Acifluorfen-Induced Isoflavonoids and Enzymes of Their Biosynthesis in Mature Soybean Leaves[1]

WHOLE LEAF AND MESOPHYLL RESPONSES

ERIC G. COSIO[2], GOTTFRIED WEISSENBÖCK, AND JERRY W. MCCLURE*
Department of Botany, Miami University, Oxford, Ohio 45056 (E.G.C., J.W.Mc.); and Botanisches
Institut I der Universität Köln, D-5000 Köln 41, Federal Republic of Germany (G.W.)

ABSTRACT

Mature soybean (*Glycine max* L. cv Harosoy 63) leaves normally contain kaempferol-3-glycosides but they accumulate no other flavonoids. Whole leaves sprayed with the diphenyl ether herbicide Acifluorfen and maintained in the light developed small necrotic lesions and accumulated isoflavone aglycones, isoflavone glucosides, and pterocarpans. Isoflavonoid accumulation was preceded by induced activity for chalcone synthase (CHS) and by increased activity for phenylalanine ammonia-lyase (PAL) and UDP-glucose:isoflavone 7-*O*-glucosyl transferase (IGT). PAL and CHS activity was highest between 24 and 30 hours after treatment, isoflavone aglycones and pterocarpans at 48 hours, IGT at 72 hours, and isoflavone glucosides at 96 hours.

Mesophyll cells isolated from control leaves contained no activity for PAL, CHS, or IGT and no flavonoids of any class. Cells isolated from treated leaves at the stage of maximal enzyme activity or isoflavonoid content contained PAL (12% of the whole leaf activity), CHS (24%), IGT (20%), and 25% of the whole leaf isoflavone glucosides, but only traces, presumably as contaminants, of the other flavonoids. We suggest that the isoflavone glucosides were synthesized and accumulated in intact mesophyll cells as soluble detoxification products, while the isoflavone aglycones and pterocarpans accumulated in the epidermis or extracellularly within the mesophyll. To our knowledge this is the first report of tissue-specific induction of isoflavonoid glucosides and key enzymes of their biosynthesis in any plant.

Soybean leaves normally accumulate appreciable levels of kaempferol and quercetin glycosides (5) but no flavonoids of any class were detected in isolated soybean mesophyll cells (7). Flavonoids appear to be restricted to the epidermis of pea leaves (11), and presumably of soybean leaves (7), where they may function as UV screens and as preformed defensive compounds (18). A number of isoflavonoids (isoflavone aglycones, isoflavone glucosides, pterocarpans, and coumestans) can be induced to accumulate in soybeans by host-pathogen interactions (10, 12, 14, 20, 21) or by abiotic elicitors such as the diphenyl ether

herbicide Acifluorfen (15). The tissue distribution of these induced isoflavonoids is unknown.

Mesophyll cells isolated from control leaves of soybean (7) and pea (11) have no activity for several key enzymes of flavonoid biosynthesis, but the tissue distribution of these enzymes has not been previously investigated in leaves induced to accumulate isoflavonoids.

The present paper examines the accumulation of isoflavonoids and the activity for PAL[3], CHS, and IGT in whole leaf homogenates and mesophyll cells isolated from Acifluorfen-treated soybean leaves.

MATERIALS AND METHODS

Chemicals. Acifluorfen was a gift from the Rohm and Haas Co. Authentic samples of daidzein, formononetin, genistein, and their 7-*O*-glucosides were a gift from J. Köster, Münster, West Germany; and the glyceollins and glyceofuran a gift of N. T. Keen, Riverside, CA. Pectolyase Y-23 was purchased from Sheishin Pharmaceutical Co., Tokyo, Japan. Silica gel GHL and Avicel microcrystalline cellulose TLC plates (250 μm coating) were from Analtech Inc. Silica gel 60 F_{254} (250 μm coating) plates were from Merck, Darmstadt, West Germany; [2-^{14}C]malonyl-CoA (58 Ci/mol) was from Amersham; and the L-[U-^{14}C]phenylalanine (450 Ci/mol) and UDP-[U-^{14}C]glucose (343 Ci/mol) were from New England Nuclear. *p*-Coumaroyl CoA was prepared using the *N*-hydroxisuccinimide ester method of Stöckigt and Zenk (24).

Plant Material. Soybean seeds (*Glycine max* L. cv Harosoy 63) were from the USDA soybean collection, Urbana, IL. Plants were grown in environmental chambers under 10 klux of cool white fluorescent light on a 13-h 24°C day and 11-h 24°C night regime at a constant 70% RH. Plants were grown individually in 10-cm diameter pots containing Metromix 250 (W. R. Grace Co., Cambridge, MA) and watered daily with full strength Hoagland solution containing 25 mg/l Sequestrene 330 (CIBA-Geigy).

Fully expanded primary leaves (averaging 4.5 cm in length) on 14-d-old plants were sprayed on the lower surface with a solution of 100 mg/l Acifluorfen (Na salt) in 0.01% (v/v) Triton X-100. Controls were sprayed with 0.01% Triton X-100 alone or with distilled H_2O. The leaves were sprayed until they were covered with fine droplets.

Mesophyll Cell Isolation. Cells were isolated from leaves cut into 1 to 2 mm strips by the enzymic technique previously described (7, 8) modified by gently agitating the strips with a Teflon-coated magnetic spin bar throughout the isolation period

[3] Abbreviations: PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; IGT:UDP-glucose, Isoflavone 7-*O*-glucosyltransferase.

and by adding 0.1% (w/v) methylcellulose and 0.1% (w/v) BSA to the isolating medium. These modifications reduced the time required for cell isolation from 2 h to 15 min. This technique liberated an average of 30% of the mesophyll cells from the leaf based on Chl, and the cells were more than 90% intact as shown by their exclusion of Evans blue due. Phase microscopy showed that the mesophyll cell preparations were less than 1% contaminated by epidermal or vascular tissues.

Extraction and Quantitation of Flavonoids. For whole leaf samples, 12 primary leaves (approximately 4 g) were ground in liquid N_2 and the frozen powder extracted 2 times with 50 ml of 45% aqueous methanol. The extracts were centrifuged, the supernatant filtered through Macherey-Nagel 615 paper, and the filtrate evaporated to 50 ml at 40°C under reduced pressure to remove most of the methanol. Isoflavone aglycones and pterocarpans were extracted from this aqueous sample with 2 × 50 ml of diethyl ether.

Daidzein- and formononetin-7-O-glucosides remaining in the aqueous phase were identified by co-chromatography with authentic standards on silica gel F_{254} plates using a solvent of chloroform:acetone:methanol (20:6:5, v/v) (21). For ease of quantitation, the isoflavone glucosides and kaempferol glycosides (7) in the aqueous fraction were converted to their aglycones by hydrolysis in 1 N H_2SO_4 for 1 h at 100°C. These aglycones were extracted from the hydrolysate with 2 × 50 ml of ethyl acetate.

Isolated mesophyll cells were twice resuspended in 45% methanol (1 mg Chl/20 ml), stirred for 10 min, centrifuged to pellet insoluble residues, and the methanolic extracts processed as above.

The organic phases from the initial and hydrolysate extractions were separately concentrated to small volumes and chromatographed on silica gel 60 F_{254} plates in chloroform:acetone:24% aqueous ammonia (50:50:1, v/v) (12). Average R_F values were 0.09 for daidzein, 0.16 for formononetin, 0.34 for glyceofuran, and 0.57 for the glyceollins (a mixture of glyceollins I, II, and III). The regions containing these compounds were located under short-wave UV light, scraped from the plates, and eluted with 10 ml of methanol.

The TLC eluates were evaporated to dryness under reduced pressure, redissolved in 0.4 ml of 90% acetonitrile, and quantitated by reversed phase HPLC on a 4.7 × 250 mm Merck RP-8 column (7 μm particles). The compounds were sharply resolved using a 15 min linear gradient of 30 to 72% aqueous acetonitrile with 1% aqueous phosphoric acid as the second solvent and at a flow rate of 1.5 ml/min. Detection was at 280 nm and quantitation was done with a Spectra Physics SP 4270 recording integrator. Retention times were 4.8 min for daidzein, 5.8 min for glyceofuran, 9.5 min for formononetin, and 11.2 min (double peak) for the glyceollins. Molar absorptivities used for quantitation were 10,500 for daidzein and formononetin (19), 10,000 for the glyceollins (1), and 7,200 for glyceofuran (12; modified from 280 nm). Kaempferol was quantitated by the isocratic HPLC system previously described (7).

Identity of the isoflavonoids was verified by co-chromatography with authentic standards using TLC and HPLC, and by a comparison of UV absorption spectra of purified samples with those of authentic standards.

Preparation of Samples for Enzyme Assays. Four leaves (about 1.3 g fresh weight) were ground to a fine powder in liquid nitrogen using a mortar and pestle. For PAL and CHS extraction, the frozen powder was stirred for 15 min in 10 ml of a pH 8 buffer containing 0.1 M K-phosphate, 20 mM potassium ascorbate, 15 mM cysteine, and 0.5 g Dowex 1 × 2 (phosphate form). For IGT extraction, the powder was stirred in 0.1 M Tris-HCl (pH 8.5) buffer containing 40 mM 2-mercaptoethanol and 0.5 g of Dowex 1 × 2 (Cl⁻ form). The preparations were filtered through Miracloth, centrifuged at 30,000g for 20 min, and the supernatants

used as a source of enzyme. To measure Chl in these powders for comparison of enzyme activities of whole leaf preparations with those of isolated cells, the frozen powder was resuspended in buffer without Dowex, ground in a Tenbroeck homogenizer, and an aliquot taken for Chl determination. The remaining sample was treated with Dowex, filtered, and centrifuged as above.

Isolated mesophyll cells were washed twice with 30 ml of cell isolation medium and resuspended in 7 ml of the K-phosphate (for PAL and CHS) or Tris (IGT) buffers described above. The cells were passed twice through a French pressure cell at 4000 p.s.i. and the lysates used directly for enzyme and Chl determinations. Dowex treatment of the cell lysates was omitted since it was found to have no effect on enzyme activities.

Enzyme Assays. PAL activity was measured by following the formation of radioactive trans-cinnamic acid in a reaction mixture containing enzyme (100 μl), 0.1 M K-phosphate (pH 8) and 2 mM L-[U-^{14}C]phenylalanine (0.5 Ci/mol) in a total volume of 150 μl. The mixture was incubated for 30 to 60 min at 30°C and the reaction terminated by adding 30 μl of concentrated acetic acid containing 20 μg of trans-cinnamic acid. Toluene (300 μl) was added and the samples were centrifuged for 2 min in a microcentrifuge. The upper 200 μl of the toluene phase was spotted onto a cellulose TLC plate and developed in 2% HCOOH. The trans-cinnamic acid was located by examining the plates under a short-wave UV lamp. This area (R_F 0.5) was scraped from the plate and counted by liquid scintillation in 5 ml of Aquasol.

CHS activity was determined by the technique of Schröder et al. (23) modifed by adding BSA (0.4 mg/ml). The reaction mixture was incubated for 15 min. The enzyme product co-chromatographed with an authentic sample (Sigma) of naringenin on TLC plates using either chloroform:methanol:water (65:25:4, v/v) on silica gel, or in benzene:acetic acid:water (115:72:3, v/v) on cellulose. Naringenin was the only radioactive product detected in this reaction.

IGT activity was determined using the method of Köster and Barz (16). Daidzein was the isoflavone substrate unless otherwise specified. Assay time was 30 min, although the reaction was linear for up to 1 h in the assays which contained from 80 to 200 μg protein.

Minimum detectable quantities (13) of product for the enzyme assays were 0.4 pmol of naringenin for CHS, 100 pmol of cinnamic acid for PAL, and 380 pmol of isoflavone 7-O-glucoside for IGT.

Chl and Protein Determinations. Chl was extracted into methanol and its concentration determined as described before (8, 17). Soluble protein was quantitated by Bradford's dye binding technique (3) using Sigma fraction V BSA as a standard. Protein samples were stored at −20°C in 20% (v/v) glycerol until analyzed.

RESULTS

Aqueous solutions of Acifluorfen under the product name Blazer are used as a postemergent contact herbicide to control broadleaf weeds in soybeans (26). Light is required for herbicidal activity of this diphenyl ether (26). We found that Acifluorfen caused the appearance of necrotic lesions, and (as described below) altered flavonoid biosynthesis and accumulation only when applied with low concentrations of a surfactant such as Triton X-100 to plants subsequently exposed to light.

Soybean leaves sprayed with 100 mg/l Acifluorfen and 0.01% (v/v) Triton X-100 developed randomly distributed necrotic lesions within 24 h after treatment. These lesions ranged in size from less than 1 mm to approximately 5 mm in diameter. Microscopic examination of free-hand sections of treated leaves showed that about half of the lesions penetrated only as far as
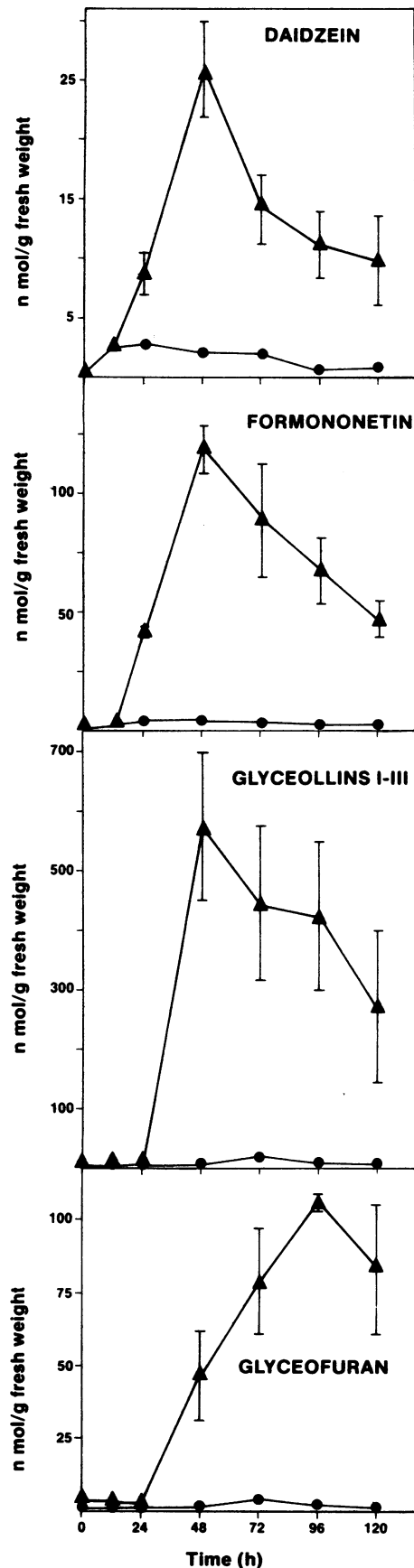
FIG. 1. Time-dependent accumulation of isoflavone aglycones (daidzein and formononetin) and pterocarpans (glyceollins and glyceofuran)

Table I. *Acifluorfen-Induced Isoflavonoids, and Kaempferol Glycosides, in Whole Primary Leaves and in Isolated Mesophyll Cells from Soybean*

Samples were taken 72 h after spraying the leaves of 14-d-old plants with an aqueous solution of 100 mg/l Acifluorfen and 0.01% (v/v) Triton X-100. Plant materials and techniques of flavonoid analysis are described in "Materials and Methods." Values are means ± SE of six independent experiments.

Compound	Whole Leaves	Mesophyll Cells	% Whole-Leaf Compound in Mesophyll Cells
	nmol/mg Chl[a]		%
Aglycones			
Daidzein	10 ± 1.8	0.41 ± 0.16	4.0 ± 1.0
Formononetin	71 ± 28.0	3.10 ± 1.0	5.2 ± 3.4
Glyceollins	205 ± 80.0	7.90 ± 6.3	3.1 ± 2.4
Glyceofuran	44 ± 9.3	0.52 ± 0.27	1.2 ± 0.5
Glycosides			
Daidzein 7-O-glucoside	98 ± 33	25.0 ± 8.6	26.0 ± 1.4
Formononetin 7-O-glucoside	16 ± 5	3.6 ± 0.65	22.0 ± 2.8
Kaempferol glycosides	615 ± 46	9.0 ± 5.5	1.4 ± 0.8

[a] The leaves had an average of 2 mg Chl and 22 mg soluble protein per g fresh weight. These values permit a comparison of units in this table with those in Figures 1 and 2.

the second cell layer of the mesophyll while the rest reached the other side of the leaf. Acifluorfen is not transported within the leaf (26) and other regions of the leaf appeared to be undamaged. Leaves sprayed with Acifluorfen and Triton X-100, with Triton X-100 alone, or with distilled H_2O, all senesced (yellowed) about 4 or 5 weeks after treatment.

Accumulation of Isoflavone and Pterocarpan Aglycones in Acifluorfen-Treated Leaves and the Absence of Significant Levels of These Compounds in Isolated Mesophyll Cells. The accumulation of daidzein, formononetin, glyceollin, and glyceofuran was determined in whole leaves over a 120-h period following Acifluorfen treatment (Fig. 1). Maximal accumulation of daidzein, formononetin, and the glyceollins was at 48 h, but glyceofuran levels peaked at 96 h. This lag in glyceofuran accumulation has been previously reported (12). Optimal concentrations of Acifluorfen for the induction of these aglycones were from 50 to 100 mg/l (not shown).

Mesophyll cells isolated 72 h after Acifluorfen treatment had only traces of isoflavone or pterocarpan aglycones; about 5% of the formononetin and 1% of the glyceofuran of the whole leaf (Table I).

Isoflavone Glucosides and Flavonol Glycosides of Whole Leaves and in Mesophyll Cells Isolated from Acifluorfen-Treated Plants.

The only flavonoids detected in control leaves of this soybean cultivar were kaempferol glycosides. Acifluorfen treatment caused no appreciable changes in total kaempferol glycoside production (not shown), but the treated leaves also accumulated 7-O-glucosides of daidzein and formononetin (Table I). The accumulation of these isoflavone glucosides lags considerably behind that of their respective aglycones. Maximal aglycone accumulation was found at 48 h (Fig. 1) but the level of isoflavone

in whole leaf extracts from soybean primary leaves sprayed on the lower surface with a 100 mg/l Acifluorfen solution in 0.01% (v/v) aqueous Triton X-100 (▲) as described in the text. Controls were sprayed with 0.01% Triton X-100 alone (●). Bars represent SE; $n = 3$.

glucosides was highest between 96 and 120 h (Fig. 2). The ratio of daidzein to formononetin glucoside in these leaves was approximately 8:1 while their aglycones accumulated in the ratio of about 1:4 (Figs. 1 and 2).

Mesophyll cells isolated from the treated leaves contained about one-fourth of the isoflavone glucosides of the whole leaf but only traces (about 1.2%) of the kaempferol glycosides (Table I).

Enzyme Activity in Whole Leaves Treated with Acifluorfen. Light is required for the expression of Acifluorfen responses (15, 26), and Table II shows that whole leaf activity for PAL and CHS increased only in treated plants maintained in the light.

Figure 3 shows changing activities for PAL, CHS, and IGT in whole leaf preparations from leaves sprayed with Acifluorfen. Activities for PAL and CHS were highest between 24 and 30 h after treatment, but IGT activity was highest after 72 h. CHS
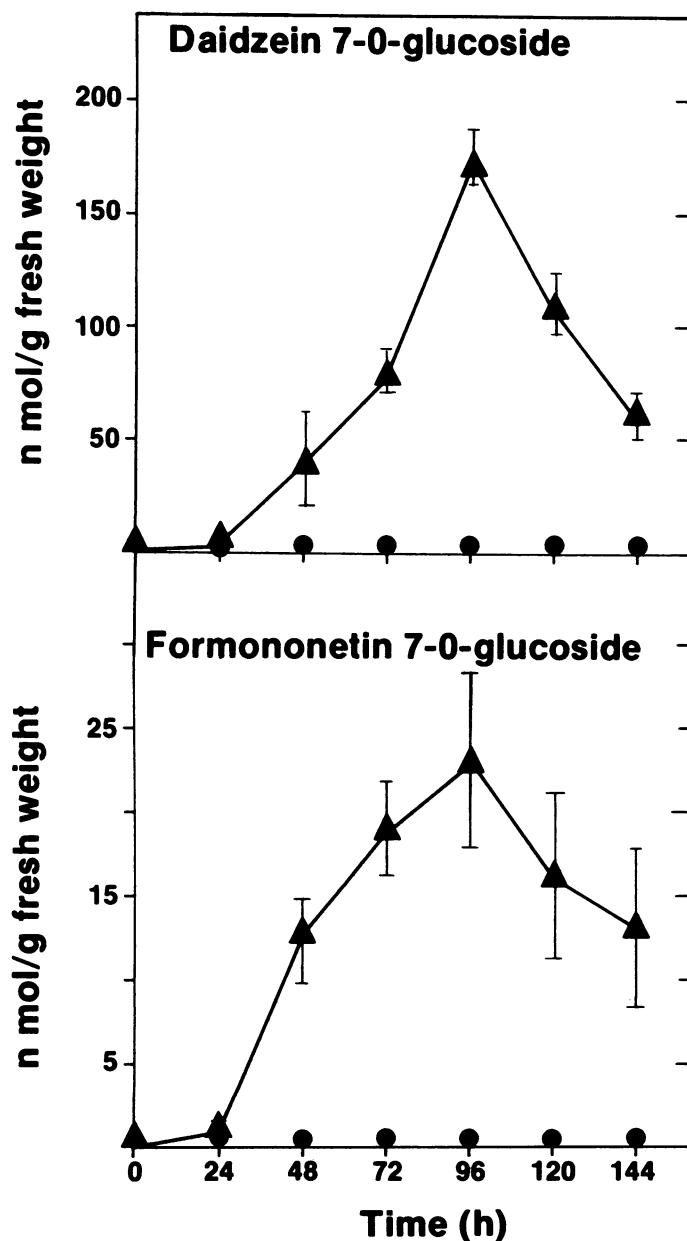
Table II. *Light Requirement for Acifluorfen Effects on Enzyme Activity in Whole Primary Leaves of Soybean*

At the onset of a light period (see "Materials and Methods"), leaves were sprayed with Acifluorfen as described in the legend to Figure 1 and either placed in a dark chamber at 24°C, or returned to the illuminated culture chamber (24°C) and maintained on the 13-h light and 11-h dark photoperiod. Leaves were harvested 30 h after spraying and enzymes activities determined as described in "Materials and Methods." Values are averages of three independent experiments.

Treatment	PAL	CHS
	μkat/kg protein	
Dark		
Control	0.085	0.004
Treated	0.095	0.008
Light		
Control	0.220	0.003
Treated	2.520	0.880

FIG. 2. Time-dependent accumulation of the 7-*O*-glucosides of daidzein and formononetin in whole leaf extracts from soybean primary leaves after Acifluorfen treatment as described in the legend to Figure 1. Bars represent SE; *n* = 3.
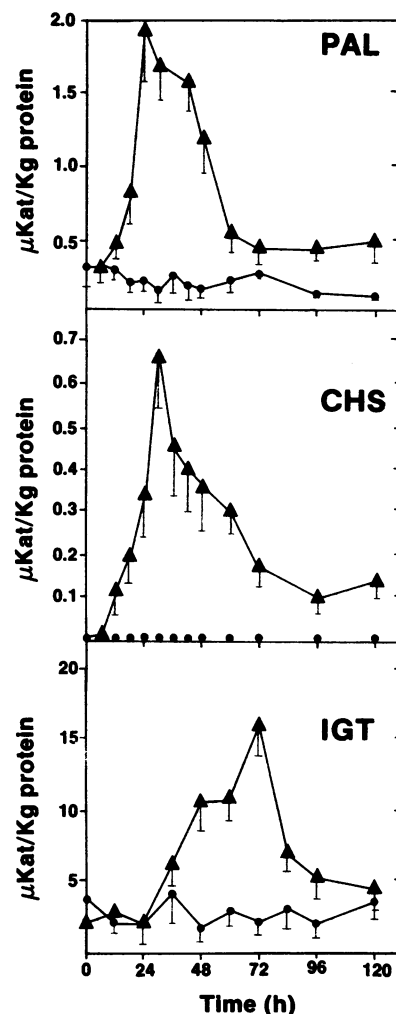

FIG. 3. Changes in PAL, CHS, and IGT activities in whole leaf homogenates from soybeans treated with Acifluorfen (▲) or with Triton X-100 alone (●) as described in the legend to Figure 1. Half bars represent SE; *n* = 3.

activity of 0.67 μkat/kg protein in whole mature soybean leaves is similar to that reported in soybean hypocotyls inoculated with *Phytophthora megasperma* (2), but PAL activity in the leaves is only 1.98 μkat/kg protein and that is about ⅓₀th of the activity

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reported in the hypocotyls (Fig. 3; Ref. 2). Maximal IGT activity in treated leaves was 16 μkat/kg protein, but untreated leaves had an activity of approximately 3 μkat/kg protein (Fig. 3). Since isoflavone glucosides were not found in untreated leaves this activity may have been due to nonspecific glucosylation of isoflavone aglycones by flavonol glucosyltransferases.

Crude extracts from treated leaves showed high glucosylating activity towards several isoflavone aglycones. Maximal activity for each aglycone tested was found 72 h after treatment. Using 100 μM substrate concentrations, IGT activity was consistently 15.7 (SE ± 2.5) and 16.1 (SE ± 2.9) μgkat/kg protein for daidzein and genistein, respectively, but only 9.6 (± 1.4) μkat/kg protein for formononetin. These differences in the ability of the enzyme preparations to glucosylate daidzein and formononetin are reflected in the ratios of aglycone to glucoside accumulating within the leaf (Figs. 1 and 2).

Activities of PAL, CHS, and IGT in Mesophyll Cells Isolated from Acifluorfen-Treated Leaves.

Mesophyll cells isolated by the procedures described above are over 90% intact, have low levels of contamination by other tissues, and retain high activities for various marker enzymes (8). No activity for PAL, CHS, or IGT could be detected in mesophyll cells isolated from untreated soybean leaves.

Table III shows that there is appreciable activity for PAL, CHS, and IGT in mesophyll cells isolated from Acifluorfen-treated leaves. At the stage of highest activity for each enzyme in whole leaves, 30 h for PAL and CHS and 72 h for IGT, mesophyll cells from treated leaves contained about 14% of the whole leaf activity for PAL, 24% of the CHS, and 20% of the IGT (Fig. 3). Enzyme activities were from 3 to 15 times the minimum detectable quantities (see "Materials and Methods"). French pressure cell treatment apparently did not diminish the activity of any enzyme assayed; in fact, passing filtered but uncentrifuged whole leaf homogenates through the pressure cell increased the activity of each enzyme by about 5%. This increase was probably caused by the release of enzymes trapped in cell fragments or vesicles. Dowex treatment did not increase the enzyme activity of cell lysates. No inhibition of enzyme activity was detected in assays containing both mesophyll cell lysates and whole leaf homogenates.

DISCUSSION

Recent investigations indicate that the mesophyll is not normally involved in flavonoid metabolism in peas (11) or in soybean (7). Flavonoids appear to be restricted to the epidermis

Table III. *Enzyme Activities in Whole Leaves and in Isolated Mesophyll Cells of Acifluorfen-Treated Soybeans*

Fully expanded primary leaves of 14-d-old plants were sprayed on the lower surface with 100 mg/l Acifluorfen in 0.01% (v/v) aqueous Triton X-100 and returned to the illuminated culture chamber. Samples were taken 30 h (PAL and CHS) or 72 h (IGT) after treatment, at the stage of highest activity for each of these enzymes in whole-leaf preparations (Fig. 1). Values are means ± SE of five independent experiments.

Enzyme	Whole Leaf	Mesophyll Cells	% Whole Leaf Activity in Mesophyll Cells
	μkat/kg Chl[a]		%
PAL	24.7 ± 5.8	3.50 ± 1.3	14 ± 3.3
CHS	6.5 ± 1.6	1.35 ± 0.7	24 ± 4.5
IGT	94.0 ± 18	18.0 ± 4.0	20 ± 6.0

[a] The primary leaves contained an average of 11 kg soluble protein/kg Chl. This factor permits a comparison of values in Table III with those in other tables and figures.

of these plants and their mesophyll cells normally lack certain key enzymes of flavonoid biosynthesis (7, 11; this report). In marked contrast; mesophyll cells isolated from oats and barley contain flavonoids, synthesize flavonoids from labeled precursors, and have high activity for several enzymes of flavonoid biosynthesis (22, 27; Netzley, Weissenböck, and McClure, unpublished). From this limited survey it appears that there may be basic differences in the tissue distribution of flavonoid metabolism in legumes and cereals.

In our attempt to induce flavonoid biosynthesis and accumulation in soybean mesophyll cells, we used the herbicide Acifluorfen which was reported to induce flavonoid phytoalexins in soybean leaves (15). To understand the role of the mesophyll, it was first necessary to characterize the process in whole leaves.

Several isoflavonoids (isoflavone aglycones, isoflavone glucosides, and pterocarpans) not detected in control plants were found in whole leaves sprayed with Acifluorfen. These effects seem to be directed primarily towards the isoflavonoid branch of the flavonoid pathway since there was no measurable change in the level of kaempferol-3-glycosides in the leaf. Between 24 and 48 h after the leaves were treated, their total flavonoid content was approximately doubled due entirely to the accumulation of isoflavonoids. About half of this increase was attributable to glyceollin which increased from almost zero to about 500 nmol/g fresh weight. At this stage the whole leaves contained approximately 2 μmol/g fresh weight total flavonoid (calculated from Figs. 1 and 2 and Table I).

This treatment also induced activity for CHS and markedly increased activity for PAL and for IGT in the whole leaf. Stages of highest enzyme activities correlated well with rates of product accumulation. PAL produces phenylpropanoid precursors for chalcone biosynthesis and its activity peaked at 24 h. CHS forms chalcones which are precursors for isoflavonoids and this activity was maximal at about 30 h. Isoflavone aglycones accumulated maximally at 48 h. IGT converts isoflavone aglycones into glucosides and its maximal activity was found at 72 h. The highest concentration of isoflavone glucosides was found at 96 h. Control leaves had slight activity for PAL which may have been associated with lignification of the vascular strands, and for IGT which might be attributed to nonspecific glucosylation by flavonol glucosyltransferases in the epidermis (11).

Daidzein is a precursor for other classes of isoflavonoids (9), and the Acifluorfen-treated leaves had higher IGT activity for daidzein than for formononetin (Table II). The preferential glucosylation of daidzein may explain why treated leaves accumulated far less daidzein than formononetin aglycone (Fig. 1).

Acifluorfen treatment had no effect on the qualitative or quantitative patterns of kaempferol-3-glycosides in whole soybean leaves, and these flavonol glycosides were absent from mesophyll cells isolated from control or treated leaves.

Mesophyll cells isolated from treated leaves contained about 25% of the total isoflavone glucosides of the leaf, but they had no kaempferol glucosides and only traces of the isoflavone aglycones and pterocarpans. Thus, Acifluorfen appears to specifically induce the isoflavone pathway and only the water-soluble isoflavone glucosides accumulate within the mesophyll.

We do not know where within the leaf the major fraction of these glycosides were accumulated. They may have accumulated primarily in the epidermis, but we have been unsuccessful in our attempts to obtain either intact epidermal peels or epidermal protoplasts from mature soybean leaves.

The presence of only trace amounts of isoflavone aglycones and pterocarpans in the mesophyll cells is not surprising since they are not known to accumulate within vacuoles or in any other cytoplasmic compartment (9, 18; G. Hrazdina, personal communication), although they do accumulate in extracellular droplets of liquid produced by fungal-infected plants and in the

medium of induced suspension cultures (1, 4, 9, 25). We suspect that these traces were contaminants liberated from extensively damaged cells in the small necrotic lesions and adsorbed onto the mesophyll cell walls when the leaf was cut into small strips during sample preparation.

Isoflavone phytoalexins generally accumulate in necrotic areas of infected organs (9), and preliminary experiments showed that when treated leaves were cut into lesion-containing and lesion-free areas, pterocarpans were detected only in the fractions containing the lesions (Cosio and Weissenböck, unpublished). Thus, we suggest that isoflavone aglycones and pterocarpans were produced primarily in or around the necrotic lesions caused by Acifluorfen, and that the isoflavone glucosides represent soluble detoxification products which accumulated in the vacuoles of mesophyll cells outside the area of the lesions.

We also found appreciable activity for PAL, CHS, and IGT in mesophyll cells isolated from treated leaves. About one-fourth of the whole leaf activity for CHS and IGT, and about 14% of the activity for PAL, was retained in the isolated mesophyll cells. Since we do not know where within the leaf the majority of activity for these enzymes was localized, and we have no direct evidence that the activity of these particular enzymes is rate-limiting for the accumulation of isoflavone glucosides, we ascribe less significance to the relative percentage of enzyme activity than to their induction in a tissue in which they are normally absent.

We previously reported that mesophyll cells isolated from mature soybean leaves contained no flavonoids and had no activity for PAL (7). In this paper we have shown that Acifluorfen induced mesophyll activity for PAL, CHS, and IGT, and led to the accumulation of the 7-O-glucosides of diadzein and formononetin in this tissue. To our knowledge this is the first report of tissue-specific induction of isoflavonoids and key enzymes of their biosynthesis in any plant.

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