here (which were overloaded for comparative purposes), at least four bands in this group were discernible. The top band was induced mainly in lower cell layers in response to wounding. It is most apparent in Figure 4B. The other three were induced in both cell layers, but their induction was stronger and much more rapid in the upper cell layers (Fig. 3). These three lower bands of the group 2 peroxidases responded more or less coordinately, although the bottom band was often slightly delayed in relation to the other two. In both cell layers, wounding alone was sufficient for their induction, but PMG wall glucan significantly enhanced the rate and intensity of their induction.

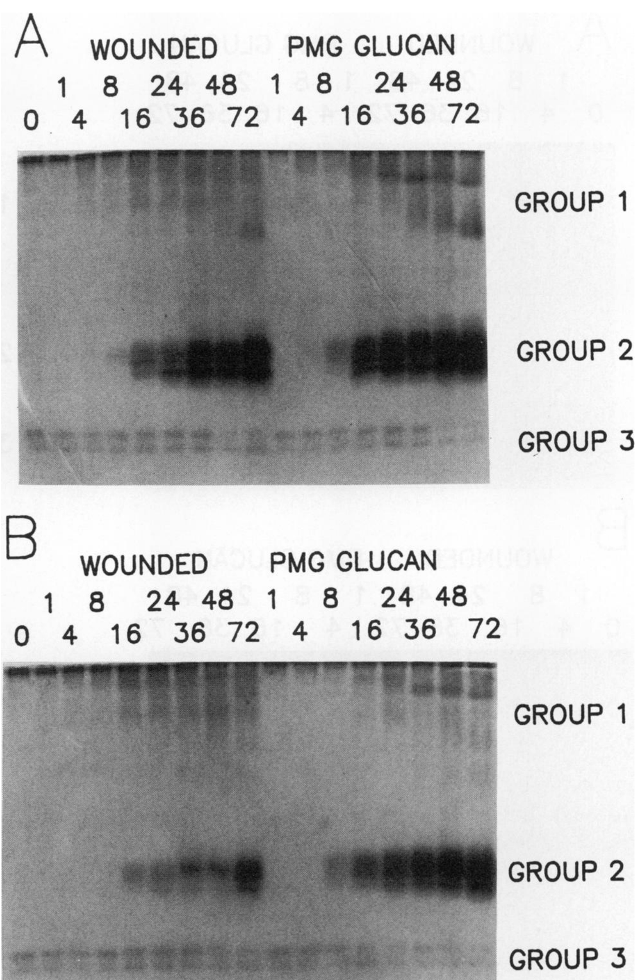

Figure 3. Anionic peroxidase isozyme pattern in upper cell layers of wounded or elicitor-treated cotyledons. Tissues were harvested from the same experiment described in Figure 2. The upper cell layers from light incubated (A) or dark incubated (B) cotyledons were extracted and subjected to electrophoresis as described in the text. Lane 1, labeled 0, is from freshly harvested cotyledons. Lanes 2 to 9 are from wounded control cotyledons harvested at the indicated hour. Lanes 10 to 17 are from elicitor-treated cotyledons at the same time points. Similar result were obtained from two separate experiments.

In elicitor-treated upper cell layers, these group 2 peroxidases could be detected as early as 4 h in light-incubated tissues and were clearly stained by 8 h (Fig. 3A). After 8 h, their induction was very rapid, reaching near saturation on the gels by 16 h. In comparison, the induction of these same peroxidases in light-incubated, wounded tissues was delayed by an additional 4–8 h, after which they showed a more gradual increase. In the dark, the induction of the group 2 peroxidases, both by wounding or PMG glucan in upper cell layers, was more gradual and of lower magnitude (Fig. 3B). Thus, the induction of the group 2 enzymes correlated very well temporally with the accumulation of phenolic polymers in wounded and elicitor-treated tissues both in the light and in the dark (Fig. 2).

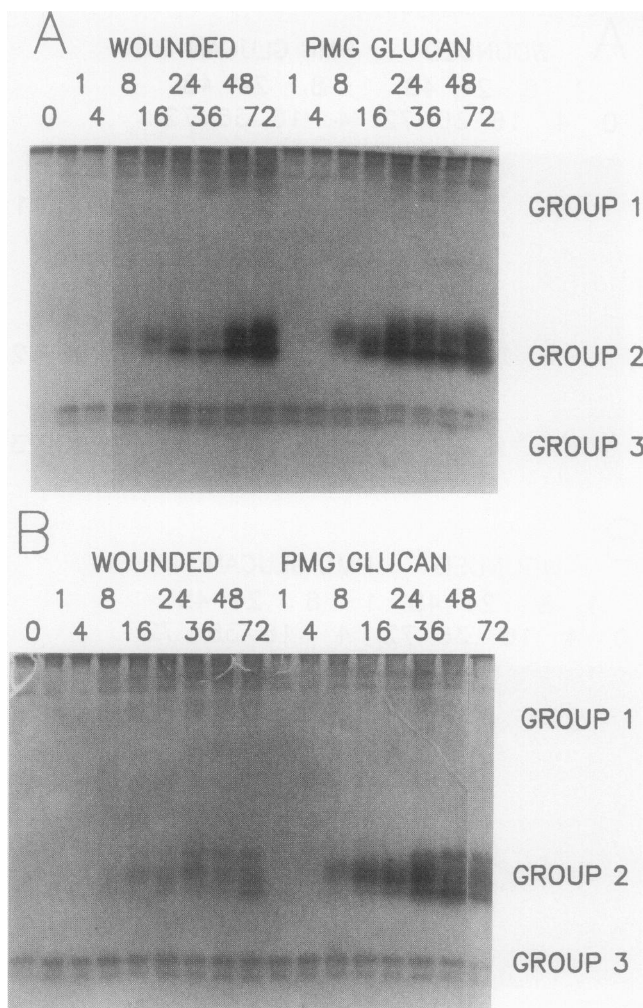

Figure 4. Anionic peroxidase isozyme pattern in lower cell layers of wounded or elicitor-treated cotyledons. Tissues were harvested from the same experiment described in Figure 2. The lower cell layers from light (A) or dark (B) incubated cotyledons were extracted and subjected to electrophoresis as described in the text. Lanes are as described in Figure 3.

Increases in activity of the group 2 enzymes was also apparent in lower cell layers, especially in the light (Fig. 4A). The correlation with phenolic polymer deposition in these cell layers was not as clear as that in the upper cell layers. Possibilities for this lack of correlation are discussed in more detail below. To gain a better perspective on the induction of peroxidases in these distal cell layers, we examined peroxidase and phenolic polymer deposition in a separate experiment in which the lower cell layers were further sectioned into two equal portions. Once again, light and dark incubations were included. The results of this experiment were very similar to those reported above. Induction of the group 2 peroxidases again correlated very well with phenolic polymer deposition in the upper cell layers under all conditions. Peroxidase induction, although delayed and of considerably lower magnitude, also occurred in the section farthest from wounding or the elicitor signal (data not shown).

Phenolic Polymer and Peroxidase Induction in PMG Resistant Near-Isoline of Williams

Although infection leads to race-specific accumulation of the glyceollins in resistant plants, purified PMG wall glucan elicitor induces glyceollin in a non-race specific manner (7). That is, the elicitor from any given race of the pathogen stimulates glyceollin accumulation in all cultivars of soybean, regardless of the presence or absence of specific *Rps* resistance genes to that race. Thus, the *Rps* genes condition glyceollin accumulation in infected tissues but are not a prerequisite for its induction by purified elicitor. We wished to determine if similar characteristics may govern the PMG wall glucan-induced accumulation of phenolic polymers.

Williams soybeans (used for the studies reported above) possess no *Rps* genes for resistance and are universally susceptible to PMG. The near isoline of Williams, Williams 79, carries the *Rps* 1c gene for resistance to race 1. Nonetheless, the responses of these two isolines to wounding and the PMG wall glucan from Race 1 were identical in all respects. The peroxidase isozymes induced were the same as those reported above and no differences were seen in the timing or magnitude of peroxidase induction or phenolic polymer deposition. Importantly, the induction of the group 2 peroxidases again correlated nearly perfectly with phenolic polymer deposition in upper cell layers of either cultivar. Thus, the PMG wall glucan elicitor also induces phenolic polymer deposition in a non-race-specific manner.

Effect of ABA on Phenolic Polymer Deposition and Peroxidase Induction

Suberin-specific anionic peroxidases, which are induced by wounding and ABA treatment, have been characterized in potato and tomato (6, 33). The tomato peroxidase is also induced by a cell-free elicitor from *Verticillium* (30). Thus, we examined a range of concentrations of ABA (10^{-6} to 10^{-2} M) for its effect on peroxidase and phenolic polymer deposition in soybean cotyledons. ABA treatment had little effect on soluble aromatics and only subtle effects on phenolic polymer deposition or the induction of the group 2 peroxidases. As shown in Figure 5, ABA (10^{-4} M) had no discernible effect on phenolic polymer deposition in the light. Consistent with this, there was little effect on the timing or intensity of induction of the group 2 peroxidase isozymes under these conditions (Fig. 6A). In the dark, ABA enhanced phenolic polymer deposition slightly, particularly at later time points (Fig. 5). This was accompanied by somewhat earlier and more intense induction of the group 2 peroxidases in the ABA-treated upper sections (Fig. 6B). Thus, even the very subtle positive effect of ABA on peroxidase in the dark serves to confirm the correlation between the extent of group 2 peroxidase induction and phenolic polymer deposition we saw in previous experiments.

To address the possibility that ABA effects on peroxidase and phenolic polymer accumulation were masked by the wound response, we also examined unwounded cotyledon tissues incubated under light for 44 h after uptake of 2×10^{-4} M ABA through the cotyledonary node. Neither excision of the cotyledons nor ABA uptake caused detectable increases in peroxidase or phenolic polymers.

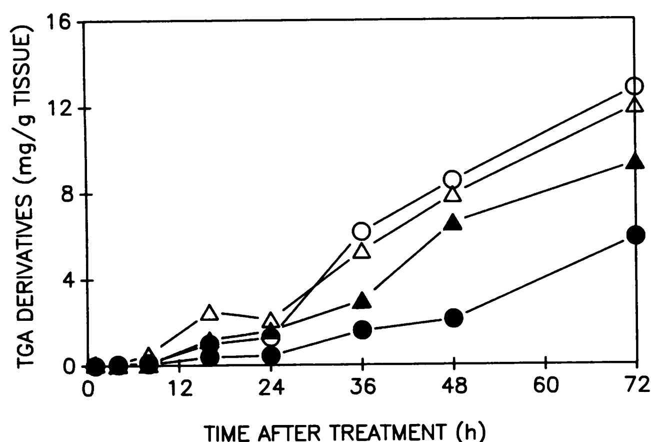

Figure 5. Phenolic polymer deposition in upper cell layers of wounded and ABA-treated cotyledons. Cotyledons were treated with either water (○) or 10^{-4} M ABA (△) in the light or in the dark (●, ▲). Tissues were harvested and assayed as in Figure 2. TGA, thioglycolic acid.

Perhaps the most pronounced effect of ABA *per se* was the enhanced induction of the fastest moving peroxidase band in group 1 (Fig. 6). This effect was more obvious in the light than in the dark. Inasmuch as it did not correspond temporally to the accumulation of phenolic polymers in these tissues, however, it was not pursued further.

Spectral and Chemical Characterization of Phenolic Polymers

The spectrum of the purified preparative sample of the thioglycolic acid derivatives from elicitor-treated cotyledon tissues is shown as an insert to Figure 1.

Unlike the thioglycolic acid derivatives from castor bean cell cultures, which show a relatively simple spectrum with a single major absorbance maximum at 280 nm (4), the soybean derivatives showed only a slight shoulder at 280 nm. Their spectrum (Fig. 1) is more complex and is dominated by a very sharp maximum at 257 nm and a broader maximum at 335 nm. As with all phenolic polymers, this ultraviolet spectrum is undoubtedly a composite of the absorption bands of its individual constituents (12). The absorbance maxima of individual aromatic residues within phenolic polymers can vary markedly depending on the nature of their linkage in the polymer and the degree and nature of substitution on the aromatic ring (12). Thus, it is not possible to assign the absorbance maxima to specific residues on the basis of ultraviolet spectra alone.

To gain further preliminary information on the nature of the polymers elicited in soybean tissues, we employed a series of histochemical stains which have traditionally been used to differentiate lignin and suberin polymers. To complement these studies, we also quantified the phenolic acids present in simple ester linkages to wall components and the individual aldehydes obtained after nitrobenzene and copper oxide oxidations of the intact polymers. In addition to confirming the phenylpropanoid nature of the wall-bound phenolics, these chemical quantitations allowed us to obtain an estimate of the specific phenylpropanoid residues present and the extent of their incorporation.

Positive histochemical staining of the phenolic polymers induced by PMG wall glucan in light-incubated cotyledons was seen as early as 4 h after treatment. At this time, both Nile Blue (which reacts with the aliphatic side chains of suberin) and phloroglucinol (which reacts with free aldehyde groups in the phenolic polymers) stained the walls of cells in the uppermost cell layer of treated cotyledons, but not of wounded control cotyledons. Within 8 h, phloroglucinol no longer stained the cell walls of surface cells, but the Nile Blue reaction continued to increase in intensity until 24 h. By 24 h, the first two cell layers in elicitor-treated cotyledons stained intensely with Nile Blue, while the uppermost cell layer of wounded cotyledons was just beginning to stain. These results suggest that at least some of the polymeric substances depos-

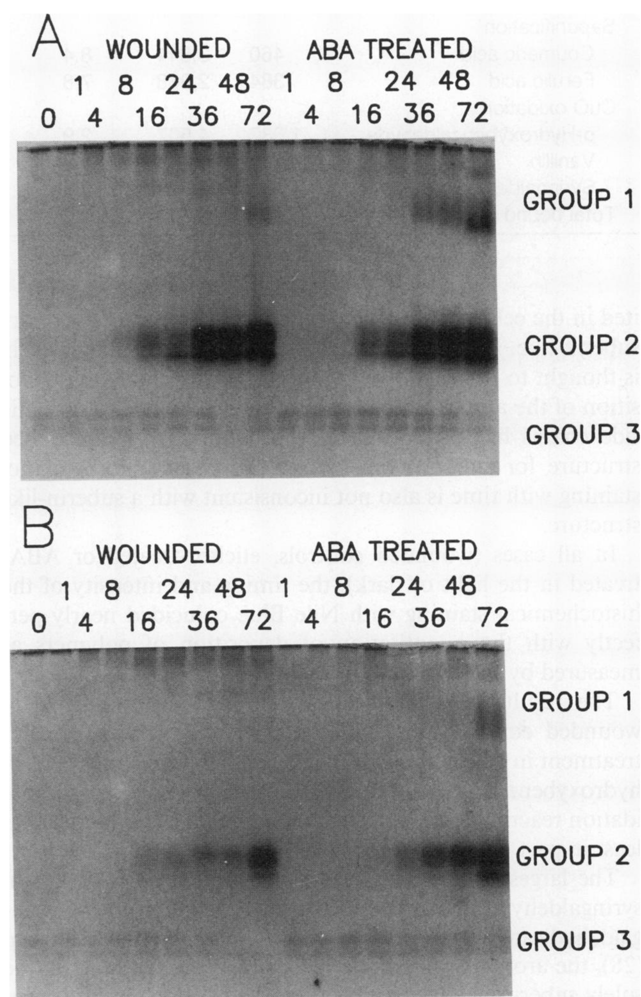

Figure 6. Anionic peroxidase isozymes induced in upper cell layers of wounded or ABA treated cotyledons. Tissues were treated as in Figure 5. Treated cotyledons were incubated in the light (A) or dark (B). Sections were extracted and subjected to electrophoresis as described in the text. Lane 1 is from freshly harvested cotyledon tissue. Lanes represent the same time points as described in Figure 3.

Table I. *Residues Released from Cell Walls following Saponification or Oxidative Cleavage*

Upper cell layers of light incubated wounded control and PMG wall glucan (30 μg/mL) treated cotyledons (20 replicates/treatment) were harvested at 24 h. After extraction as described in "Materials and Methods," the residue was saponified in cold alkali to release esterified phenolic acids. The residue from the saponification was then divided into equal aliquots which were oxidized in alkaline CuO or nitrobenzene at 130°C to cleave the phenolic polymers and release constituent aldehydes. Phenolic acids and aldehydes were quantitated by HPLC after extraction of the reaction mixtures with ethyl acetate. Concentrations of reaction products are given in nmol/g fresh weight tissue. Results from nitrobenzene oxidation (not shown) were similar to those for CuO oxidation. Similar results were obtained in two separate experiments.

Reaction and Product	Residues Recovered		Ratio of PMG to Control
	Control	PMG	
	nmol/g fresh wt		
Saponification			
Coumaric acid	460	3,844	8.4
Ferulic acid	384	2,988	7.8
CuO oxidation			
p-Hydroxybenzaldehyde	1,532	4,507	2.9
Vanillin	704	2,689	3.8
Syringaldehyde	280	2,778	9.9
Total bound phenylpropanoids	3,360	16,806	5.0

ited in the cell walls in response to wounding and elicitor are suberin-like. During suberin deposition, the phenolic matrix is thought to be laid down before or concurrently with deposition of the aliphatic side chains (26, 27). Since the aliphatic side chains block free aldehyde functions in the proposed structure for suberin, the loss of the weak phloroglucinol staining with time is also not inconsistent with a suberin-like structure.

In all cases (wounded controls, elicitor-treated or ABA-treated in the light or dark), the timing and intensity of the histochemical staining with Nile Blue coincided nearly perfectly with the quantitation of deposition of polymers as measured by the thioglycolic acid procedure (Figs. 2 and 5).

The results of saponification and oxidative cleavage of wounded control and PMG induced polymers 24 h after treatment in the light are given in Table I. The presence of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde in the oxidation reactions confirmed that the phenolic polymers are at least in part phenylpropanoid derived.

The largest induced change in constituent aldehydes is in syringaldehyde (Table I). Since substantial amounts of syringaldehyde are normally associated with lignin and not suberin (28), the aromatic polymers which are deposited may not be solely suberin-like, as suggested by their histochemical staining. It is possible that the polymers deposited in response to PMG wall glucan are mixed in nature and that the distinct lack of phloroglucinol staining after 4 h is due to the hydrophobic nature of the aliphatic domains of suberin which preclude effective staining by this more hydrophilic reagent.

The presence of relatively large amounts of simple esterified phenolic acids (ferulic and coumaric, Table I) in the wall preparations is interesting. It suggests that the deposition of phenolic polymers, as measured by the thioglycolic acid procedure (Fig. 2) or the quantitation of their constituent aldehydes, is accompanied by the incorporation of phenolic monomers.

Changes in Soluble Phenylpropanoid-Derived Metabolites Relative to the Deposition of Wall-Bound Phenolics

As noted in "Materials and Methods," the phenolic polymer analyses and the chemical characterization of the phenolic polymer constituent aldehydes were carried out on the residue from the same samples analyzed by HPLC for soluble aromatics. This allowed us to examine the relative flow of phenylpropane units into the various alternative phenylpropanoid-derived end products with time.

The changes in isoflavone conjugate and glyceollin levels for the same samples analyzed in the experiments presented in Figure 2 are shown in Figure 7. The timing and magnitude of the responses were similar to those reported previously (16). For simplicity, we show these data only for the upper sections of PMG wall glucan-treated cotyledons in the light. Total wall-bound phenylpropanoids (esterified phenolic acids and constituent aldehydes) are also shown in Figure 7. This allows a direct molar comparison of the total accumulations into the soluble and insoluble phenylpropanoid-derived pools. It should be pointed out that neither saponification nor oxidative cleavage would be expected to give 100% yield of the monomeric constituents in the wall (34). Thus, the levels of total wall-bound phenylpropanoids shown in Figure 7 is a conservative estimate.

The results of Figure 7 underscore the fact that the commitment of phenylpropanoid precursors to wall polymers and esterified phenolic acids is a major and early event. The accumulation of wall-bound phenolics began within 4 h and was nearly saturated by 24 h when it amounted to several times the later peak accumulations of the isoflavone conju-

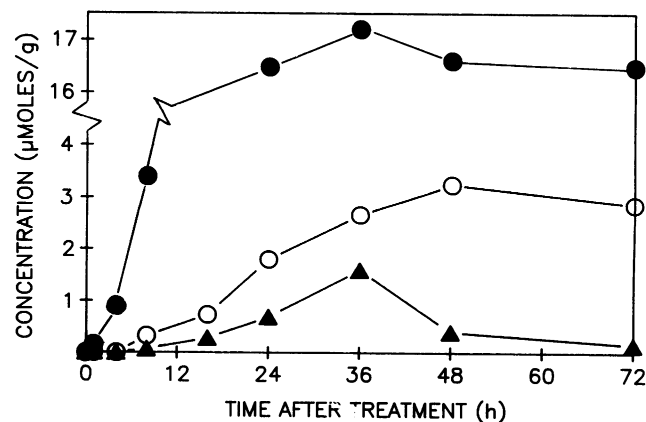

Figure 7. Isoflavonoid accumulation in the upper cell layers of elicitor-treated cotyledons in the light. HPLC was used to quantitate total daidzein conjugates (○), glyceollin (▲), and total wall bound phenolic aldehydes and esters (●) in the same tissues harvested for the data in Figure 2A.

gates and glyceollin combined. The first increases in soluble metabolites of PMG wall glucan-treated tissues were seen in the isoflavone conjugates; increases in these pools were apparent by 8 h and continued until 48 h. Glyceollin accumulation was a less rapid and a more transient response, beginning only after 12 h and actually falling off after 36 h.

DISCUSSION

As reported above, wounding of soybean cotyledon tissues leads to the induction of anionic peroxidases and to the gradual deposition of cell wall phenolic polymers, particularly in cell layers immediately below the wound surface. Although various peroxidases are induced by wounding, among them three specific anionic bands of intermediate electrophoretic mobility are strongly and coordinately induced and correlate very well in timing and amplitude to the deposition of phenolic polymers. Both the magnitude of induction of these isozymes and phenolic polymer deposition are substantially higher in light-incubated cotyledons.

PMG wall glucan markedly enhances both of these wound-induced responses. The rate of induction of the anionic peroxidases which correlate with phenolic polymer deposition in wounded tissues is significantly increased by the elicitor. In the light, these isozymes are detectable as early as 4 h after elicitor treatment. This is consistent with the much earlier deposition of phenolic polymers in elicitor-treated tissues (also detectable within 4 h both histochemically and by measurement with the thioglycolic acid procedure).

An array of histochemical stains initially suggested that the polymers possess strongly staining lipid domains and few free aldehyde groups, suggesting that the polymers were more suberin than lignin-like. However, the more reliable oxidative cleavage of the polymers led to the release of even larger amounts of syringaldehyde (considered to be more characteristic of lignin) than *p*-hydroxybenzaldehyde and vanillin (found in both polymers). It is likely that the polymers deposited have characteristics of both suberin and lignin. It is quite possible that the lipid components of the suberin-like polymers preclude effective staining by the more hydrophilic stains used to detect lignin.

The complex and heterogeneous nature of the wall-bound phenolics deposited in response to wounding or PMG wall glucan treatment is further supported by the presence of substantial quantities of the simple phenylpropanoic acids, coumaric and ferulic, in ester linkages to wall components. Esterified coumaric and ferulic acids are a common feature of angiosperm cell walls, where they have been shown to be linked to lignin, suberin, and carbohydrate polymers (11, 39). Although they have been proposed to function in cross-linking of various wall polymers (39), little direct evidence for this exists. Since they accumulate in response to infection in some species, they have also been suggested to play a potential role in disease resistance (19). Although the accumulation of coumaric and ferulic acid derivatives is an early step in the accumulation of the oxidized phenolic polymers, their esterification in the wall does not involve the action of peroxidases. The relative role, if any, of these esters in soybean disease resistance will require further study.

Preliminary evidence suggests that the coordinately induced peroxidase bands, which correlate tightly in all experiments with phenolic polymer deposition, are likely wall-bound and thus could participate in polymer deposition. However, we do not know if they are products of different genes or are the result of post-translational modifications (*e.g.* glycosylation) of the same protein. Moreover, our use of the term "induction" simply refers to an increase in the stainable activities of these bands. We currently do not know the level of regulation at the molecular level. This is of obvious interest for further study.

The induction of peroxidases in soybean cotyledons forms a decreasing gradient from upper to lower cell layers. Although the deposition of phenolic polymers correlates very well with the accumulation of the peroxidases in upper cell layers, there is little phenolic polymer deposition in lower cell layers despite the induction of moderately high levels of peroxidases. It may be that peroxidase is not the limiting factor in these cells. Two additional factors which could limit phenolic polymer deposition in the lower cells are a lack of suitable phenylpropanoid precursors or the relatively low oxygen potential (and thus peroxide availability) of these tissues. Further research will be needed to clarify this issue.

In previous work (16), we demonstrated that the PMG wall glucan elicitor induces the accumulation of glyceollin in cells proximal to the site of elicitor treatment and an even more massive buildup of isoflavone conjugates in proximal and distal cell populations. Both of these responses, however, are delayed and of lower magnitude when compared to the phenolic polymer deposition. The rapid deposition of phenolic polymers induced by this elicitor in proximal cell populations thus adds an important new perspective to our understanding of its action. We are currently investigating the molecular nature of the differential regulation of these alternative molecular and cellular responses.

Although ABA has been implicated in the induction of suberin-specific anionic peroxidases in solanaceous hosts (6, 30, 33), the role of ABA, if any, in these responses in soybean is unclear. Treatment with ABA alone had only subtle effects above the wound response on phenolic polymer deposition or on the central group of anionic peroxidases which correlate with polymer deposition. Under conditions where wounding was minimized (uptake of ABA through the petiole of excised cotyledons), no peroxidase or phenolic polymer accumulation was seen. Our results, then, do not support a positive role for ABA in induced resistance in soybean. Consistent with this, Ward and co-workers have reported that elicitor treatment or PMG infection of soybean tissues actually causes a rapid decrease in ABA levels (5) and that ABA may actually suppress phenylalanine ammonia-lyase induction in soybean (37).

Our results differ in several important ways from those reported previously for soybean cell cultures. Farmer (10) concluded that PMG wall glucan elicitor actually suppressed the deposition of lignin in soybean cell cultures, despite the fact that the elicitor triggered an earlier increase in phenylalanine ammonia-lyase. Importantly, in these studies, lignin was measured only indirectly by its reaction with phloroglucinol, which reacts with free aldehyde groups. As noted above, the phloroglucinol staining of the phenolic polymers deposited in our studies decreased rapidly with time, suggesting either a

lack of free aldehydes or an inaccessibility of the polymers to this relatively hydrophilic stain. Although the presence of vanillin in nitrobenzene oxidations of the control lignin in cell cultures was reported by Farmer (10), these measurements were not reported for the PMG wall glucan treatments.

Our results are also different in several respects from those of Bruce and West (4) with castor bean. First of all, the responses we have characterized are triggered by the β-1,3/β-1,6 cell wall glucan preparation from PMG rather than by oligogalacturonides. We have not examined this latter class of elicitor for it effects on peroxidase or phenolic polymer deposition. Secondly, in soybean tissues treated with the PMG wall glucan, it is the anionic, and not the cationic, peroxidases which respond to elicitor and correlate with phenolic polymer deposition. Finally, the phenolic polymers deposited in PMG wall glucan-treated soybean tissues are considerably more complex than the simple lignin-like polymers reported in cultured cells of castor bean.

In preliminary studies, we have also investigated the deposition of phenolic polymers in several soybean tissues in response to infection by both PMG and *Pseudomonas syringae* pv. *glycinea* (TL Graham, unpublished results). The results of these studies suggest that the deposition of the phenolic polymers may also be a rapid and specific response to incompatible infection. Thus, it is possible that phenolic polymer deposition may play an important early role in the hypersensitive containment of the incompatible pathogen which is complementary to the later accumulations of soluble antibiotic phenylpropanoid metabolites.

ACKNOWLEDGMENT

The authors are grateful to Dr. Pappachan Kolattukudy for his enthusiasm, encouragement, and for coordination of the Midwest Plant Biotechnology Consortium projects and funds under which part of this work was performed.

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