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Role of Constitutive Isoflavone Conjugates in the Accumulation of Glyceollin in Soybean Infected with *Phytophthora megasperma*

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We report for the first time that the closely related isoflavones daidzein and genistein are present constitutively in large quantities as conjugates in all seedling organs of the soybean isolines Williams and Williams 79. The conjugates have been identified as the 7-O-glucosyl- and 6″-O-malonyl-7-O-glucosyl-isoflavones. Since daidzein is an immediate precursor of the pterocarpan phytoalexins, the glyceollins, we have examined the role of the daidzein conjugates in glyceollin accumulation in soybean tissues infected with *Phytophthora megasperma* f. sp. *glycinea*. The conjugates are present in both Williams isolines at levels far in excess of the amount required for glyceollin accumulation in infected tissues. During incompatible infections of cotyledon tissues (*P. m.* f. sp. *glycinea* race 1, Williams 79), the conjugates are rapidly hydrolyzed to free daidzein. High levels of glyceollin subsequently accumulate and infection is arrested within 48 hr. In compatible infections (*P. m.* f. sp. *glycinea* race 1, Williams), release of the free isoflavones is delayed; only low levels of glyceollin accumulate and then only 48 hr after the infection front has passed. These results suggest that 1) glyceollin biosynthesis may not be solely dependent on the induction of enzymes of early phenylpropanoid and flavonoid metabolism in this organ and 2) resistance to *P. m.* f. sp. *glycinea* race 1, as defined by the Rps_1^c gene in this organ, may reside partly in the rapid release of the isoflavone aglycones from their conjugates and/or in the later steps of glyceollin biosynthesis.

Additional keywords: glucosides, *Glycine max*.

The fungal pathogen *Phytophthora megasperma* Drechs. f. sp. *glycinea* (Hildeb.) Kuan & Erwin can infect all vegetative soybean organs so far examined (Sinclair 1982). It causes symptoms ranging from rapid preemergence and postemergence seedling damping off to slowly spreading lesions on older plant tissues. Genetic resistance to *P. m.* f. sp. *glycinea* in soybean exists in the form of major dominant *Rps* genes occurring at seven loci, with several allelic forms at two of these loci. There are at least 25 known races of *P. m.* f. sp. *glycinea* that are characterized by their different specific interactions with these *Rps* genes (Schmitthenner 1985). Where a given *Rps* gene provides effective resistance to a given race of *P. m.* f. sp. *glycinea*, the interaction between host and pathogen is termed incompatible. Where a given *Rps* gene is lacking or provides no resistance to a given race of *P. m.* f. sp. *glycinea*, the interaction is termed compatible.

Antibiotic pterocarpan phytoalexins have generally been accepted as playing a role in the *Rps* gene-mediated restriction of the spread of *P. m.* f. sp. *glycinea* in incompatible interactions (Keen and Yoshikawa 1982). Research to date has led to the characterization of four isomeric pterocarpan antibiotics now referred to as glyceollins I–IV (Sims *et al.* 1972; Burden and Bailey 1975; Lyne *et al.* 1976; Partridge and Keen 1977; Lyne and Mulheirn 1978). Both the timing and magnitude of the accumulation of the glyceollins differ markedly in compatible and incompatible infections and are consistent with the proposed role of the glyceollins in race-specific resistance (Keen and Yoshikawa 1982; Darvill and Albersheim 1984; Ebel 1986).

Several recent reviews (Hahlbrock and Grisebach 1979; Hahlbrock 1981; Ebel and Hahlbrock 1982; Ebel 1986) summarize much of our current knowledge on the steps leading to isoflavonoid and glyceollin biosynthesis. Synthesis of the glyceollins begins in the aromatic amino acid pathway. Through the action of enzymes including phenylalanine ammonia-lyase (PAL), chalcone synthase (CS), chalcone isomerase (CI), and a newly characterized reductase involved in the biosynthesis of 6′-deoxychalcone (Welle and Grisebach 1989), the flavanones naringenin and its 5-deoxy derivative are formed from phenylalanine and three molecules of malonyl-CoA. *In vitro* data suggest that the isoflavone genistein is formed from naringenin through a two-step 2,3-aryl migration (Hagmann and Grisebach 1984; Kochs and Grisebach 1986). Presumably, daidzein is also formed directly by a similar aryl migration from the corresponding 5-deoxyflavanone. Since the glyceollins lack the hydroxyl group on the A-ring at position 5, daidzein rather than genistein is thought to be the immediate precursor of the glyceollins (Ebel 1986).

Research on the later steps in glyceollin biosynthesis, from the isoflavone precursor daidzein, includes initial work on three enzyme activities: pterocarpan 6a-hydroxylase (Hagmann *et al.* 1984), dimethylallylpyrophosphate: 3,6a,9-trihydroxypterocarpan dimethylallyltransferase (Zahringer *et al.* 1979; Leube and Grisebach 1983), and the enzyme leading to cyclization of the prenylated pterocarpans to glyceollin isomers (Welle and Grisebach 1988).

Investigations on the regulation of isoflavonoid and glyceollin biosynthesis have focused primarily on the three specific enzymes of early phenylpropanoid and flavonoid metabolism noted above (PAL, CS, and CI). An early

research report suggested that increased activity of these enzymes may not be required for glyceollin accumulation in infected tissues (Partridge and Keen 1977). However, later studies, using direct assays of enzymatic activity (Borner and Grisebach 1982; Bonhoff *et al.* 1986a), radiolabel incorporation from early precursors (Zahringer *et al.* 1978; Yoshikawa *et al.* 1979; Moesta and Grisebach 1981), and messenger RNA measurements (Esnault *et al.* 1987; Schmelzer *et al.* 1984; Habereder *et al.* 1989), suggested that glyceollin biosynthesis is accompanied by increased transcription and activity of these enzymes and thus *de novo* synthesis of isoflavone precursors. Recently, race-specific induction of several of the later enzymes in glyceollin biosynthesis has also been reported in infected soybean roots (Bonhoff *et al.* 1986a; Bonhoff *et al.* 1986b).

In this study, we describe recent results from our laboratory on the race-specific interactions of *P. m.* f. sp. *glycinea* race 1 with the soybean cultivars Williams (with no known *Rps* resistance genes and susceptible to race 1) and Williams 79 (with the $Rps_1{}^c$ gene conferring resistance to race 1). We report the presence of large and previously unreported constitutive pools of conjugates of daidzein and the closely related metabolite genistein in all seedling tissues from these Williams isolines. Two conjugates of both daidzein and genistein have been characterized. They are present in all soybean seedling organs at levels far in excess of the amount required for the synthesis of the glyceollin that accumulates in infected tissues.

We show that during incompatible infections of cotyledon tissues (*P. m.* f. sp. *glycinea* race 1, Williams 79), the daidzein and genistein conjugates are rapidly hydrolyzed at the infection front to free daidzein and genistein. Subsequently, glyceollin accumulates to levels as high as 700 nmol per gram of tissue at that site, and complete containment of the pathogen occurs within 48 hr. In compatible infections (*P. m.* f. sp. *glycinea* race 1, Williams), release of the free isoflavones begins only 24 hr after the infection front has passed. Despite the eventual release of as much as 1,300 nmol of daidzein per gram of tissue, only low levels of glyceollin accumulate and then only 48 hr after the infection front has passed. As noted below, our results suggest that glyceollin accumulation in infected soybean cotyledon tissues carrying the $Rps_1{}^c$ gene may not be solely dependent on the *de novo* synthesis of daidzein. The results also suggest that resistance to *P. m.* f. sp. *glycinea* race 1, as defined by the $Rps_1{}^c$ gene in this organ, may in part reside in the rate of hydrolysis of the constitutive isoflavone conjugates and/or in the later steps in glyceollin biosynthesis.

MATERIALS AND METHODS

Chemicals. Daidzein and genistein were obtained from Atomergic Chemetals Corporation, Plainview, NY. A mixture of glyceollins I–III was purified by high pressure liquid chromatography (HPLC) using the conditions described below from infected seeds treated according to Keen (1975). The identity and purity of the glyceollins were confirmed by comparison of their ultraviolet and mass spectra and thin layer and high pressure liquid chromatographic behaviors to published values (Sims *et al.* 1972; Burden and Bailey 1975; Lyne *et al.* 1976; Partridge and Keen 1977; Lyne and Mulheirn 1978).

Growth of *P. m.* f. sp. *glycinea* cultures and soybean seedlings. *P. m.* f. sp. *glycinea* race 1 was obtained from A. F. Schmitthenner (The Ohio State University and the Ohio Agricultural Research and Development Center, Wooster). Stock cultures were maintained at 4° C on lima bean agar slants. Cultures of *P. m.* f. sp. *glycinea* for inoculum were obtained by transferring fresh agar plugs from the stocks to lima bean agar plates and allowing the *P. m.* f. sp. *glycinea* to grow at 25° C to the periphery of the plate (approximately 7 days). Zoospores were prepared according to F. W. Schwenk and C. D. Nickell (1984. Factors affecting production of *Phytophthora megasperma* f. sp. *glycinea* zoospores, *P. m.* f. sp. *glycinea* Workshop, Kemp Station, WI, September 1984). Zoospores were counted using a hemacytometer (Reichert Scientific Instruments, Buffalo, NY). Alternatively, 1-mm plugs of mycelia from *P. m.* f. sp. *glycinea* were taken from the periphery of 7-day-old agar plates and then used for inoculation as described below.

Certified Williams and Williams 79 soybean seeds were obtained from Countrymark, Delaware, OH. Seeds were sorted to obtain those that were uniform, undamaged, and disease-free. These were then surface-sterilized in 5% hypochlorite solution for 10 min and washed with deionized water before planting in flats of vermiculite. The seedlings were grown at 23° C with 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light at the top of the seedlings and a 14-hr photoperiod. Plants were watered every other day and fertilized once weekly with Ra-pid-gro plant food (23-19-17 with micronutrients).

Inoculation of soybean cotyledons. Unblemished cotyledons from 10-day-old seedlings were harvested and used immediately. The cotyledons were surface-sterilized by soaking in 70% ethanol for 1 min followed by two washes in sterile distilled water. The cotyledons were then placed bottom side up in petri plates containing moistened filter paper (10 cotyledons per plate). The epidermis of the center of the cotyledon was just barely broken at one point using a sterile dissecting needle, and inoculation was achieved by placing a 20-μl droplet of a suspension of 5×10^4 *P. m.* f. sp. *glycinea* zoospores per milliliter or a 1-mm agar plug of mycelium directly over the pinprick lesion. Although wounding was not essential for infection, it greatly increased the synchrony and reproducibility of infection. Four replicate plates containing 10 cotyledons each were inoculated per treatment. Inoculated cotyledons were incubated in the closed petri plates at 25° C in 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light or in the dark as noted below. The position of the infection front and the concentration of individual metabolites were determined as described below.

HPLC analysis and quantitation of isoflavonoids. Two replicate sets of 10 cotyledons each were harvested for HPLC analysis. The tissues were harvested in sections using cork borers of increasing diameter. Section S1, representing the point of inoculation, was harvested by removing a plug of tissue, centered on the point of inoculation, with a No. 2 cork borer. Concentric sections outward from S1 (S2 and S3) were subsequently harvested with Nos. 4 and 6 cork borers. In this way, each concentric section represents a 3-mm progression away from the point of inoculation.

The tissues were extracted either immediately in 400 μl of 80% ethanol per 0.1 g fresh weight or immediately upon thawing after storage at −80° C. Extraction was achieved by grinding the tissues directly in microfuge tubes using a polypropylene pestle (Kontes Glass Co., Vineland, NJ) at 600 rpm. The crude extract was centrifuged for 4 min at 18,000 × *g*, and the supernatant was taken directly for HPLC.

Injections of 20 μl were made on a 4.6 mm i.d. × 250 mm Hibar Ec Cartridge containing Merck Lichrosorb RP-18 10 μm C18 reverse-phase packing (Alltech Associates, Deerfield, IL). A guard column containing the same packing was used to protect the analytical column. Chromatography was achieved at 25° C using a gradient of 0–55% acetonitrile over 25 min, followed by a step increase to 100% acetonitrile for 2 min before a step return to water. A flow rate of 1.5 ml/min was used. Spectrophotometric detection was at either 236 or 280 nm.

Under these conditions, baseline resolution was achieved on all of the metabolites of interest, allowing their quantitation as follows. Calibration was achieved using authentic standards employing a Kratos Spectroflow 783 Absorbance Detector coupled to a Spectra-Physics SP4290 Integrator. Glyceollin standards were prepared using a molar extinction coefficient of 10,300 in ethanol at 285 nm (Ayers *et al.* 1976). Under the conditions described above, the glyceollins cochromatograph as a single peak at 25.6 min. Thus the concentrations quantitated in infected tissues represent total glyceollin.

Daidzein and genistein were similarly quantitated using molar extinction coefficients of 25,400 in methanol at 252 nm and 38,460 in methanol at 260 nm, respectively (Ganguly and Sarre 1970). The conjugates of daidzein and genistein were quantitated by hydrolysis to the free aglycones in 1 N HCl at 100° C for 1 hr.

Retention times for the isoflavones and their conjugates (Fig. 1) were as follows: DZ1 (13.8 min), DZ2 (15.8 min), GT2 (17.3 min), daidzein (18.9 min), and genistein (21.9 min).

Characteristics of compatible and incompatible cotyledon infections. Inoculation of cotyledons of the cultivars Williams (susceptible) and Williams 79 (Rps_1^c gene for resistance) with *P. m.* f. sp. *glycinea* race 1 as described above led to clearly delineated compatible and incompatible infections. The temporal and spatial aspects of the development of symptoms in both reactions were followed in the light and dark.

In the dark, the compatible infection (Williams, *P. m.* f. sp. *glycinea* race 1) was characterized by water-soaked lesions at the point of inoculation by 12 hr. By 24 hr, the water-soaked lesions had coalesced, and the infection had spread into the tissues directly under the point of inoculation (S1). The beginning of tissue maceration was evident at 24 hr, and droplets oozing out of the surface of S1 were apparent on some cotyledons. Between 24 and 48 hr, the infection began to spread very rapidly. By 48 hr, water-soaking and droplets of ooze were evident on the surfaces throughout section S2 and the infection had progressed into S3. The tissues were infected and partially macerated to all depths within these sections. At 72 hr, all but the most peripheral tissues were water-soaked and partially macerated with droplets of ooze across all sections. Within the next 24 hr, the tissue maceration continued to the point that the entire cotyledon was water-soaked and grayish-green. The compatible reaction in the light was characterized by very similar symptoms. Infection was only slightly slower in development.

In the dark, the incompatible infection (*P. m.* f. sp. *glycinea* race 1, Williams 79) was characterized by the development of tiny dark brown necrotic flecks at the point of inoculation by 12 hr. These necrotic flecks began to coalesce within the innermost portions of S1 by 24 hr, with considerable damage to surface cells. There was little internal infection and where this occurred, the tissues were not water-soaked, but showed a necrotic reddish-brown reaction. By 48 hr, mycelia had grown along the outside surface of the cotyledon, causing a spreading surface necrosis that had reached the outermost portions of section S2. This slowly spreading necrotic lesion was highly characteristic of the incompatible reaction in the dark. It was accompanied by limited necrotic infection of internal

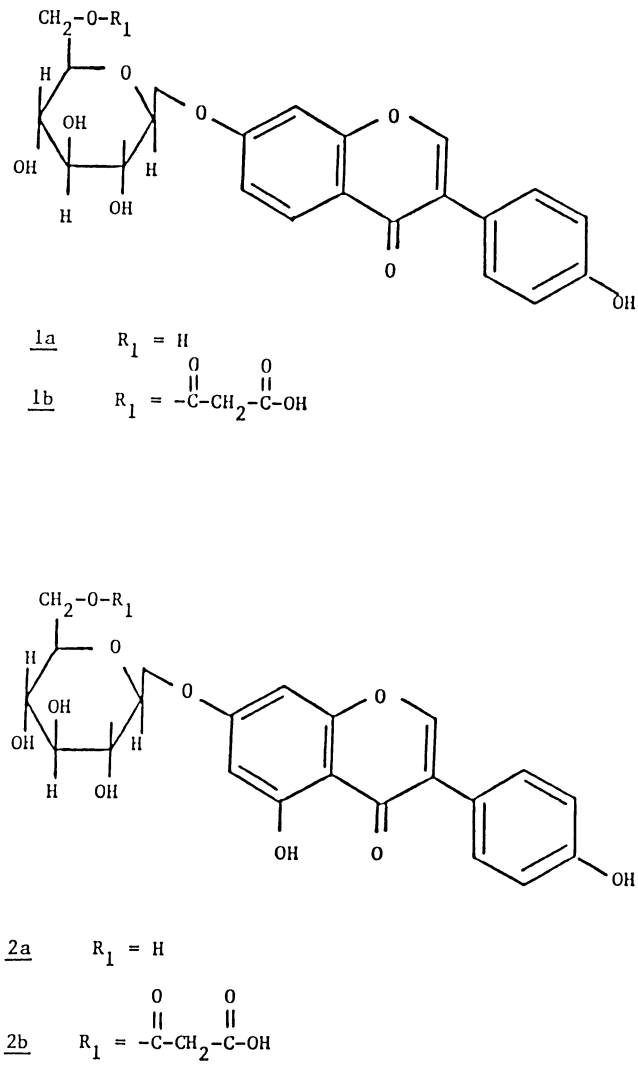

Fig. 1. Structures and identities of soybean isoflavone conjugates. 1a, 7-O-glucosyl daidzein (DZ1); 1b, 6″-O-malonyl-7-O-glucosyl daidzein (DZ2); 2a, 7-O-glucosyl genistein (GT1); and 2b, 6″-O-malonyl-7-O-glucosyl genistein (GT2).

tissues, which remained turgid. After 48 hr, lesion development largely ceased; in 2 replicates of 20 the lesion had just barely progressed into S3.

The incompatible infection in the light was characterized by similar symptoms during the first 12 hr. However, the initial necrotic flecks enlarged only slightly by 24 hr. As in the dark, lesion development was necrotic at all times and involved only limited internal necrosis. Lesion development ceased by 48 hr, by which time it had spread to the outermost portions of S1 and just barely into S2. Thus, although spread of the lesion in the light ceased at the same time as in the dark, it developed more slowly and was contained earlier in a spatial sense.

There were several clear distinctions, then, between the compatible and incompatible reactions. Compatible infections were largely internal with few surface symptoms except for water-soaking and the oozing droplets. Extensive maceration occurred, particularly after 72 hr. At no time was necrosis evident externally or internally. Incompatible infections were limited to necrotic lesions on the surface and in the uppermost tissues of the cotyledons. Lesion development ceased at 48 hr. There was little tissue maceration and the cotyledons remained turgid.

These visual observations of symptom development were complemented by harvesting and individually culturing sections S1–S3 for each of the same 20 replicate cotyledons on lima bean agar. This was done when the other replicate plates of cotyledons were harvested for HPLC analysis. The emergence of *P. m.* f. sp. *glycinea* mycelia from a given cultured section was taken as an indication that the infection front had progressed into that section. Results from the cultured sections agreed remarkably well with the observed symptoms and established that the infection front in the compatible infections was just barely ahead of the visible water-soaked lesions. In the incompatible infections, the infection front was indistinguishable from the position of the leading edge of the necrotic lesions. Taken together these data were used to establish the position of the infection front with time in the tissues extracted for HPLC analysis.

RESULTS AND DISCUSSION

Characterization of the isoflavone conjugates. To facilitate our investigations of the role of various aromatic secondary metabolites in race-specific and non-race-specific resistance in soybean, we developed an HPLC profiling procedure that allowed us to more completely examine metabolic events in infected and elicitor-treated tissues (Graham *et al.* 1982). Recent examinations of such HPLC profiles (Graham 1988) resulted in the identification of several major constitutive aromatic peaks, which increased or decreased along with glyceollin in a manner highly correlated to elicitor treatment or discrete infection events. Isolation and characterization of these peaks led to their identification as free daidzein and genistein and acid labile conjugates of both. Since daidzein and genistein were not previously reported as major constitutive metabolites in soybean seedling tissues, we further characterized these molecules.

Daidzein and genistein were found to exist as free aglycones and as multiple glycosyl conjugates (Fig. 1) in all soybean seedling organs examined. The conjugates have been identified as 7-O-β-D-glucosyl-daidzein (DZ1, Fig. 1, 1a), 7-O-β-D-glucosyl-genistein (GT1, Fig. 1, 2a), and the corresponding 6″-O-malonyl conjugates (DZ2 and GT2, Fig. 1, 1b and 2b). The full details of the structural characterization of these molecules will be presented elsewhere. Some of their properties are summarized below.

DZ1 and GT1 hydrolyzed under strong acid conditions (1 N HCl, 100° C, 1 hr) stoichiometrically to free daidzein and genistein, respectively. The fact that the released compounds were daidzein and genistein was established by comparison of their ultraviolet spectra with authentic daidzein and genistein and by their coelution upon HPLC, under several conditions, with daidzein and genistein. DZ1 and GT1 were also hydrolyzed stoichiometrically to daidzein and genistein in the presence of almond β-glucosidase. Hydrolysis in strong acid or by β-glucosidase led to the release of one molar equivalent of glucose for each mole of genistein or daidzein. Bathochromic ultraviolet spectral shifts occurring in the presence of aluminum chloride and sodium ethylate (Mabry *et al.* 1970) were diagnostic of a 7-O-substitution. Fast atom bombardment mass spectra of DZ1 and GT1 confirmed both the molecular weights and the presence of daidzein and genistein, respectively. Proton nuclear magnetic resonance (NMR) spectroscopy was used to further confirm their identities.

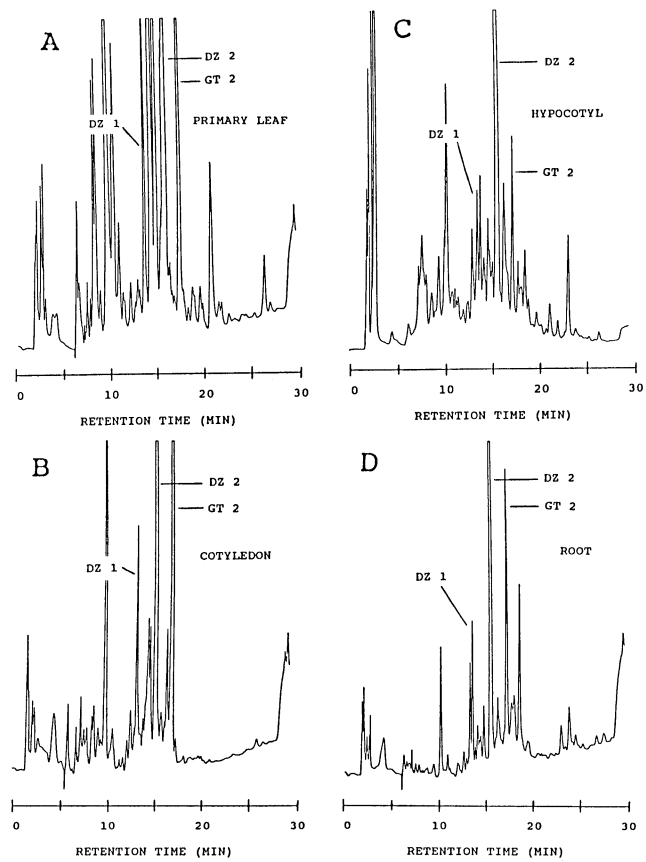

Fig. 2. High pressure liquid chromatography profiles of aromatic metabolites in various soybean organs. The 7-O-glucosyl conjugates of daidzein (DZ1) and the 6″-O-malonyl-7-O-glucosyl conjugates of daidzein and genistein (DZ2 and GT2, respectively) are identified.

DZ2 and GT2 (the malonylated conjugates of daidzein and genistein) hydrolyzed stoichiometrically to DZ1 and GT1, respectively, under mild acid conditions (0.1 N HCl, 70° C, 1 hr). The retention times of DZ2 and GT2 on HPLC shifted sharply and stoichiometrically at pH 3. This behavior is consistent with an acid titratable group. DZ2 and GT2 were also hydrolyzed by β-glucosidase, but the products were free daidzein and genistein and an anionic sugar that released free glucose upon mild acid hydrolysis. These results led us to suspect that DZ2 and GT2 were the 6″-O-malonyl conjugates of daidzein and genistein (Fig. 1). This was confirmed by the fast atom bombardment mass spectrum and proton NMR.

To our knowledge, the 6″-O-malonylated conjugates of daidzein and genistein (which are by far the major conjugates of daidzein and genistein in soybean seedling tissues) have not been previously reported from any plant species. Malonylated conjugates of other flavonoids and isoflavonoids, however, are quite common (Hahlbrock 1981; Wong 1975).

The simple 7-O-glucosides of daidzein and genistein (DZ1 and GT1) have been reported previously as constituents of soybean seeds (Wong 1975) and as induced metabolites of leaves (Osman and Fett 1983; Cosio *et al.* 1985). However, they have never been described as constitutive metabolites in soybean seedling organs.

In addition, the 6″-O-acetyl-7-O-glucosyl conjugates of daidzein and genistein have been reported as dietary components of defatted soybean meal (Ohta *et al.* 1979; Ohta *et al.* 1980) and toasted soybean flakes (Farmakalidis and Murphy 1985). We have not detected the acetates in any living soybean tissues. Since the malonyl group is subject to decarboxylation upon heating, it is possible that the 6″-O-acetyl conjugates arose as a result of heat during the processing of the soybean meal examined by these researchers. Alternatively, the malonyl group may have

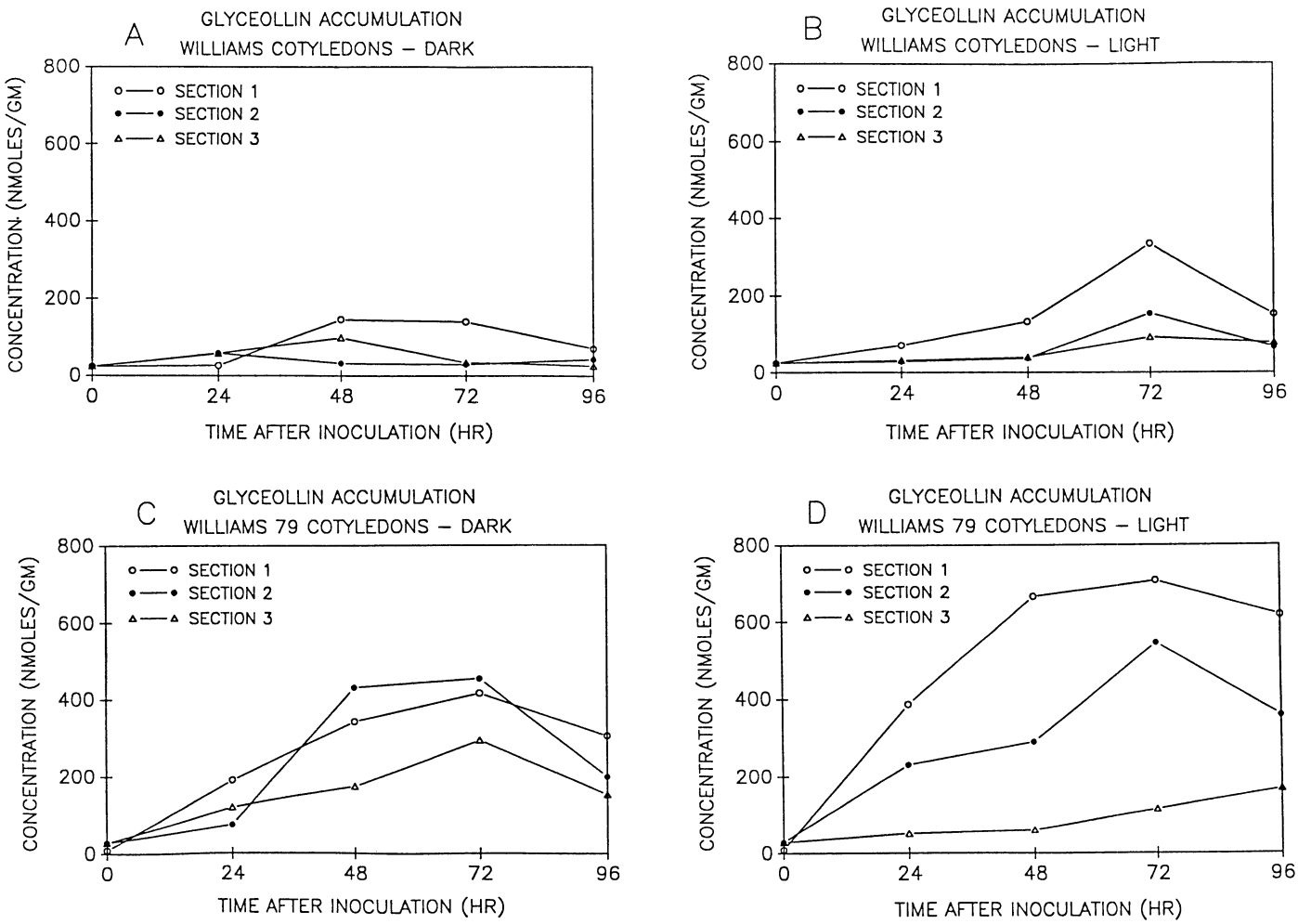

Fig. 3. Glyceollin accumulation in infected soybean cotyledons. Cultivars Williams (no *Rps* gene for resistance) or Williams 79 (Rps_1^c gene for resistance) were infected by placing a 1-mm agar plug of *Phytophthora megasperma* f. sp. *glycinea* race 1 mycelia on the lower surface of excised 10-day-old cotyledons. The inoculated cotyledons were then incubated lower surface up on moist filter paper in petri plates at 25° C in the dark or in the light with a 14-hr photoperiod. At the indicated times after inoculation, tissues were harvested using cork borers of increasing diameter to give concentric rings of tissue, each representing a 3-mm increase in radius. Section S1 is the inner 3 mm of tissue representing the point of inoculation and the 2 mm of surrounding tissue. Sections S2 and S3 represent consecutive 3-mm sections outward from this point. The tissues were extracted in 80% ethanol and their glyceollin content (nanomoles per gram fresh weight tissue) measured by high pressure liquid chromatography. Each point represents the average of two separate determinations including 10 replicate cotyledons per determination. Nearly identical data were obtained in a second experiment in which a zoospore inoculum was used.

been decarboxylated during the conventional mass spectral analyses used by these authors, destroying the parent ion. We chose the fast atom bombardment mass spectral techniques used in our analyses because they involve minimal sample heating.

Distribution of daidzein, genistein, and their conjugates in seedling organs. As shown in Figure 2, the conjugates DZ1, DZ2, and GT2 are major soluble aromatic metabolites in root, hypocotyl, cotyledon, and primary leaf tissues. DZ2 is a predominant metabolite in all tissues, whereas the distribution of DZ1 and GT2 is more organ-specific. We have detected GT1 only as a minor metabolite in hypocotyl tissues (data not shown).

Depending on the organ, the conjugates of daidzein alone are present at 700–1,700 nmol/g fresh weight tissue. The ED_{50}, for a mixture of the glyceollins, for inhibition of *P. m.* f. sp. *glycinea* mycelial growth *in vitro* is 33–50 μg/ml (Yoshikawa *et al.* 1978; Bhattacharyya and Ward 1985) or 100–150 nmol/ml. From experiments in which we have protected Williams cotyledons against *P. m.* f. sp. *glycinea* infection by prior application of *P. m.* f. sp. *glycinea* wall glucan, we have estimated an ED_{50} for mixed glyceollins *in vivo* of 80–90 nmol/g of tissue (Lundry *et al.* 1981). Thus the daidzein conjugates are present in all seedling tissues at concentrations far in excess of that needed for an ED_{50} accumulation of glyceollin.

Race-specific resistance to *P. m.* f. sp. *glycinea* has been reported in infected cotyledon tissues (Morrison and Thorne 1978). We chose to begin our examination of the role of the conjugates of daidzein and genistein in glyceollin accumulation in cotyledon tissues because they possess several advantages. First is the ease of acquisition and handling of these tissues and their comparative cellular simplicity (they are largely composed of mesophyll parenchyma cells). Cotyledons are also the classical and most commonly used organ for molecular studies of glyceollin elicitation (Frank and Paxton 1971; Ayers *et al.* 1976; Partridge and Keen 1977). Finally, cotyledons possess substantial pools of genistein as well as daidzein conjugates, allowing us to assess the relative role of these two closely related isoflavones in glyceollin accumulation and/or other disease resistance responses in this organ.

Spatial accumulation of glyceollin in cotyledon tissues relative to the compatible and incompatible infection front. Despite the fact that race-specific reactions to infection have been reported in cotyledons (Morrison and Thorne 1978), the spatial and temporal aspects of accumulation of the glyceollins in cotyledon tissues in relation to the infection front have not been described previously. A clear demonstration of race specificity in glyceollin accumulation has required strict attention to these spatial and temporal events in other tissues (Yoshikawa *et al.* 1978; Hahn *et al.* 1985). For this reason, we established these parameters in cotyledons to allow us to relate the possible role of the conjugates in more defined race-specific events. The symptoms and timing of symptom development for Williams (susceptible) and Williams 79 (resistant) to infection by race 1 of *P. m.* f. sp. *glycinea* have been described previously in the text. Clear compatible (internal water-soaked) and incompatible (surface necrotic) responses were observed. Graphs A–D in Figure 3 show the accumulation of the glyceollins in infected cotyledons, in a spatial and temporal manner, for compatible and incompatible infections in the dark and light.

In the dark, the compatible reaction is characterized by very low levels of glyceollin accumulation (Fig. 3A). In all sections at all times, glyceollin accumulation is well below or just approaching the ED_{50} value (80–90 nmol/g of tissue). Since the cotyledons are completely macerated by 96 hr, there is virtually no response to the presence of the pathogen as infection progresses.

In the light, the compatible reaction is only slightly different (Fig. 3B). Although there is an accumulation of glyceollin to 230 nmol/g of tissue at the point of inoculation (S1), this occurs nearly 48 hr after the infection front has progressed beyond this section.

The incompatible reactions are dramatically different. In cotyledons incubated in the light (Fig. 3D), glyceollin accumulates at the point of inoculation to more than five times the ED_{50} value within the first 24 hr, reaching up to eight times the ED_{50} value in 48 hr. This is consistent with the lack of lesion development after 48 hr and the necrotic containment of the infection largely in the section originally inoculated (S1). The lower response in the adjacent section (S2) is probably due to the fact that the infection front had progressed just barely into the innermost portions of S2 in 20% of the replicate cotyledons by 48 hr. Consistent with the lack of infection symptoms in the outermost section (S3), there is little accumulation of glyceollin in this section.

The incompatible reaction in the dark is shown in Figure 3C. Although glyceollin accumulates to as much as 400 nmol/g of tissue, it is delayed by 24–48 hr as compared to the incompatible reaction in the light. This is consistent with the fact that the infection was not contained in S1, as it was in the light, and had progressed into S2 by 48 hr. The accumulation of more than 400 nmol/g of tissue (five times the ED_{50} for glyceollin) in S2 at 48 hr is consistent with arrested lesion development in S2 and the lack of progression of infection into S3. The accumulation of low levels of glyceollin in S3 at 72 hr is consistent with the fact that the infection front had just barely passed into section S3 in 10% of the replicate cotyledons.

The results of these cotyledon infection experiments show a rapid and specific accumulation of high levels of glyceollin at the infection front in incompatible but not compatible reactions. Our results complement previous *in vivo* studies with hypocotyl and root tissues (Yoshikawa *et al.* 1978; Hahn *et al.* 1985). Together they consistently suggest that both the timing and magnitude of the glyceollin response are critical to pathogen containment.

Changes in daidzein, genistein, and glyceollin levels in compatible and incompatible infections. The concentrations of daidzein, DZ1, DZ2, genistein, GT1, and GT2 were also determined in the above experiments. For the sake of simplicity, we report here only those results at the point of inoculation (section S1). Results with sections S2 and S3 were very useful in that they allowed additional correlations of conjugate turnover to glyceollin accumulation as the infection front progressed through these tissues. However, the results in all cases were similar to and simply served to confirm those described below for

section S1.

Graphs A–D in Figure 4 show the changes in the levels of daidzein and its conjugates in relation to glyceollin in the compatible and incompatible reactions in the light and dark. There were no appreciable differences in the nature or timing of changes in the levels of DZ1 and DZ2, so they are expressed as total conjugates (DZ1 + DZ2).

In the incompatible reactions (Fig. 4, graphs C and D), there is a marked and immediate reduction in levels of the conjugates. These fall to less than half their original levels by 24 hr and to nearly nondetectable levels within just 48 hr. Since this occurs in tissues that are for the most part still healthy (the necrotic lesions even at 48 hr in the dark are largely surface lesions), it would appear to represent a specific event rather than the result of widespread tissue disruption. Concurrent with this, there is an accumulation of free daidzein and glyceollin. The larger initial buildup and slower consequential disappearance of free daidzein in the dark are consistent with the kinetics of later accumulation of glyceollin in these tissues. The eventual decrease in glyceollin is consistent with the half-life of 100 hr reported for glyceollin in soybean tissues (Moesta and Grisebach 1981).

The compatible reaction in the dark (Fig. 4A) is characterized by a delayed disappearance of the daidzein conjugates, a nearly stoichiometric accumulation of daidzein, and little to no accumulation of glyceollin. In the compatible reaction in the light (Fig. 4B), there is again a delayed and then rapid reduction of the total conjugate pools, with an almost stoichiometric accumulation of daidzein and little glyceollin. Thus, in both compatible reactions, only low levels of glyceollin are synthesized despite the accumulation of very large amounts of free daidzein.

The susceptible cultivar Williams (Fig. 4A) actually contains higher constitutive levels of the daidzein conjugates than does the resistant cultivar Williams 79 (Fig. 4C), suggesting that the presence of the Rps_1^c gene has no apparent relationship to the presence or amounts of the conjugates.

The breakdown of the daidzein conjugates must be viewed very differently in the compatible infected tissues. The rapid phase of conjugate hydrolysis occurs 24 hr after the infection front has progressed out of this tissue. The plant tissues in which hydrolysis is occurring are largely macerated and have lost much of their cellular integrity. It is possible, then, that these degradative events in the

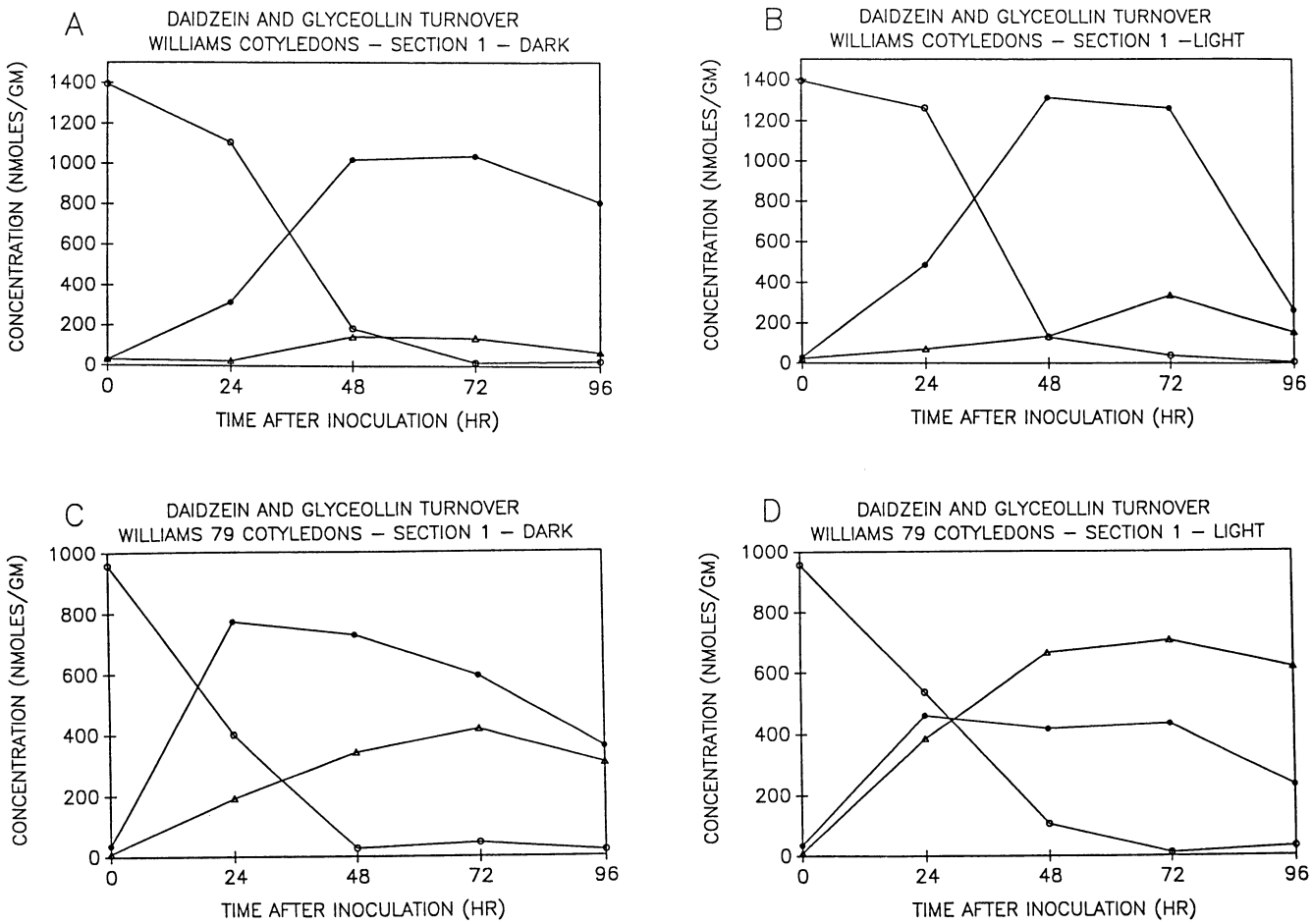

Fig. 4. Changes in the levels of daidzein and glyceollin in infected soybean cotyledons. The experimental procedure is as described in Figure 3 except that only data from section S1 are shown. The levels of free daidzein (●), total daidzein conjugates (○), and glyceollin (△) were determined by high pressure liquid chromatography and represent nanomoles per gram fresh weight tissue. Each data point represents the average of two separate determinations including 10 replicate cotyledons per determination. Nearly identical data were obtained in a second experiment in which a zoospore inoculum was used.

compatible reaction are the result of nonspecific cellular disruption and the mixing of the vacuolar conjugates with hydrolases and other enzymes of host and/or pathogen origin. Regardless, by the time daidzein is released in the compatible reactions, the majority of the cells appear to be either unstimulated by any elicitors present or incapable of glyceollin biosynthesis.

Figure 5 shows similar data for genistein. We show this data only for section S1 and only in the incompatible reaction in the light. Since the glyceollins lack a hydroxyl group on the A-ring at position 5, daidzein, and not genistein, is thought to be the immediate precursor of the glyceollins. It is intriguing to note, however, that the genistein conjugate, GT2, is present in both Williams and Williams 79 cotyledons at even higher levels than the daidzein conjugates. This fact alone compelled us to examine the steady-state levels of genistein and its conjugates relative to those of daidzein upon infection (Fig. 5).

We observed that hydrolysis of GT2 occurs over a time course similar to the one for the hydrolysis of the daidzein conjugates in both Williams and Williams 79 infections. Thus, large quantities of genistein are also released before glyceollin biosynthesis. Since genistein metabolism has not been monitored in infected tissues before, it is conceivable that previously uncharacterized mechanisms for the conversion of genistein to daidzein exist under conditions of infection. However, as illustrated in Figure 6, the pools of total daidzein and total genistein (free aglycones plus conjugates) both decline steadily and gradually over the course of the experiments. Thus, although a gradual conversion of genistein to daidzein cannot be ruled out, there is no evidence for a concerted or programmed shift in genistein to daidzein. We do not know the metabolic fate of the genistein released from its conjugates; however, from our present data, from purely structural considerations, and from what we know of the enzymology of the pathway (Welle and Grisebach 1989), it seems unlikely that the released genistein is acting as a glyceollin precursor.

Figure 6 illustrates several other important points. The conjugates of daidzein are present in Williams 79 at levels of more than 12 times that required for an ED_{50} glyceollin response. The presence of such massive levels of constitutive isoflavone conjugates and their early and nearly complete hydrolysis during the incompatible infection (Fig. 4D) raise the obvious question of their role, relative to that of *de novo* synthesis of the isoflavones, in the glyceollin response for these tissues. When one examines the data of Figure 4D, but considers the total pools of 5-deoxyisoflavonoids (total daidzein and glyceollin) during the course of infection as shown in Figure 6, an early but transient net increase of 30–40% in these metabolites is seen. This increase is likely due to *de novo* synthesis as observed in other organs in response to incompatible infections (see studies outlined earlier in the text). Assessment of the relative contributions of *de novo* synthesis and conjugate hydrolysis to specific spatial and temporal phases of the incompatible response, however, will require careful and detailed pulse-labeling experiments in the future. It is intriguing to speculate that *de novo* synthesis and conjugate hydrolysis may play important complementary roles which will only be understood when we examine more discrete cellular events.

Conclusions. In this research article we have reported spatial and temporal aspects of compatible and incompatible infections of soybean cotyledons as they relate to the accumulation of glyceollin and the release of its isoflavone precursors from previously unreported constitutive conjugates. Our results show that these constitutive conjugates of the isoflavones daidzein and genistein are present in all soybean seedling organs of the cultivars Williams (with no known *Rps* gene for *P. m.* f. sp. *glycinea* resistance) and Williams 79 (carrying the Rps_1^c gene for resistance) at levels which far exceed those required for an ED_{50} glyceollin response. The presence of greater levels of the

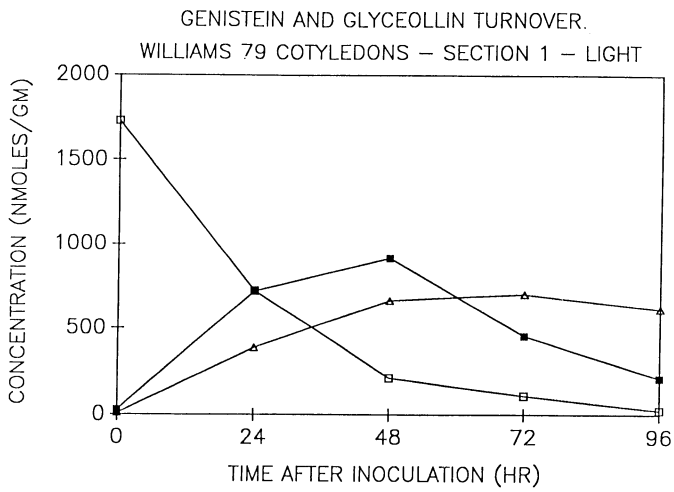

Fig. 5. Changes in the levels of genistein and glyceollin in infected cultivar Williams 79 cotyledons incubated in the light. The experimental procedure is as described in Figure 3 except that only data from section S1 are shown. The levels of free genistein (■), total genistein conjugates (□), and glyceollin (△) were determined by high pressure liquid chromatography and represent nanomoles per gram fresh weight tissue. Each data point represents the average of two separate determinations including 10 replicate cotyledons per determination.

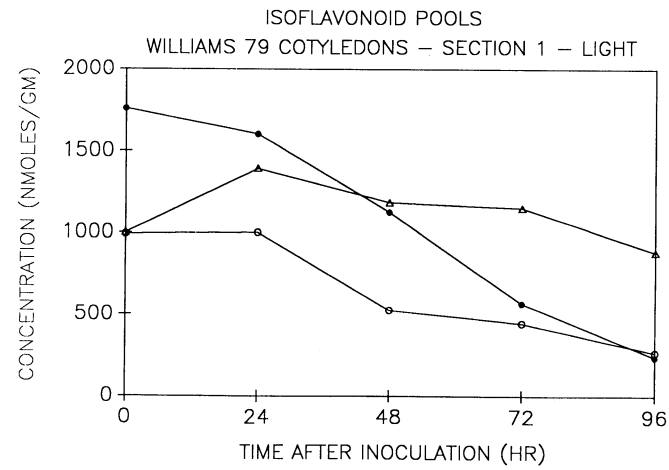

Fig. 6. Changes in isoflavonoid pools in infected cultivar Williams 79 cotyledons incubated in the light. The experimental procedure is as described in Figure 3 except that all data are from section S1. The plots represent total daidzein (○, free daidzein and its conjugates), total genistein (●, free genistein and its conjugates), and total 5-deoxyisoflavonoids (△, the total of daidzein, its conjugates, and glyceollin). Each data point represents the average of two separate determinations including 10 replicate cotyledons per determination.

conjugates in the cultivar Williams makes it unlikely that the Rps_1^c gene is responsible for the presence of or levels of these constitutive conjugates in soybean tissues.

The infection of Williams 79 cotyledon tissues with an incompatible race of *P. m.* f. sp. *glycinea* results in typical necrotic incompatible lesions and rapid glyceollin accumulation at the infection front. The compatible reaction in Williams cotyledons is characterized by rapid, unrestricted, and complete tissue maceration with little or no glyceollin accumulation.

In incompatible infected cotyledon tissues, the constitutive conjugates of daidzein are broken down to free daidzein over a time frame consistent with their possible utilization for glyceollin biosynthesis. Both the early hydrolysis of the daidzein conjugates and the subsequent accumulation of glyceollin appear to represent specific events occurring in largely intact incompatible infected tissues. Although the genistein conjugates are also broken down, our data provide no evidence for the conversion of genistein to daidzein and thus for the utilization of genistein for glyceollin biosynthesis.

Both genistein and daidzein conjugates are also eventually broken down in compatible infections, but only after the infection has passed well beyond those tissues that have become macerated. There is little to no accumulation of glyceollin. It is possible, then, that isoflavone release in the compatible infection is a nonspecific event due to severe tissue disruption. It is also possible that by the time the isoflavones are released, the majority of cells in this tissue are no longer capable of glyceollin biosynthesis.

We thus propose that the early release of daidzein from its conjugates and/or the induction or activation of later enzymes in the biosynthesis of glyceollin may play potentially important roles in the race-specific accumulation of the glyceollins in tissues carrying the Rps_1^c gene. How these proposed events relate to the well-documented *de novo* synthesis of the isoflavones and of glyceollin in infected and elicitor-treated tissues will require further investigation.

It is important to point out that the results reported here could be unique to cotyledon tissues. As we have shown, the distribution of specific conjugates varies somewhat in an organ-specific manner. Organ-specific and tissue developmental effects on the race-specific elicitation of glyceollin have been shown in hypocotyl, root, and leaf tissues (Paxton and Chamberlain 1969; Lazarovits *et al.* 1981; Bhattacharyya and Ward 1986a; Bhattacharyya and Ward 1986b). It is also possible that daidzein release from its conjugates may depend both on the conditions of the assay and the specific cultivar being examined. Thus, further work will be necessary to more fully determine the potential role of these processes under varying conditions in soybean cultivars carrying this and other *Rps* genes.

Given the presence of the conjugates, it will be of interest to reexamine the various molecular elicitors of the phytoalexin response in soybean in regard to their effects on the *de novo* synthesis of the isoflavones, on the release of the isoflavones from their constitutive conjugates, and on enzymes in the later steps in glyceollin biosynthesis. A preliminary report of these investigations has been presented (Graham and Graham 1989).

It will also be of obvious interest to compare the possible role of constitutive conjugates of isoflavones in the accumulation of pterocarpan phytoalexins in other legumes. Early work of Olah and Sherwood (1971, 1973) showed that glycosyl conjugates of several isoflavones are synthesized and subsequently hydrolyzed in alfalfa plants infected by *Ascochyta imperfecta* Peck. However, the possible role of these changes in isoflavone metabolism with respect to the accumulation of pterocarpan phytoalexins was not examined.

Research more directly related to what we report here has been conducted in chick-pea. From studies of susceptible and resistant chick-pea tissues infected with *A. rabiei* (Pass.) Labrousse, Weigand *et al.* (1986) concluded that constitutive conjugates of the isoflavones formononetin and biochanin A are not hydrolyzed substantially in infected tissues and do not appear to contribute to the biosynthesis of the pterocarpan phytoalexins medicarpin and maackiain. The apparent lack of involvement of isoflavone conjugates in the resistant response of chick-pea to *A. rabiei* may reflect differences in either the host or the pathogen. Although a number of possibilities can be identified, perhaps the most significant difference is that the two pathogens are in completely different taxonomic groups. As such, they possess very different life cycles and are very distinguishable in both the manner in which they infect host tissues and the symptoms they cause.

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