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Glyceollin Elicitors Induce Major but Distinctly Different Shifts in Isoflavonoid Metabolism in Proximal and Distal Soybean Cell Populations

T. L. Graham and M. Y. Graham

Department of Plant Pathology, The Ohio State University, Columbus 43210 U.S.A.
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Biotic and abiotic glyceollin elicitors were examined for their effects on isoflavonoid metabolism in discrete soybean cotyledon cell populations proximal and distal to the point of elicitor application. *Phytophthora megasperma* f. sp. *glycinea* wall glucan elicited glyceollin to levels as high as 1,800 nmoles/g of tissue, but only in the uppermost cell layers of treated cotyledons. In these same cell layers, *P. m.* f. sp. *glycinea* wall glucan elicited even larger accumulations (>4,000 nmoles/g of tissue) of conjugates of the glyceollin precursor daidzein and the related isoflavone genistein. In underlying cell populations, 5-20 cells away from the point of elicitor application, *P. m.* f. sp. *glycinea* wall glucan induced accumulations of daidzein and genistein conjugates as high as 9,000 nmoles/g of tissue, but no glyceollin. Accumulation of the isoflavone conjugates, but not of glyceollin, was highly light-dependent in all sections. Glyceollin accumulation induced in the upper cells by abiotic elicitors was accompanied by large decreases in existing daidzein and genistein conjugates, suggesting that abiotic elicitors may act, in part, by releasing the precursor daidzein from preexisting pools. Although abiotic elicitors also caused a buildup of isoflavone conjugates in underlying cell layers, the response was far less dramatic when compared with the biotic elicitors.

Additional keywords: legume, *Glycine max*, disease resistance, flavonoids.

Phytoalexins are low molecular weight antibiotics that accumulate in plant tissues in response to infection. They are thought to play an important role in the restriction of pathogen growth in resistant host tissues. The phytoalexins that accumulate in soybean tissues, the glyceollins, are pterocarpans derived from the isoflavonoid precursor daidzein (Ebel 1986).

The glyceollins accumulate in soybean tissues in response to a number of stimuli other than pathogen attack. These include physical stimuli, such as ultraviolet light (Bridge and Klarman 1973), abiotic chemical elicitors, such as mercuric chloride and silver nitrate (Yoshikawa 1978), and biotic elicitors of both host and pathogen origin (Darvill and Albersheim 1984). The biotic elicitors, which may be responsible for glyceollin elicitation in infected tissues, include $\beta 1 \rightarrow 3, \beta 1 \rightarrow 6$-linked cell wall glucans from *Phytophthora megasperma* Drechs. f. sp. *glycinea* (Hildeb.) Kuan & Erwin (Ayers *et al.* 1976b; Sharp *et al.* 1984) and α-1,4-D-galacturonides from the plant cell wall with an optimal size of 12 residues (Nothnagel *et al.* 1983). A synergistic interaction between the biotic elicitors of host and pathogen origin has been reported (Davis *et al.* 1986).

We have developed, in our laboratory, a highly sensitive HPLC profiling method that allows us to simultaneously monitor a broad range of soluble aromatic soybean metabolites (Graham *et al.* 1982; T. L. Graham, unpublished). The technique has been particularly useful in monitoring induced alterations in the flavonoid and isoflavonoid pathways (Graham 1988). Using these profiles, we were able to show the presence of previously unreported constitutive conjugates of the isoflavones daidzein and genistein in soybean seedling tissues and their potential role in glyceollin accumulation in infected cotyledon tissues (Graham *et al.* 1990). The possible metabolic relationships of these conjugates, their free aglycones, and glyceollin are summarized in Figure 1 (Ebel 1986; Welle and Grisebach 1989; Graham *et al.* 1990). We have shown (Graham *et al.* 1990) that the preexisting conjugates of both daidzein and genistein are rapidly hydrolyzed in cotyledon tissues infected with *P. m.* f. sp. *glycinea* and large quantities of free daidzein and genistein are released. The free daidzein may play a role as a precursor in the subsequent accumulation of the glyceollins (Graham *et al.* 1990), whereas free genistein is directly toxic to *P. m.* f. sp. *glycinea* (Graham 1989).

In this study, we have further employed these same HPLC profiling methods to examine the effects of biotic and abiotic glyceollin elicitors on the various metabolic alternatives summarized in Figure 1. Of particular importance is that we have taken advantage of the remarkable sensitivity of the HPLC profiling protocol (as little as 100 fmoles of a given metabolite can be measured) to examine the responses of discrete cell populations to elicitor application. This approach has allowed us to show that cell populations proximal and distal to the point of elicitor application respond very differently to elicitor treatment. Although glyceollin accumulates only in the uppermost cell layers of treated tissues, conjugates of the isoflavones daidzein and genistein accumulate to levels as high as 20 times those of glyceollin in underlying cell layers. This massive buildup of isoflavone conjugates in cells distal to the point of elicitor application is intriguing and may relate to previously observed long-term induced disease resistance of these tissues.

MATERIALS AND METHODS

Chemicals. The *P. m.* f. sp. *glycinea* cell wall preparation was prepared from race 1 of *P. m.* f. sp. *glycinea* according to Ayers *et al.* (1976a). Its composition, as determined by elemental, protein, lipid, and sugar analyses, was essentially the same as reported by Ayers *et al.* (1976b). The elicitor for application to the cotyledons was prepared according to Ayers *et al.* (1976a) by autoclaving the wall fragments for 3 hr in deionized double-distilled water.

All other chemicals were reagent grade and obtained from Sigma Chemical Co., St. Louis, MO.

Cotyledon elicitor assay. We chose to use cotyledon tissues for our elicitor studies for several reasons. First of all, this tissue is readily available in quantity and is easily manipulated. Second, it has been one of the classical tissues used for elicitor studies (Frank and Paxton 1971; Ayers *et al.* 1976a; Partridge and Keen 1977). Perhaps most important for our current studies, however, is the fact that soybean cotyledons have a relatively simple cellular structure. Other than the epidermis and a few major vascular elements, they are made up nearly completely of aligned columns of tightly packed mesophyll parenchyma cells (Hadley and Hymowitz 1973). The individual cells are remarkably homogeneous throughout the organ and average 150 × 50 μm. This simple cellular architecture offered us distinct advantages in the sampling of tissues and in the subsequent interpretation of local and distal cellular responses to a localized elicitor treatment.

Certified soybean cultivar Williams (or Williams 79) seed were obtained from Countrymark, Delaware, OH. Seedlings were grown as reported previously (Graham *et al.* 1990). Unblemished cotyledons were harvested from 9- or 10-day-old seedlings unless otherwise noted. At this time the unifoliate leaf had emerged, but was only partially expanded. The lower surface of each cotyledon was individually surface-sterilized by wiping briefly with an alcohol swab (Becton-Dickinson & Co., Franklin Lakes, NJ), which was first washed in 70% ethanol and wrung out until it was moist, but not dripping. The cut cotyledon assay was then performed according to Ayers *et al.* (1976a). Elicitors were applied in double-distilled deionized water as 30-μl droplets to the cut cotyledon surface. Treated cotyledons were incubated in petri plates containing moistened filter paper in the dark or at 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. Ten cotyledons were incubated per petri plate, and two replicate plates were assayed per treatment. Although the droplets applied to the surfaces of most cotyledons had evaporated by 24 hr, the cut cotyledon surface remained moist for several days. If droplets remained on the cotyledons at the time of harvest, they were dried before harvest by placing the open petri plates in a laminar flow of air in a sterile transfer hood.

Tissues were harvested from the cotyledons as diagrammed in Figure 2. Cylindrical plugs of tissue were

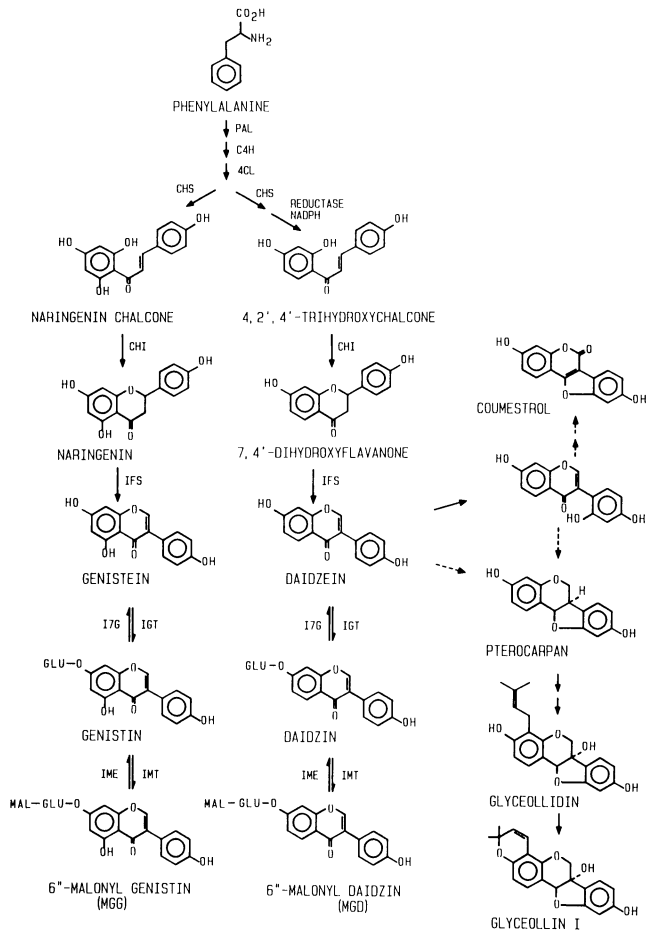

Fig. 1. Formation and alternative metabolic fates of isoflavones in soybean. Abbreviations: GLU, β-D-glucosyl; MAL, 6″-malonyl; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, "isoflavone synthase;" IGT, isoflavone 7-O-glucosyltransferase; IMT, isoflavone 7-O-glucoside-6″-malonyltransferase; IME, isoflavone 7-O-glucoside-6″-malonate malonylesterase; and I7G, isoflavone 7-O-glucoside β-glucosidase. The enzymes designated IGT, IMT, IME, and I7G have not been characterized in soybean. Their existence is hypothesized based on parallel enzyme activities that have been described for closely related conjugates of formononetin and biochanin A in chickpea (Hösel and Barz 1975; Köster *et al.* 1983; Hinderer *et al.* 1986).

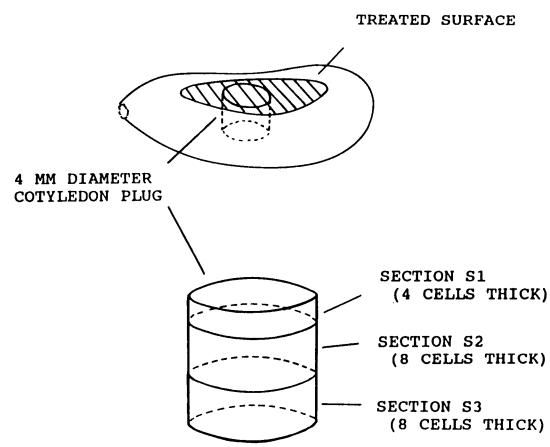

Fig. 2. Protocol for harvest of discrete cell layers from elicitor-treated soybean cotyledons for HPLC analysis. A vertical column of cells was harvested from the individual inverted cotyledons using a No. 1 cork borer (4-mm-i.d.) centered on the treated surface. In some experiments, the intact column of cells was extracted for HPLC analysis. Alternatively, the column of cells was carefully sliced into cross sections that averaged in thickness as follows: S1, the uppermost 0.6 mm of tissue, including the treated surface; S2, the next 1.2 mm of tissue; and S3, the final 1.2 mm of tissue containing the upper epidermis of the cotyledon.

removed from the center of the treated surface of each cotyledon using a No. 1 cork borer (4-mm-i.d.). The use of tissue plugs for analysis ensured that each replicate represented a treated surface area of equal size. In some experiments the entire tissue plug was extracted for analysis. In those experiments in which we examined proximal and distal effects of the elicitors, the tissue plugs were carefully sliced into three sections, each progressively farther from the cut surface as shown in Figure 2. The first section, S1, represented the treated surface and the first four cell layers from the surface. Sections S2 and S3 were cross sections containing progressively underlying tissues. Each was an average of eight cell layers thick.

The tissue plugs or sections were pooled for a given replicate, extracted, and subjected to HPLC as described previously (Graham *et al.* 1990). Briefly, HPLC was performed at 25° C on a 4.6 mm i.d. × 250 mm Hibar Ec Cartridge containing Merck Licrosorb RP-18 10 μm C18 reverse-phase packing (Alltech Associates, Deerfield, IL). The column was eluted at 1.5 ml/min with a linear gradient of 0–55% acetonitrile in water, pH 3.0, for 25 min followed by a 2-min wash with 100% acetonitrile and a return to water. Under these conditions the various glyceollin isomers elute as a single peak at 25.8 min.

RESULTS

Effects of *P. m.* f. sp. *glycinea* wall glucan on isoflavone and glyceollin accumulation. A representative HPLC profile of the effects of *P. m.* f. sp. *glycinea* wall glucan on soybean cotyledon tissues is shown in Figure 3B. In addition to the accumulation of glyceollin, effects on a number of other aromatic metabolites are apparent. These are summarized in Table 1 at various *P. m.* f. sp. *glycinea* wall glucan concentrations, along with the identities of the induced metabolites where known. As illustrated in Table 1, the major changes induced by the wall glucan are the accumulations of glyceollin and the malonylated conjugates of daidzein and genistein. (The data in Fig. 3B and Table 1 represent values obtained from extraction of the entire cotyledon plug [Fig. 2] 48 hr after treatment.)

At 10 μg/ml of *P. m.* f. sp. *glycinea* wall glucan, the amount of glyceollin accumulating in tissues incubated in the light (Table 1) represents only 8.7% of the newly formed 5-deoxyisoflavonoids (including only daidzein, its conjugates, coumestrol, and glyceollin) and only 6.4% of the total newly accumulated isoflavonoids (including genistein, daidzein, their conjugates, coumestrol, and glyceollin). At higher *P. m.* f. sp. *glycinea* wall glucan levels, the amount of glyceollin elicited relative to total 5-deoxyisoflavonoids increases up to 20%, suggesting that glyceollin accumulation is somewhat more favored over conjugate formation at higher wall glucan concentrations.

The data of Table 1 also illustrate the different responses to *P. m.* f. sp. *glycinea* wall glucan when cotyledon tissues are incubated after treatment in constant light or constant darkness. In cotyledons incubated in the dark, the increase of total isoflavonoids is much less than that in the light. This effect is most pronounced for the daidzein and genistein conjugates; their accumulation in the dark is an order of magnitude less than that in the light. In contrast, the accumulation of glyceollin in the dark is still one-half that in the light.

Since these experiments measure steady state levels of the various compounds, we cannot define the precise effects of *P. m.* f. sp. *glycinea* wall glucan on each separate event

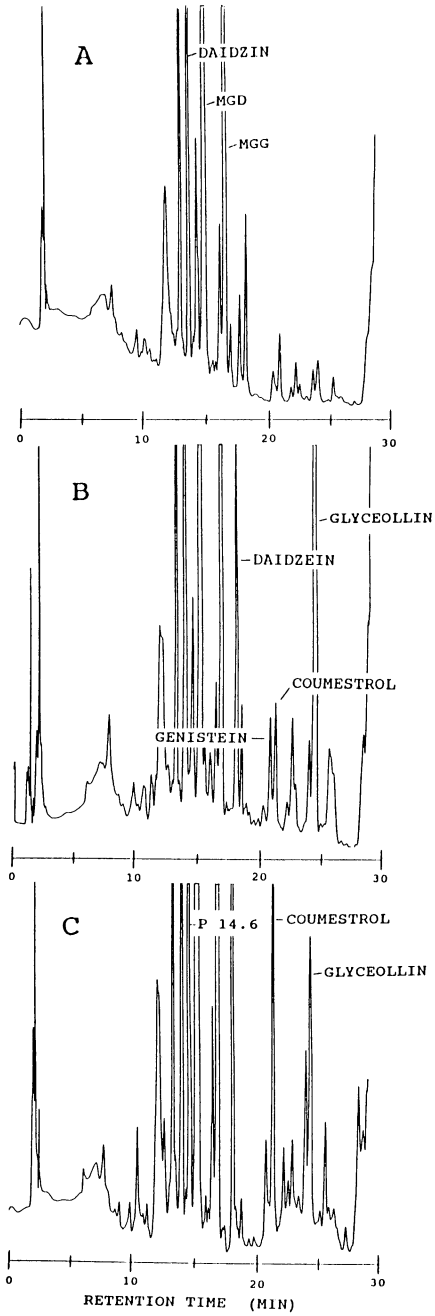

Fig. 3. HPLC profiles of elicitor-treated soybean cotyledon tissues. The cut cotyledon assay was performed as described in the text, with 20 replicate cotyledons per treatment. The entire column of cells, as described in Figure 2, was harvested for each replicate, and the pooled replicate cell columns were extracted and subjected to HPLC as described in the text. Treatments were wounded control (**A**), 50 μg/ml of *Phytophthora megasperma* f. sp. *glycinea* wall glucan (**B**), and 1 mM silver nitrate (**C**). Note that these data were selected as representative to emphasize qualitative differences in the profiles, particularly of the metabolites other than the isoflavone conjugates. Quantitative values are given for a wider range of treatments in Tables 1 and 2.

Table 1. Dose response of soybean cultivar Williams cotyledons to *Phytophthora megasperma* f. sp. *glycinea* wall glucan in the light and dark

		Increase in metabolite concentration[a]							
		Fungal wall concentration (μg/ml)							
Condition	**Metabolite**	**0**	**5**	**10**	**20**	**40**	**60**	**80**	**100**
Light	Daidzein	(15)	2	11	32	59	78	93	100
	Daidzin	(122)	19	80	203	317	382	389	402
	MGD[b]	(1,112)	206	918	2,498	3,803	4,000	4,105	4,303
	Genistein	(0)	0	2	8	20	28	47	59
	MGG[c]	(2,150)	101	404	797	1,490	1,701	1,796	1,802
	Coumestrol	(3)	1	9	32	51	60	73	85
	Glyceollin	(6)	18	97	398	900	1,041	1,163	1,213
	Glyceollin (%)[d]	···	7.3	8.7	12.6	17.5	18.7	20.0	19.9
Dark	Daidzein	(12)	0	5	13	30	44	52	61
	Daidzin	(108)	2	9	38	57	80	93	102
	MGD	(997)	5	20	122	148	188	213	221
	Genistein	(0)	0	0	5	8	20	28	42
	MGG	(2,015)	6	10	42	89	122	153	160
	Coumestrol	(2)	2	4	9	23	36	40	47
	Glyceollin	(0)	4	19	150	318	489	622	653
	Glyceollin (%)	···	30.7	33.3	45.2	55.2	58.4	61.0	60.2

[a]Concentrations are in nanomoles per gram (fresh weight) of tissue. Values at *P. m.* f. sp. *glycinea* wall glucan (0) are those present in control tissues; all other values are the increase above those control values.
[b]6″-Malonyldaidzin.
[c]6″-Malonylgenistin.
[d]The glyceollin (%) values are the percent that glyceollin represents the total 5-deoxyisoflavone response.

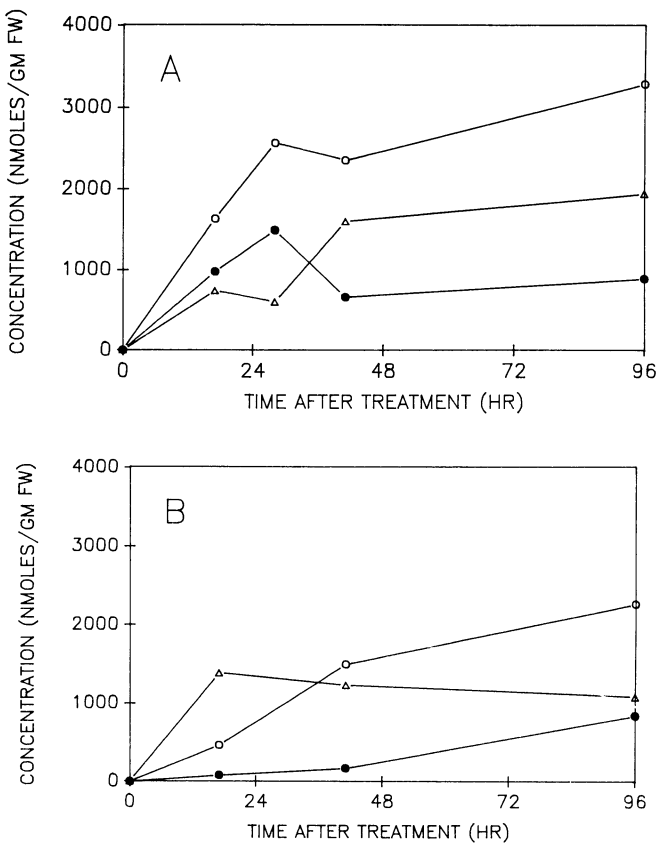

Fig. 4. Time course of response of uppermost cell layers of soybean cotyledon tissues to *Phytophthora megasperma* f. sp. *glycinea* wall glucan in the light and dark. Cotyledons were treated with *P. m.* f. sp. *glycinea* wall glucan (20 μg/ml). Treated tissues were then incubated in constant light (**A**) or constant darkness (**B**). At the indicated times after treatment, the uppermost cell layers, S1 (Fig. 2), were harvested and pooled for 20 replicate cotyledons for each data point. The levels of 6″-malonyldaidzin (○), 6″-malonylgenistin (●), and glyceollin (△) were determined by HPLC. The levels of the metabolites at time zero were subtracted from all data points. Similar results were obtained in a second experiment.

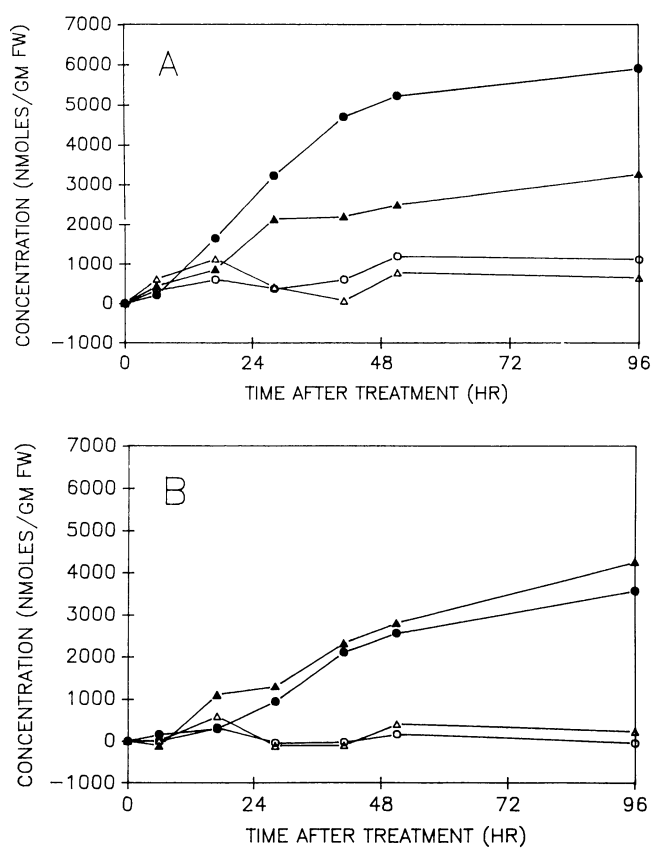

Fig. 5. Time course of accumulation of isoflavone conjugates in soybean cotyledon cell layers below the elicitor-treated surface. Cotyledons were treated with *Phytophthora megasperma* f. sp. *glycinea* wall glucan (20 μg/ml) and incubated in constant light or in constant darkness. At the indicated times after treatment, sections S2 (**A**) and S3 (**B**) were harvested as shown in Figure 2; individual sections from 20 replicate cotyledons were pooled for each data point. The levels of 6″-malonyldaidzin (●, light; ○, dark) and 6″-malonylgenistin (▲, light; △, dark) were determined by HPLC. The levels of the metabolites at time zero were subtracted from all data points. Similar results were obtained in a second experiment.

in Figure 1. The data in Figure 3 and Table 1 represent the steady state amounts of glyceollin and the other metabolites at or near their peak levels (48 hr). At later time points, free daidzein, free genistein, and glyceollin levels gradually decrease (data not shown). The isoflavone conjugates, however, do not show appreciable decreases with time, even after 8 days of incubation, suggesting that they represent more stable end products.

Although coumestrol also accumulates in tissues treated with *P. m.* f. sp. *glycinea* wall glucan (Fig. 3B and Table 1), this is a relatively minor response. The accumulations of the isoflavone genistein and its conjugates are also smaller relative to those of the 5-deoxyisoflavonoids.

Local and distal cellular responses to *P. m.* f. sp. *glycinea* wall glucan. The alternative formation of glyceollin and the daidzein conjugates could be explained if they were truly alternative metabolic fates of newly synthesized daidzein within a given cell or if they were accumulating differentially in separate cell populations. To begin to address the possibility of differential responses of separate populations of cells within the cotyledon tissues, we examined the effects of *P. m.* f. sp. *glycinea* wall glucan with time on metabolite profiles in different sections away from the treated surface (Fig. 2). The results, presented in Figures 4 and 5, and explained below in detail, show that the accumulation of glyceollin is highly localized to the uppermost cell layers, while the accumulation of the isoflavone conjugates also occurs to significant levels in underlying tissues.

Graphs A and B in Figure 4 illustrate the time course of response of section S1 (first four cell layers) to *P. m.* f. sp. *glycinea* wall glucan application when cotyledons were incubated in the light or in the dark. Consistent with the results of Table 1, the accumulation of total isoflavonoids is greater in the light (compare graphs A and B in Fig. 4). Once again, the lowered accumulation in the dark is much more pronounced for the daidzein and genistein conjugates than for glyceollin, particularly at the earlier time points after elicitor application (Fig. 4B).

Graphs A and B of Figure 5 illustrate the responses of underlying cell layers (sections S2 and S3) to treatment with *P. m.* f. sp. *glycinea* wall glucan from the same experiments. Since there was no significant glyceollin accumulation in these lower sections, we have shown only the data for the daidzein and genistein conjugates and have presented it somewhat differently to emphasize the differences between incubations in the light and dark.

In the light, daidzein conjugates accumulate in section S2 to levels as high as 6,000 nmoles/g of tissue (Fig. 5A). This is a sixfold increase over the constitutive levels of daidzein conjugates (1,020 nmoles/g of tissue) present in these tissues. Genistein conjugates also accumulate substantially in S2 in the light (up to 3,000 nmoles/g of tissue, Fig. 5A). Accumulations of daidzein and genistein conjugates occur in section S3 as well (Fig. 5B), although these accumulations are somewhat delayed compared to those in S2.

Although conjugates of both daidzein and genistein accumulate in the underlying tissues, the daidzein conjugates accumulate maximally in the middle section of the cotyledon (S2, Fig. 5A), and those of genistein accumulate maximally in the lowest section of the cotyledon (S3, Fig. 5B). This latter difference, though less dramatic than the strict spatial limitation in glyceollin accumulation, was also a reproducible result. It suggests that the accumulation of these alternative, but closely related isoflavones may also be subject to somewhat different cellular regulation.

Incubation of cotyledons treated with *P. m.* f. sp. *glycinea* wall glucan in the dark leads to dramatically lower accumulations of daidzein and genistein conjugates in all sections (Fig. 5, graphs A and B). This data, taken together with that of Table 1 and Figure 4, suggest that the accumulation of isoflavone conjugates is particularly influenced by light. Although light markedly enhances conjugate accumulation in all sections, the effect is most obvious in the underlying tissues.

Thus, as shown above, by sectioning cotyledon tissues according to the proximity to the treated surface and analyzing these sections separately, we uncovered differential responses of proximal and distal cell populations to *P. m.* f. sp. *glycinea* wall glucan. However, the choice of the sectioning protocol *a priori* was for operational reasons. Thus, we achieved an enrichment, but not a truly clean separation of different cell types in each of the sections. The fact that section S1 could still contain mixed cell populations may in part explain the more complex pattern of the data in graphs A and B of Figure 4.

Effects of abiotic elicitors on isoflavone conjugate and glyceollin levels. To evaluate the effects of abiotic elicitors on these same aspects of isoflavonoid metabolism, we chose a broad range of abiotic agents, including representatives of various elicitor classes. Included were ultraviolet light (Bridge and Klarman 1973), the heavy metal ions mercuric chloride and silver nitrate (Yoshikawa 1978), the detergent Triton X-100 (Keen and Bruegger 1977), and the sulfhydryl reagent iodoacetate (Ingham *et al.* 1981; Osman and Fett 1983). The molecular elicitors were applied at various levels from 10 μM to 1 mM. As noted by other researchers, relatively high concentrations were required for a response. We thus present results for comparative purposes for all elicitors at 1 mM. All of these elicitors have been reported to induce glyceollin and, in some cases, additional isoflavonoids.

Table 2 shows the effects of these abiotic elicitors on isoflavone conjugate and glyceollin pools in cell layer S1 and in pooled cell layers S2 and S3. Their effects are very different from the *P. m.* f. sp. *glycinea* wall glucan elicitor. Although glyceollin accumulation is apparent with all elicitors and net accumulations of total isoflavonoids (the total of daidzein and genistein and their conjugates, coumestrol, and glyceollin) are apparent with some of the elicitors, there is no net accumulation of isoflavone conjugates in surface tissues. Instead, although the extent varies, the abiotic elicitors as a class all cause marked decreases in isoflavone conjugate pools in the treated surface cells and in some cases the accumulation of relatively large quantities of free daidzein and genistein. Like *P. m.* f. sp. *glycinea* wall glucan, abiotic elicitors (with the exception of mercuric chloride) also induce the accumulation of isoflavone conjugates in untreated underlying tissues, although they are severalfold less effective than *P. m.* f. sp. *glycinea* wall glucan in this effect.

As shown in Figure 3C, the abiotic elicitors are also far less specific in their effects on aromatic metabolism. In addition to glyceollin, considerable quantities of coumestrol and of the unknown metabolite at 14.6 min are induced. Although coumestrol is also induced upon treatment with *P. m.* f. sp. *glycinea* wall glucan, it is only a minor response. We do not yet know the identity of the metabolite with a retention time of 14.6 min, but it also appears to be a flavonoid conjugate. It is not a conjugate of any of the known soybean flavonoids or isoflavonoids that we have examined (including daidzein, genistein, formononetin, biochanin A, quercetin, kaempferol, or isorhamnetin), and it is not the glucosyl conjugate of coumestrol reported by Le-Van (1984).

Isoflavonoid and glyceollin responses in other soybean cultivars. Since the response of soybean cultivars to *P. m.* f. sp. *glycinea* wall glucan has previously been reported to be neither cultivar- nor race-specific (Darvill and Albersheim 1984), we did not expect to see qualitative differences in the responses of various cultivars to this elicitor preparation. Above we have reported results for the soybean cultivar Williams. In each of the studies, we also examined the cultivar Williams 79. Although minor quantitative differences were seen, the responses of Williams 79 closely paralleled those for Williams in terms of all the major observations discussed above. Thus, the Williams 79 data are not shown due to their redundancy.

DISCUSSION

In earlier research (Graham *et al.* 1990), we reported the constitutive presence of multiple conjugates of the isoflavones daidzein and genistein in all soybean seedling tissues. Furthermore, we showed that the conjugates are hydrolyzed in both incompatible and compatible infected cotyledon tissues. However, the rate of release of free daidzein was more rapid in the incompatible infection, and the subsequent accumulation of glyceollin was both faster and of much higher magnitude in incompatible infections. This work suggested that the release of daidzein from preexisting conjugates, as outlined in Figure 1, might play a role complementary to that of *de novo* synthesis in providing precursors for glyceollin accumulation in infected soybean cotyledon tissues. Moreover, genistein, released from its conjugates over a time course similar to that of daidzein, may play a complementary role to glyceollin in antibiotic containment of *P. m.* f. sp. *glycinea* (Graham 1989).

In this study, we have examined several biotic and abiotic elicitors of glyceollin for their effects on the steady state levels of the various metabolites shown in Figure 1. We used a highly sensitive protocol that allowed us to separately examine the responses of discrete soybean cotyledon cell populations proximal and distal to the point of elicitor application.

We have shown that, in addition to glyceollin, *P. m.* f. sp. *glycinea* wall glucan induces a major accumulation of conjugates of the isoflavones daidzein and genistein in soybean cotyledon tissue incubated in the light. Although accumulations of glyceollin approaching 1,800 nmoles/g of tissue were seen, these responses were transient and strictly localized to the uppermost four cell layers of treated cotyledon tissues. Conjugates of daidzein and genistein accumulate in the same cell section to levels exceeding 4,000

Table 2. Effects of abiotic elicitors on soybean cultivar Williams metabolites

		Increase in metabolite concentration[a]					
		Elicitor[b]					
Section	Metabolite	CK[c]	UV	$AgNO_3$	$HgCl_2$	TX-100	Iodoacetate
S1	Daidzein	(294)	24	157	663	−37	−34
	Daidzin	(328)	−59	−276	−281	−261	−253
	MGD[d]	(2,895)	−1,530	−2,015	−2,319	−1,455	−2,184
	Genistein	(167)	−95	152	187	1,245	−18
	MGG[e]	(4,233)	−1,709	−3,317	−3,486	−2,945	−3,216
	P14.6[b]	(87)	366	4,422	1,727	2,323	191
	Coumestrol	(50)	78	352	4	409	−13
	Glyceollin	(32)	1,140	6,096	3,657	3,435	145
	Total Isf[f]	(7,999)	−1,785	1,149	−1,575	391	−5,573
S2 + S3	Daidzein	(41)	0	18	218	7	37
	Daidzin	(120)	99	211	−10	116	146
	MGD	(1,518)	811	1,437	−119	1,075	985
	Genistein	(27)	2	20	79	10	10
	MGG	(2,822)	357	348	−1,355	327	229
	P14.6[b]	(19)	39	163	1,238	136	112
	Coumestrol	(2)	5	14	59	6	3
	Glyceollin	(4)	11	69	1,041	26	−4
	Total Isf	(4,534)	1,285	2,117	−87	1,567	1,406

[a] Abiotic elicitors were tested at 1 mM except for Triton X-100 (TX-100), which was tested at 0.01%. UV exposure was for 30 min (660 microwatts per square centimeter at 254 nm). Tissues were harvested at 48 hr. Sections S2 and S3 were combined for these analyses.

[b] To allow better comparison to the other metabolites, values for the unknown P14.6 (see Fig. 3C) were calculated assuming an average integration response factor of 500,000 IU/nmole.

[c] CK stands for wounded control tissues. The values in parentheses represent constitutive levels of the metabolites in the control tissues. These values have been subtracted from values for treated tissues.

[d] 6″-Malonyldaidzin.

[e] 6″-Malonylgenistin.

[f] Total Isf is the total isoflavonoids.

nmoles/g of tissue. In underlying consecutive cell layers (each averaging eight cells thick), there is no accumulation of glyceollin, instead massive and metabolically more stable accumulations of daidzein and genistein conjugates occur. The total accumulations of the isoflavone conjugates in these lower sections range from 7,000–9,000 nmoles/g of tissue. When one considers that together these tissue layers are four times the weight of the uppermost layer, the overall accumulation of the isoflavone conjugates is more than 20 times that of glyceollin.

We do not yet know the basis of the differential responses of surface and underlying tissues to elicitor. It could be a reflection of heterogeneity in the *P. m.* f. sp. *glycinea* wall glucan elicitor preparation or in the signals generated after elicitor treatment. Alternatively, it could reflect physiological differences in the cells perceiving the elicitor signal(s).

Light is at least one parameter that dramatically affects the local and distal cell responses. Although the accumulation of glyceollin is relatively unaffected by incubation of treated cotyledons in the dark, the accumulation of the isoflavone conjugates is greatly diminished, particularly in the underlying tissues. Further investigations of this response, including the specific wavelengths required, are underway in our laboratory.

The massive buildup of isoflavone conjugate pools in tissues distal to the point of treatment with *P. m.* f. sp. *glycinea* wall glucan is intriguing. We have previously reported the induction of both short- and long-term protection against *P. m.* f. sp. *glycinea* infection by pretreatment of soybean cotyledon tissues with *P. m.* f. sp. *glycinea* wall glucan (Lundry *et al.* 1981; Lambert and Graham 1987). The short-term protection afforded by *P. m.* f. sp. *glycinea* wall glucan correlated well with a transient increase in glyceollin in the pretreated tissues. The second phase of protection (the long-term protection response), however, was effective for several weeks and did not correlate to prestimulated levels of glyceollin. One possibility is that the stable buildup of isoflavone conjugates in tissues treated with *P. m.* f. sp. *glycinea* wall glucan contributes to the long-term protection response by presensitizing these tissues to further attack by *P. m.* f. sp. *glycinea*. If this mechanism were operating, we would predict that *P. m.* f. sp. *glycinea* infection of these tissues would lead to a much greater release of genistein and daidzein and a subsequently more rapid or effective antibiotic containment.

Particularly intriguing to us is the fact that the massive buildup of isoflavone conjugates which we have described in this work occurs throughout the entire cotyledon within 48 hr. That is, the isoflavone buildup is a long-range as well as a long-term response. We are currently examining whether this response occurs in other soybean organs and whether it is systemically induced.

The relationship of these results with the *P. m.* f. sp. *glycinea* wall glucan elicitor to our previous results with infected cotyledon tissues is not yet clear. Of great importance, however, is that the major effect of *P. m.* f. sp. *glycinea* wall glucan is a net increase in isoflavonoids; it does not stimulate the net hydrolysis of isoflavone conjugates that is seen in tissues infected with *P. m.* f. sp. *glycinea* (Graham *et al.* 1990). This suggests that factors in addition to the *P. m.* f. sp. *glycinea* wall glucan must play important roles in the responses of these tissues to actual infection.

The abiotic elicitors show a very different mode of action than does *P. m.* f. sp. *glycinea* wall glucan. Although net accumulations of total isoflavones are also induced in response to some of the abiotic agents, a major net hydrolysis of the isoflavone conjugates occurs in response to all abiotic elicitors and the accumulation of glyceollin, though massive, is not a specific event. Both coumestrol and an unknown flavonoid conjugate accumulate to levels approaching, or in some experiments even exceeding that of glyceollin. In contrast to their effects on treated cells, the abiotic elicitors also trigger a small but positive net accumulation of isoflavone conjugates in underlying tissues. This distal buildup of isoflavones does not occur in wounded tissues, suggesting that it may be the result of a common secondary plant signal which is generated by both biotic and abiotic elicitors in a local elicitation event.

Relatively high concentrations (0.5–1 mM) of the abiotic elicitors are required for elicitation (Yoshikawa 1978; Keen and Bruegger 1977; Osman and Fett 1983). Moreover, in our hands, at concentrations where the abiotic elicitors are active, cellular toxicity is apparent in the surface tissues (data not shown). We hypothesize that some of the effects of the abiotic elicitors on surface cells may be due in part to cell damage. For example, if the tonoplasts were damaged, leakage and subsequent hydrolysis of the major pool of malonylated isoflavone conjugates could occur. The fact that the free isoflavone aglycones and coumestrol accumulate to such high levels suggests that the last reactions in the pathway to glyceollin may be either not totally effectively induced or are impaired. Taken together with the diverse and nonspecific effects of the abiotic elicitors, these considerations suggest that they may act through multiple and nonspecific cellular targets as opposed to the more specific and programmed cellular response to the *P. m.* f. sp. *glycinea* wall glucan.

Both the presence of the isoflavone conjugates and the starkly different local effects of biotic and abiotic elicitors on isoflavone conjugate pools may help to explain several previous anomalies in different laboratories regarding the biosynthetic mechanisms underlying abiotic and biotic elicitation of glyceollin (Yoshikawa 1978; Moesta and Grisebach 1981). We will address this question more fully in future studies describing radiolabel incorporation analyses.

As shown in Figure 1, coumestrol is also derived from daidzein (Dewick *et al.* 1970; Berlin *et al.* 1972; Dewick and Martin 1979; Martin and Dewick 1980). Coumestrol has been reported to accumulate in the hypersensitive response of soybean leaves to incompatible races of *Pseudomonas syringae* pv. *glycinea* (Coerper) Young *et al.* (Keen and Kennedy 1974) and has been reported to be toxic to various bacterial plant pathogens (for a more recent summary, see Fett and Osman 1982). Recently, we have found that coumestrol is also toxic to *P. m.* f. sp. *glycinea* growth *in vitro* (L. I. Rivera-Vargas and T. L. Graham, unpublished results), suggesting that it could potentially play a role complementary to that of glyceollin and genistein

in antibiotic containment of *P. m.* f. sp. *glycinea*. As discussed above, however, its induction is more pronounced in response to abiotic elicitors. Its role in infected tissues remains to be examined.

Chickpeas contain analogous 7-O-glucosyl and 6″-malonyl-7-O-glucosyl conjugates of the isoflavones formononetin and biochanin A (Köster *et al.* 1983), which differ respectively from daidzein and genistein only in the presence of a methoxy rather than a hydroxy group at the 4′ position. Kessmann and Barz (1986) examined the effects of fungal elicitor preparations from *Ascochyta rabiei* (Pass.) Lab. on these isoflavone conjugates and on pterocarpan phytoalexin accumulation in chickpea cotyledons. Our results with soybean cotyledon tissues differ in several important ways from those of Barz and co-workers. First of all, the isoflavone conjugates in soybean cotyledons are present in dry seed and do not increase appreciably upon germination (T. L. Graham, unpublished), whereas the chickpea isoflavone conjugates (those of biochanin A and formononetin) accumulate only after germination. Second, although both wounding and elicitor treatment induce the accumulation of the pterocarpan phytoalexins medicarpin and maackiain in chickpea, they do not induce an accumulation of isoflavone conjugates. In contrast, soybean cotyledon tissues respond to wounding with no accumulation of glyceollin, and elicitor causes a dramatic local and distal accumulation of isoflavone conjugates in addition to the local accumulation of glyceollin.

In conclusion, the results from our current studies on molecular elicitors of glyceollin complement our previous results from infected tissues in suggesting that complex molecular and cellular events may regulate the various metabolic alternatives outlined in Figure 1. The isoflavone conjugates appear to represent a dynamic reservoir that may be enlarged or depleted depending on the physiological status of a given cell population and the signals it perceives. The regulation of these processes may have important implications in terms of the response of soybean to its microbial associates.

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