Dihydroflavonol Reductase Activity in Relation to Differential Anthocyanin Accumulation in Juvenile and Mature Phase *Hedera helix* L.[1]

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ABSTRACT

Juvenile phase English ivy (*Hedera helix* L.) plants accumulate anthocyanin pigment in the hypodermis of stems and petioles, whereas genetically identical plants of the mature phase do not. The objective of this work was to assess which enzyme(s) might limit anthocyanin accumulation in mature phase ivy. Leaf discs of both juvenile and mature phase ivy accumulated comparable levels of the flavonols kaempferol and quercetin, whereas only juvenile phase discs accumulated anthocyanin. The accumulation of quercetin, but lack of accumulation of leucocyanidin or anthocyanin in mature phase discs, suggested that mature discs lacked dihydroflavonol reductase activity. There was no detectable dihydroflavonol reductase activity in mature phase discs, whereas there was an induction of activity in juvenile phase discs in response to sucrose, or photosynthetically fixed carbon, and light as a photomorphogenic signal. Phenylalanine ammonia-lyase, an enzyme early in the anthocyanin biosynthetic pathway, was induced above its basal level by sucrose and light in discs of both phases of ivy, with greater activity in mature phase discs. Phenylpropanoids, a class of compounds that are precursors to flavonoids, accumulated in leaf discs of both phases, with greater levels in mature phase discs. These results indicate that the lack of dihydroflavonol reductase activity limits the accumulation of anthocyanin in mature phase tissue.

During the protracted juvenile phase of development of the woody perennial English ivy (*Hedera helix* L.), anthocyanin pigment accumulates in collenchyma cells of the hypodermis of stems and petioles. With the transition at the shoot apices to the mature phase (phase change), subsequently formed shoot tissue does not accumulate anthocyanin in its homologous hypodermal tissue. Under some environmental conditions we have observed that the leaf laminae of juvenile phase ivy accumulate anthocyanin, whereas laminae of mature phase ivy do not. Apparently, with the transition from the juvenile to mature phase there is a loss of competence to accumulate anthocyanin, as well as changes in competence to express other phase-dependent characters, such as adventitious rooting and flowering potential. The long term goal of this research is to understand how stable differences in competence to express phenotypes or developmental processes are established and maintained during maturation.

Anthocyanins, one class of flavonoid pigments, are derived from photosynthetically produced carbohydrates via enzyme-catalyzed reactions of the shikimic acid, phenylpropanoid, and flavonoid biosynthetic pathways (12). Phenylpropanoids, which are derived from the shikimic acid pathway metabolite phenylalanine, are precursors to a large number of derivatives in addition to flavonoids, including the cell wall polymer lignin and various low mol wt conjugates such as chlorogenic acid. The formation of flavonoids from phenylpropanoids involves the condensation of three two-carbon units from malonyl-CoA with the activated phenylpropanoid 4-coumaroyl-CoA. Anthocyanins and other flavonoids accumulate as glycosides. In the case of ivy, it was postulated that the lack of anthocyanin accumulation in mature phase tissue could result from an absence or difference in activity of one or more phenylpropanoid or flavonoid biosynthetic enzymes, or rapid anthocyanin degradation.

The biosynthesis of anthocyanin in competent tissue of light grown plants of other species is typical of a photomorphogenic "high irradiance response," for which there is a dependence upon the irradiance level and its duration of treatment, but a failure of both reciprocity and red, far red photoreversibility (17). The analysis of the high irradiance photomorphogenic effect on phenylpropanoid and flavonoid metabolism can be confounded by a photosynthetic influence on carbohydrate supply (3, 18) and enzyme activity (3). The first objective of this research was to determine whether both juvenile and mature phase ivy are competent of responding to light and carbohydrates with regard to phenylpropanoid and anthocyanin biosynthesis.

In other plant species, mutant alleles of genes encoding flavonoid biosynthetic enzymes have been utilized to deduce the identity of the wild type gene product through an analysis of the resulting difference in metabolism. For example, a white flowering *Petunia hybrida*, which is homozygous recessive for a mutant allele of the *an3* locus, accumulates flavanones but not succeeding intermediates of the anthocyanin biosynthetic pathway nor anthocyanins (5). These plants putatively lack flavanone 3-hydroxylase activity (21), the enzyme that converts flavanones to dihydroflavonols (8), the subsequent intermediate in the anthocyanin biosynthetic pathway. Using a similar rationale, the primary objective of this report was to assess which enzyme(s) might limit the accumulation of anthocyanin in the mature phase of ivy based on a comparison of the composition of related metabolites and the

activity of the implicated enzyme(s) in juvenile and mature phase tissue.

To reach these two objectives, an *in vitro* leaf disc assay was developed. The leaf disc assay permitted testing the competencies of tissues of both phases of ivy that lacked pigmentation *in situ* and facilitated the administration of substrates under controlled conditions.

MATERIALS AND METHODS

Plant Material

A single genotype of juvenile and mature phase ivy (*Hedera helix* L.) was clonally propagated and grown under greenhouse conditions with a minimum temperature of 20°C. Juvenile phase plants were grown as single stem plants in 10 and 13 cm diameter containers, while mature phase plants were grown with four plants per 16 liter container. The plants were grown in a peat, perlite, sand medium (2:1:1). Half-strength Hoagland solution was applied as fertilizer with each irrigation. Active shoot growth was maintained by periodic pruning.

Leaf Disc Assay

The youngest fully expanded leaf on juvenile and mature phase plants was detached from the plant at the base of the petiole, between 7:00 and 9:00 AM for all experiments, and maintained with the petiole in deionized water for approximately 10 min. The leaves were surface sterilized in 0.5% sodium hypochlorite for 6 min with occasional agitation, then rinsed three times (6 min each) in sterile, deionized water.

The standard assays were conducted axenically in 10 cm Petri dishes with one piece of 9 cm Whatman No. 1 filter paper and 2 mL of filter sterilized 0.2 M sucrose in 10 mM Mes, adjusted to pH 6.0 with KOH. Treatments without sucrose were 10 mM Mes buffer alone. Ten leaf discs (1.2 cm in diameter) punched from the leaf laminae using a sterile cork borer were placed adaxial side down on the filter paper. Petri dishes were wrapped with Parafilm around their edges to prevent water vapor loss. Dishes were incubated in a growth chamber at 21 ± 0.5°C with a continuous mean PPFD of 400 μmol·m^{-2}·s^{-1} from Philips cool white (T12) fluorescent lamps. The PPFD was checked beneath a Petri dish cover with a LiCor LI-185 quantum photometer before the start of each experiment. Under the standard assay conditions with or without sucrose treatment, the steady-state atmospheric CO_2 level in the Petri dish was 100 μL/liter CO_2 as determined with an Anarad AR-600 infrared gas analyzer. Dark treatments were achieved by wrapping dishes in black cloth and then placing them under aluminum foil covers.

To reduce the atmospheric CO_2 levels within the Petri dishes below the standard assay level of 100 μL/liter CO_2, a CO_2 trap was constructed by floating an open 5.5 cm × 1 cm Petri dish, containing 1 mL of treatment solution, filter paper, and five leaf discs, on 15 mL of 0.1 N KOH within a 10 cm Petri dish wrapped with Parafilm. This trap resulted in a steady-state CO_2 concentration of 20 μL/liter. Elevated levels of CO_2 were attained by floating the 5.5 cm Petri dish on 15 mL of 0.4 M sodium bicarbonate. The CO_2 atmosphere over the 4 d of this treatment declined from 1.4 × 10^4 to 3 × 10^3

μL/liter and will be referred to by the median level of 5 × 10^3 μL/liter CO_2. Ambient levels of CO_2 (300 μL/liter) were attained by floating a 5.5 cm dish on 15 mL of water within the 10 cm dish, but without Parafilm wrap.

An experimental unit consisted of 10 discs/Petri dish for the standard assays and 5 discs/dish for assays with modified atmospheric CO_2.

Spectrophotometric Quantification

For extraction, leaf discs were placed in a vial with 1 mL of −20°C methanol-HCl (0.1% HCl, v/v) per disc and held at 2°C for 24 h. Spectrophotometric analysis of the extract was conducted with a Beckman DU-50 spectrophotometer. The A_{532} due to anthocyanin (cyanidin glycoside) was normalized for the A_{532} due to Chl by subtracting 24% of the absorbance at the Chl maximum of A_{653} from the A_{532} (18, 20).

The relative level of methanol extractable phenylpropanoids was approximated by determining the A_{329} of extracts diluted 40-fold with methanol-HCl, since hydroxylated phenylpropanoids and their conjugates have characteristic absorption spectra with maxima near A_{329} (20).

The absorbance of the extracts due to anthocyanin or phenylpropanoids was assumed to be directly proportional to the molar concentration of these metabolites in the extracts. Therefore, the absorbance values have been equated with the level of accumulation of anthocyanin or extractable phenylpropanoids by the leaf discs in response to the given treatments.

To estimate the level of accumulation of leucocyanidin in mature phase leaf discs, 1 mL of the methanolic extracts was evaporated to near dryness under a stream of nitrogen gas, 1 mL of butanol-HCl (95:5, v/v) reagent was added, and the sample heated at 95°C for 30 min. The remaining disc tissue was treated with 1 mL of the butanol-HCl reagent per disc to assay for nonextractable, oligomeric proanthocyanidins. Treatment with butanol-HCl results in the acid catalyzed conversion of leucocyanidin and proanthocyanidin to cyanidin, which has a maximum A_{550} in the butanol solvent (23).

HPLC Analysis

Ten leaf discs per assay were ground in liquid nitrogen using a mortar and pestle. The tissue was transferred to vials and extracted with 10 mL of methanol-HCl at −20°C for 24 h. Five milliliters of each methanolic extract were taken to near dryness under a stream of nitrogen, then dissolved in 2 mL of 2 N aqueous HCl. The HCl solutions were heated at 100°C for 1 h to yield nonconjugated phenylpropanoids and flavonoids (14, 19). The cooled solutions were partitioned twice against 0.75 mL of ethyl acetate. Aliquots of the combined ethyl acetate fractions were used for HPLC (Waters models 590 and 510 pumps and 680 gradient controller) and on-line spectrophotometry (Hewlett Packard 1040A diode array detector). Authentic phenylpropanoids, flavanones, flavones, dihydroflavonols, and flavonols were used to establish chromatographic and spectral parameters for the identification of metabolites in the sample preparations, which excluded the cationic anthocyanidins. Phenylpropanoid analysis and a survey of flavonoids were conducted using a 24 min

linear gradient of 40 to 100% methanol in 0.005% H_3PO_4 (v/v) using a 4.6 × 150 mm Maxil 5 μm, C_{18} column. Quantitative analysis of the flavonols kaempferol and quercetin was conducted using a PRP-1 4.6 × 150 mm column and a 25 min linear gradient of 50% solvent A (0.1 N acetic acid in methanol) in solvent B (0.1 N acetic acid) to 100% solvent A. Before acid hydrolysis, an aliquot of the methanolic extracts used for flavonol analysis was utilized to estimate the cyanidin glycoside content of the extracts by spectrophotometry using a mM absorption coefficient of 30 (25).

Enzyme Extraction Procedure

An experimental unit consisted of six Petri dishes (10 cm) with 10 leaf discs (1.2 cm diameter)/dish, giving a fresh weight of approximately 1.5 g. For leaf disc assays with 300 μL/liter or a median level of 5 × 10^3 CO_2, an experimental unit consisted of 12 Petri dishes with five leaf discs per dish.

All steps of the extraction were carried out at 0 to 2°C except where noted. Leaf discs were ground in liquid nitrogen using a mortar and pestle. The tissue was transferred to graduated cylinders and homogenized with 70 mL of −20°C acetone for 30 s using a Polytron. Acetone powders were collected by filtration and washed with two additional volumes of −20°C acetone, then dried at room temperature.

Acetone powders were extracted with 4 mL of 0.1 M borate buffer, pH 8.8, containing 5 mM sodium ascorbate, 0.3 g dry Polyclar AT, and 0.5 g dry XAD-4 (23, 24). The extract was pressed through Miracloth and centrifuged at 12,000g for 10 min. Aliquots of the supernatant were used to assay for PAL[2] enzyme activity. The remaining supernatant was passed through a 1 × 5 cm Sephadex G-25 (fine) column equilibrated with 0.1 M Tris buffer, pH 7.4, containing 5 mM sodium ascorbate. Aliquots of the eluate that contained the protein fraction were used as the enzyme source for the DFR assay. Protein was estimated by the method of Bradford (2) using BSA as a standard.

Enzyme Assays

PAL

PAL was assayed according to a modified procedure of Vance and Sherwood (26). The 1.1 mL incubation mixture contained 0.1 M borate buffer, pH 8.8, 3 μmol L-[2,3,4,5,6-^{3}H]-phenylalanine (specific activity 0.02 μCi/μmol), and 0.1 mL of enzyme extract. The reaction mixture was incubated at 30°C for 1 h and the reaction was stopped by the addition of 0.1 mL of 6 N HCl. The mixture was extracted twice with 2 mL of ethyl acetate and the combined ethyl acetate fraction dried with anhydrous sodium sulfate. Ten milliliters of scintillation fluid (Ecolite by Westchem) were added to the ethyl acetate fraction and counted in a liquid scintillation spectrometer (Beckman LS 3801). Specific activity of PAL for the samples was determined after subtracting the values of control assays consisting of complete assays with heat inactivated enzyme.

DFR

DFR was assayed according to the method of Stafford and Lester (23, 24). The 1.1 mL incubation mixture contained 0.1 M Tris buffer, pH 7.4, 1 μmol (±) dihydroquercetin, 1 μmol NADPH, 0.1 to 0.3 mL of the enzyme extract, and a NADPH regenerating system consisting of 1 unit of glucose-6-phosphate dehydrogenase and 6 μmol of glucose-6-phosphate. The assay mixture was incubated at 30°C for 1 to 3 h, then immediately extracted three times with 1 mL of ethyl acetate, which was combined and then extracted three times with 0.2 mL of H_2O. The ethyl acetate fraction was evaporated to near dryness under a stream of nitrogen gas. The number of nmol of leucocyanidin in the ethyl acetate fraction was estimated from the acid catalyzed conversion of the leucocyanidin to cyanidin. This was accomplished by the addition of 1 mL butanol-HCl (95:5 v/v) reagent to the evaporated ethyl acetate fraction and heating at 95°C for 30 min. The absorbance due to cyanidin in the butanol solvent at the maximum A_{550} (mM absorption coefficient = 34.7) (23) was normalized for the A_{550} due to Chl by subtracting the A_{600} from the A_{550}. Control assays were conducted using complete assays with heat inactivated enzyme.

Experimental Design

The experiments were conducted as completely randomized, or complete randomized block designs. An analysis of variance of the data was conducted. The P values presented represent the probability of observing an outcome at least as extreme as the data observed, given the null hypothesis that there is no treatment effect. The least significant difference for mean separation was by Tukey's honest significant difference. The SE and the pooled SE are presented for the HPLC data and figures, respectively, as an estimate of variability.

RESULTS

Effect of Light and Carbohydrates on Anthocyanin and Phenylpropanoids

To test whether there was an effect of light and carbohydrates on phenylpropanoid and flavonoid metabolism in tissue of both juvenile and mature phase ivy, leaf discs were treated in the presence or absence of light and sucrose, at reduced levels of atmospheric CO_2 (20 μL/liter). For dark treated discs, there was not a significant increase of methanol-extractable phenylpropanoids in discs treated with sucrose as compared with discs treated without sucrose (Table I). There was a large accumulation of extractable phenylpropanoids in discs of both phases in response to the combination of sucrose and light, with greater accumulation in mature phase discs. Juvenile leaf discs accumulated anthocyanin in response to sucrose in the light, but not in the dark. The anthocyanin accumulated in the palisade and spongy parenchyma of juvenile phase leaf discs, but not in the epidermal or vascular tissues. Mature phase leaf discs did not accumulate anthocyanin in any combination of light and sucrose treatment, even with levels up to 600 μmol·m^{-2}·s^{-1} and 0.4 M, respectively (data not presented). Thus, leaf discs of both phases of ivy responded to light and carbohydrates in phenylpropanoid

Table I. *Effect of Light and Sucrose on Extractable Phenylpropanoids and Anthocyanin*

The mean absorbance (accumulation) of phenylpropanoids and anthocyanin in methanolic extracts of juvenile and mature phase leaf discs treated with a 20 μL/liter CO_2 atmosphere for 4 d. Extracts were diluted 40-fold for the determination of the phenylpropanoid absorbance.

Light	Sucrose	Phenylpropanoid Absorbance		Anthocyanin Absorbance	
		Juvenile	Mature	Juvenile	Mature
$\mu mol \cdot m^{-2} \cdot s^{-1}$	M	A_{329}		A_{532}-$0.24[A_{653}]$	
0	0.0	0.14	0.18	0.00	0.00
0	0.2	0.22	0.25	0.00	0.00
400	0.0	0.23	0.28	0.00	0.00
400	0.2	0.71	0.93	0.84	0.00
		$HSD_{0.05}{}^a = 0.11$		$HSD_{0.05} = 0.18$	

[a] HSD, Tukey's honest significant difference.

biosynthesis, but as observed *in situ*, mature phase discs lacked competence to accumulate anthocyanin. Analysis of variance demonstrated a highly significant interaction between sucrose and light on the accumulation of phenylpropanoids in both phases ($P < 0.001$) and anthocyanin in juvenile discs ($P < 0.001$). The lack of response to sucrose in the absence of light indicates that there was an effect of light that was independent of carbohydrate availability. The lack of response to light without sucrose, when discs were treated with low levels of atmospheric CO_2, indicates that carbohydrate levels were limiting.

Leaf discs of both phases treated with ambient CO_2 levels (300 μL/liter) in light had increased levels of phenylpropanoids as compared with dark treated discs, and juvenile phase discs accumulated anthocyanin in the light, but not in the dark. Thus, the requirement for carbohydrates could be met by photosynthetic or exogenous carbohydrates.

At concentrations of 10^{-5} M or higher, glyphosate, a specific inhibitor of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase of the shikimic acid pathway (1), prevented the accumulation of extractable phenylpropanoids in leaf discs of both phases and anthocyanin in juvenile phase leaf discs treated with 0.1 M sucrose in light (Fig. 1). The similarity of the effect of glyphosate concentration on the degree of inhibition of phenylpropanoid and anthocyanin accumulation indicates that phenylpropanoid precursors of anthocyanin were limiting, and the accumulation of anthocaynin in juvenile discs depended upon an increase in the availability of phenylpropanoid intermediates of the biosynthetic pathway.

Phenylpropanoid and Flavonoid Compositional Analysis

An analysis of leaf discs treated in light for 4 d was conducted to determine which phenylpropanoid and flavonoid moieties biosynthetically related to anthocyanins accumulated. To reduce the diversity of products that arise through conjugation, the methanolic extracts were subjected to acid hydrolysis to yield nonconjugated phenylpropanoid and fla-

vonoid moieties, except that the anthocyanin (a cyanidin glycoside) level was estimated before hydrolysis. In both phases, four phenylpropanoids were identified (Table II). There was a three- to fivefold increase in the levels of the phenylpropanoids in response to sucrose. Caffeic acid was the predominant phenylpropanoid present in discs of both phases treated with and without sucrose. Mature phase leaf discs had greater levels of caffeic acid than did juvenile phase discs.

The flavonols kaempferol and quercetin were the only flavonoids identified that accumulated at detectable levels in leaf discs treated with sucrose and light, except for the accumulation of a cyanidin glycoside in juvenile discs (Table III). Comparable levels of kaempferol and quercetin accumulated in discs of the two phases. Low or nondetectable levels of flavonols were present in juvenile and mature phase discs treated in light without sucrose (data not presented). Dihydrokaempferol and dihydroquercetin, immediate precursors of kaempferol and quercetin (Fig. 2), did not accumulate at detectable levels.
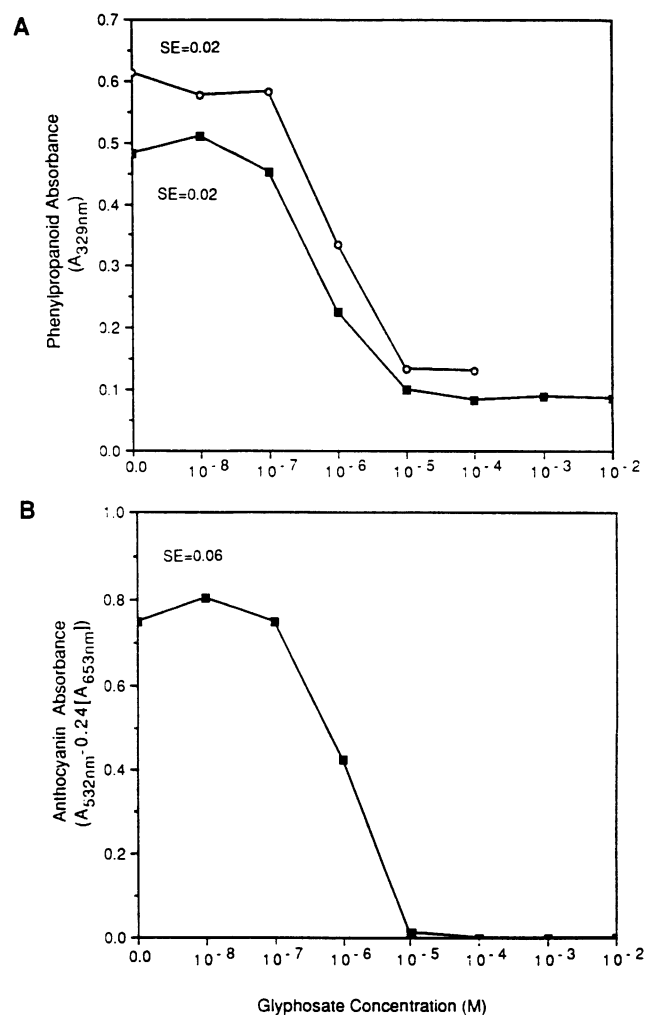

Figure 1. Effect of glyphosate on the absorbance (accumulation) of (A) extractable phenylpropanoids and (B) anthocyanin from leaf discs treated with 0.1 M sucrose for 4 d. ■, Juvenile; ○, mature.

Table II. *Extractable Phenylpropanoids of Juvenile and Mature Phase Leaf Discs*

The mean absorbance (±SE) of methanol extractable phenylpropanoids from juvenile and mature phase leaf discs treated with or without sucrose in 400 μmol·m^{-2}·s^{-1} light for 4 d. Extracts were subjected to an acid hydrolysis to yield nonconjugated phenylpropanoids before separation by HPLC and on-line spectrophotometry. Absorbance was determined at the absorption maximum of each compound. Differences in the molar extinction coefficients of the metabolites and the solvent composition in which they were eluted precludes direct molar comparisons of individual phenylpropanoids.

Phase	Sucrose	Absorbance			
		Cinnamic (A_{283})	4-Coumaric (A_{309})	Caffeic (A_{325})	Ferulic (A_{323})
	M	*milli-absorbance units*			
Juvenile	0.0	16 ± 9.3	7.0 ± 0.6	43 ± 5.2	7.3 ± 0.9
Mature	0.0	13 ± 1.5	8.0 ± 1.5	116 ± 29	11 ± 2.2
Juvenile	0.2	44 ± 13	19 ± 2.6	210 ± 22	18 ± 1.8
Mature	0.2	40 ± 7.5	27 ± 7.0	305 ± 34	36 ± 6.8

The qualitative difference in the cyanidin glycoside content between the two phases was not proportionally related to the small quantitative difference in flavonols (Table III). The accumulation of the flavonol quercetin in both phases indicates that the biosynthetic intermediate dihydroquercetin (Fig. 2) was present in both phases, even though it did not accumulate at detectable levels. The implied presence of dihydroquercetin and the actual presence of a glycoside of cyanidin in juvenile phase discs indicates that there was activity of the enzymes that catalyze the conversion of dihydroquercetin to leucocyanidin and cyanidin. Colorimetric assays of methanolic extracts of leaf discs and the residual disc tissue treated with the butanol-HCl reagent at 95°C for 30 min indicated that there was no detectable leucocyanidin nor proanthocyanidin, respectively, in the mature phase discs. The implied presence of dihydroquercetin in mature phase discs, but lack of accumulation of leucocyanidin, cyanidin, or proanthocyanidin, suggests that there was an absence of activity of DFR, the biosynthetic enzyme that catalyzes the formation of leucocyanidin from dihydroquercetin.

Enzyme Activity as a Function of Phase and Time

Juvenile and mature phase leaf discs were treated with sucrose and light to determine whether there was detectable activity of DFR and PAL in both phases as a function of treatment time. PAL catalyzes the formation cinnamic acid, the first phenylpropanoid, by the deamination of phenylalanine (13). PAL was selected to be assayed in addition to DFR to verify that mature phase tissue was competent to respond to light with regard to phenylpropanoid enzymatic activity. There was a basal level of PAL activity in leaf discs of both phases at the initiation of the time course experiment (Fig. 3A). PAL activity reached a maximum at d 2 of treatment for mature phase leaf discs and d 2 to 3 for juvenile phase discs, with subsequent declines in activity. In this experiment, mature leaf discs had twice the specific activity of PAL as

juvenile discs. DFR activity was not detectable in extracts of mature phase leaf discs sampled daily during the 5 d of treatment (Fig. 3B). In contrast, extracts of juvenile phase leaf discs had detectable DFR activity by d 2 of treatment and the activity increased up to day 4. There was no change in activity from d 4 to 5. A basal level of extractable phenylpropanoids was present in the leaf laminae of both phases of ivy at the initiation of the time course experiment (Fig. 3A). In contrast, at time zero there was no anthocyanin in the laminae of either phase (Fig. 3B). There was greater accumulation of phenylpropanoids in mature discs than juvenile discs (P < 0.001), but anthocyanin accumulated only in juvenile discs. Temporally, there was an apparent correlation between enzyme activity and the accumulation of the respective biosynthetic metabolites.

In none of the many experiments performed was there detectable DFR activity in extracts of mature phase leaf discs. The limit of detection of the enzyme assay was approximately 3 milli-absorbance units, equal to 0.086 nmol of the reaction product. The values observed for extracts of juvenile leaf disks ranged from 15 to 53 milli-absorbance units (0.43–1.5 nmol)/ assay, indicating that there was at least 5- to 18-fold greater DFR activity in juvenile discs as compared with mature discs.

When aliquots of comparable levels of protein from mature preparations were added to DFR assays of juvenile enzyme preparations, the mean specific activity of DFR was 0.32 nmol·h^{-1}·(mg protein)$^{-1}$ for assays with and without the addition of mature enzyme preparation. This result suggests that the lack of DFR activity from mature phase leaf discs was not due to an endogenous inhibitor of DFR activity. Without the addition of NADPH to the enzyme assays there was no detectable DFR activity in extracts of juvenile leaf discs, indicating that the reaction was NADPH-dependent.

Carbohydrates, Light, and Enzyme Activity

To test whether there was an effect of carbohydrates or light on the activity of PAL and DFR, leaf discs were treated with or without sucrose, in the presence or absence of light. For dark treated leaf discs, PAL activity was not significantly greater in discs incubated with sucrose as compared with discs without sucrose, nor was there a difference in PAL activity between juvenile and mature phase discs (Table IV). For leaf discs incubated without sucrose, there was not a significant

Table III. *Extractable Flavonoids from Juvenile and Mature Phase Leaf Discs*

The mean flavonoid content (±SE) of juvenile and mature phase leaf discs treated with 0.2 M sucrose in 400 μmol·m^{-2}·s^{-1} light for 4 d. Methanolic extracts were split for the determination of (A) flavonoids (hydrolyzed aglycones) excluding anthocyanidin and (B) cyanidin glycoside.

Flavonoid	Juvenile	Mature
	nmol/leaf disc	
A. Kaempferol	43 ± 4.0	31 ± 3.8
Quercetin	80 ± 6.2	70 ± 8.4
B. Cyanidin glycoside	21 ± 5.5	0.0 ± 0.0

3x Malonyl-CoA   4-Coumaroyl-CoA

Naringenin Chalcone

Naringenin ⟶ Eriodictyol

Dihydrokaempferol → Dihydroquercetin → Leucocyanidin → Cyanidin

Kaempferol   Quercetin   Flavan-3-ol Proanthocyanidin

Figure 2. A partial schematic of the flavonoid biosynthetic pathway. 4-Coumaroyl-CoA is derived from the shikimic acid pathway via phenylpropanoid intermediates.
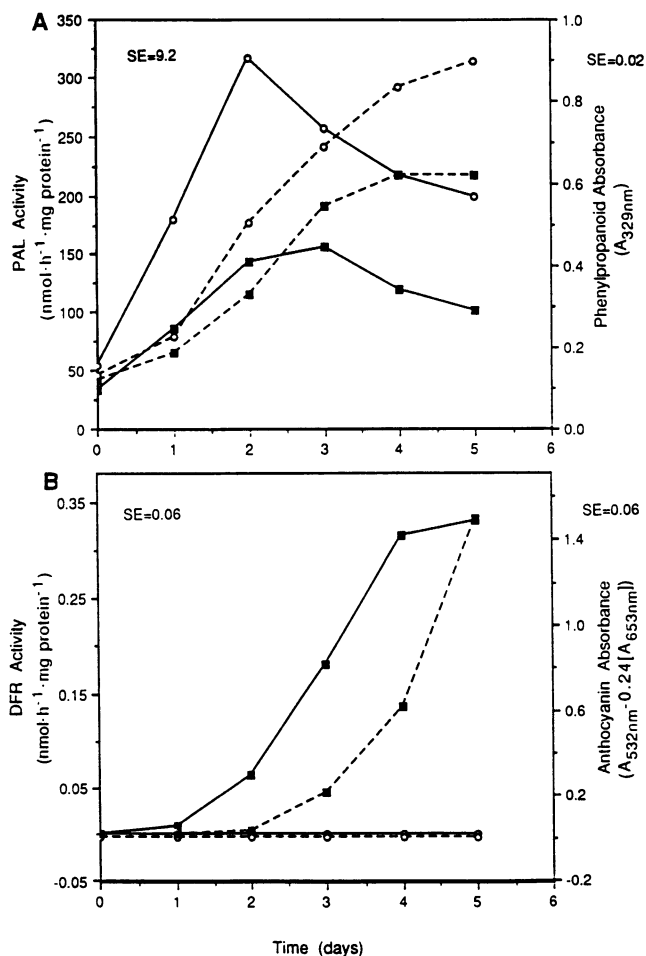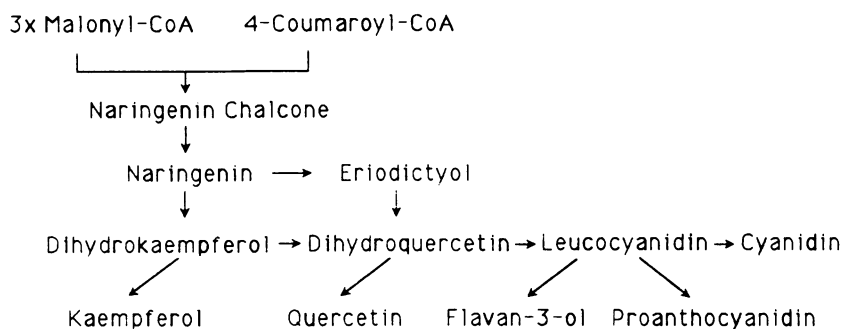

Figure 3. Effect of treatment time on (A) PAL activity and absorbance (accumulation) of extractable phenylpropanoids and (B) DFR activity and anthocyanin absorbance (accumulation) of juvenile and mature phase leaf discs treated with 0.2 M sucrose and 400 μmol·m^{-2}·s^{-1} light. —, Enzyme activity; - - - -, absorbance; ■, juvenile; ○, mature.

difference in PAL activity of discs treated in light as compared with discs treated in the dark. Treatment of leaf discs of both phases with sucrose in light increased the levels of PAL activity by greater than fivefold as compared with discs incubated without sucrose in the dark. Thus, there was a highly significant interaction ($P < 0.001$) of sucrose and light on PAL activity of leaf discs of both phases. The response to sucrose was dependent upon the presence of light, and the response to light was dependent upon the presence of sucrose. There was a significant phase effect ($P = 0.026$), with 27% greater PAL activity in mature phase leaf discs than in juvenile discs treated with sucrose and light.

Leaf discs of neither phase had detectable DFR activity when treated in the absence of light, nor when treated in light without sucrose (Table IV). Only juvenile phase leaf discs treated with sucrose in light had DFR activity. This phase-specific induction of DFR activity in juvenile discs was also dependent upon the interaction of sucrose and light, as indicated by a significant three-way interaction ($P < 0.001$).

Juvenile phase leaf discs treated with light, but without sucrose, had greater DFR activity and anthocyanin content with increased atmospheric CO_2 levels (Table V). Correlation analysis indicates a significant ($P = 0.006$) positive relationship between DFR activity and the anthocyanin content of

Table IV. *Effect of Light and Sucrose on PAL and DFR Activity*

The mean activity of PAL and DFR at d 2 and 4 of treatment, respectively, of juvenile and mature phase leaf discs.

Light	Sucrose	Enzyme Activity			
		PAL		DFR	
		Juvenile	Mature	Juvenile	Mature
μmol·m^{-2}·s^{-1}	M	nmol·h^{-1}·[mg protein]$^{-1}$			
0	0.0	34	44	ND[a]	ND
0	0.2	49	64	ND	ND
400	0.0	66	66	ND	ND
400	0.2	188	238	0.23	ND

HSD$_{0.05}$[b] = 49   HSD$_{0.05}$ = 0.02

[a] ND, not detectable.   [b] HSD, Tukey's honest significant difference.

Table V. *Effect of Atmospheric CO_2 on DFR Activity and Anthocyanin Accumulation*

The mean DFR activity and anthocyanin absorbance of extracts of juvenile phase leaf discs treated with 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for 4 d.

CO_2	DFR Activity	Anthocyanin Absorbance
$\mu L/liter$	$nmol \cdot h^{-1} \cdot [mg\ protein]^{-1}$	A_{532}-$0.24[A_{653}]$
100	ND[a]	0.00
300	0.20	0.37
5000	0.37	1.87
	$HSD_{0.05}$[b] = 0.26	$HSD_{0.05}$ = 0.71

[a] ND, not detectable.   [b] HSD, Tukey's honest significant difference.

the leaf discs. These data indicate that photosynthetically produced carbohydrates, as well as exogenous sucrose, were effective in inducing DFR activity and the correlated accumulation of anthocyanin.

DISCUSSION

The stable difference in competence to accumulate anthocyanin in the mesophyll cells of juvenile and mature phase leaf discs parallels the difference in competence to accumulate anthocyanin in the hypodermis of stems and petioles. The leaf disc assay provided a convenient system for the analysis of the composition of metabolites that were synthesized in response to light and carbohydrates.

It was postulated that a stable difference in the competence to express activity of one or more enzymes of anthocyanin biosynthesis or degradation prevents the accumulation of anthocaynin in mature phase tissue, in contrast to juvenile phase tissue. The accumulation of phenylpropanoids in mature phase discs as well as juvenile phase discs (Table I) indicated that mature phase tissue was competent to respond to light and carbohydrates. Although extractable phenylpropanoids were present in the lamina of leaves at the initiation of the leaf disc assays (Fig. 3A), the dependence of anthocyanin accumulation in juvenile phase discs on further phenylpropanoid biosynthesis was demonstrated (Fig. 1, A and B). Therefore, the greater accumulation of extractable phenylpropanoids (Table I; Fig. 1), especially caffeic acid and its conjugates (Table II), in mature than juvenile phase leaf discs could represent metabolic products that compete for precursors of flavonoid biosynthesis. However, the only slightly lower level of flavonols than juvenile phase discs that accumulated in mature discs (Table III) suggests that there was not a substantial limitation of phenylpropanoid precursors, nor malonyl-CoA. This conclusion is further supported by the comparable levels of 4-coumaric acid (Table II), the phenylpropanoid moiety that is utilized for flavonoid biosynthesis, in the hydrolyzed extracts of the two phases.

The comparable accumulation of flavonols in both phases (Table III), but the lack of detectable accumulation of other flavonoid products in mature phase discs that would compete for common biosynthetic intermediates, such as the oligo-

meric proanthocyanidins, suggests that flavonoid precursors of the anthocyanin were not limiting.

The accumulation of the flavonol quercetin and the implied presence of dihydroquercetin in mature phase discs, but lack of accumulation of leucocyanidin, cyanidin, or proanthocyanidin, led to the tentative conclusion that there was an absence of activity of DFR, the biosynthetic enzyme that catalyzes the formation of leucocyanidin from dihydroquercetin. This conclusion is based upon the assumption that the synthesis of dihydroquercetin and quercetin in mature phase discs was localized in the same cell types and intracellular compartments in which anthocyanin synthesis occurs in the juvenile phase. The lack of detectable DFR activity in extracts from mature leaf discs strongly supports the conclusion that DFR activity is limiting in mature phase tissue. This conclusion is further supported by the parallel increase in DFR activity and accumulation of anthocyanin as a function of treatment time (Fig. 3B) and the parallel response of DFR activity and anthocyanin accumulation to increased atmospheric CO_2 levels by the competent juvenile phase leaf discs (Table V). A parallel to the flavonoid metabolism found in mature phase ivy has been reported for a defined mutant of *Matthiola incana*. White flowers of *Matthiola* lines, which are homozygous for a mutant allele of the *e* locus accumulate flavonols but not anthocyanins (7), and this lack of anthocyanin is the result of the lack of DFR activity (15).

The data presented do not exclude the possibility that mature phase-dependent degradation of anthocyanin occurs in ivy. However, anthocyanins are stable in tissues of other plant species, even after the cessation of their biosynthesis (4, 16).

The enzyme that catalyzes the formation of cyanidin from leucocyanidin has not been characterized *in vitro* (22). The accumulation of the cyanidin glycoside indicates that this enzyme is expressed in the competent juvenile phase tissue. It was not possible to determine whether this enzyme is expressed in mature phase tissue; however, it may represent another enzyme that is differentially expressed in juvenile and mature phase tissues. The greater specific activity of PAL (Fig. 3; Table IV) and the greater accumulation of phenylpropanoids (Table I) in mature than juvenile phase discs represent another phase-dependent difference in metabolism, although the difference is not a qualitative one as observed with DFR and anthocyanin accumulation.

The lack of inhibition of DFR assays of juvenile preparations by the addition of mature enzyme preparation suggests that the lack of DFR activity in extracts from mature phase leaf discs was not due to an endogenous inhibitor of high mol wt. The Sephadex G-25 chromatography separated the high mol wt protein fraction used for the DFR assay from low mol wt metabolites. This separation eliminates, or greatly reduces, the possibility that the lack of DFR activity in mature enzyme preparations was due to a freely dissociable low mol wt inhibitor.

The accumulation of the flavonols quercetin and kaempferol by juvenile phase leaf discs indicates that their biosynthetic precursors, dihydroquercetin and dihydrokaempferol, were present in the tissue, although not at detectable levels. The dihydroflavonols dihydroquercetin and dihydrokaempferol are biosynthetic intermediates of the anthocyanidins

cyanidin and pelargonidin. DFR catalyzes a reduction of ring C of dihydroflavonols, the first of the two or possibly more steps in the conversion of dihydroflavonols to anthocyanidins. The accumulation of cyanidin but not pelargonidin, the anthocyanidin derived from dihydrokaempferol, in the juvenile tissue (data not presented) suggests that DFR of ivy can reduce dihydroquercetin but not dihydrokaempferol. DFR from *Petunia* catalyzes the reduction of dihydroquercetin and dihydromyricetin, another dihydroflavonol, but not dihydrokaempferol (9). DFR from *Matthiola* does not exhibit narrow substrate specificity and catalyzes the reduction of dihydrokaempferol, dihyroquercetin, and dihydromyricetin (15).

The presence of PAL activity and extractable phenylpropanoids in discs from juvenile and mature phase leaves, before experimental treatments (time 0; Fig. 3A), indicates that there was a basal level of phenylpropanoid metabolism at the stage when the leaves become fully expanded. This metabolism was likely related to the biosynthesis of lignin precursors, utilized during vascularization of the leaves, as well as the production of low mol wt conjugates. The phenylpropanoid conjugates chlorogenic acid and isochlorogenic acid accumulate in high concentrations as metabolic products in leaves of juvenile and mature phase ivy (10). The phenylpropanoid moiety of these two conjugates is caffeic acid. Therefore, the presence of caffeic acid as the predominant phenylpropanoid in the hydrolyzed extracts of both phases (Table IV) is consistent with previous findings. The level of extractable phenylpropanoids at time zero (Fig. 3) was similar to the low levels of phenylpropanoids extracted from leaf discs treated for 4 d in the dark, or in the light without sucrose (Table I), indicating that there was little or no net accumulation with these treatments. DFR activity and anthocyanin accumulation were not detected at d 0 (Fig. 3B) in the leaf laminae of juvenile and mature phase plants grown at temperatures above 20°C.

The increase of PAL activity in both phases and DFR activity in juvenile leaf discs was observed in response to a highly significant interaction of sucrose and light (Table I). This response of PAL and DFR to sucrose in light, but not in the dark, indicates that light played a role in the induction of enzyme activity. This effect of light is consistent with the photomorphogenic "high irradiance response" observed in plant tissues grown in light (17). The increase in PAL and DFR activity in response to light in the presence of sucrose, but not in the absence of sucrose when treated with limiting CO_2, indicates that sucrose had a role in the induction of enzyme activity. With an increased atmospheric CO_2 level, there was an induction of DFR activity and accumulation of anthocyanin by juvenile phase discs. Thus, photosynthetically produced or exogenously supplied carbohydrates interacted with a photomorphogenic effect of light in the induction of anthocyanin biosynthetic enzyme activity. A similar effect of carbohydrates on PAL activity is observed in strawberry leaf discs (3).

The accumulation of phenylpropanoids and anthocyanin in juvenile phase leaf discs required a sucrose-dependent increase in metabolites beyond the glyphosate sensitive step of the shikimic acid pathway (Fig. 1). Therefore, sucrose treatment has two interdependent roles in the accumulation of phenylpropanoids and anthocyanin in juvenile ivy—that

of a general substrate and that of an inducer of enzyme activity.

The light-induced accumulation of flavonoid glycosides in parsley cell suspension cultures involves the selective and coordinated induction of enzyme activity of general phenylpropanoid and flavonoid metabolism (6, 11). The patterns of changes in activity of general phenylpropanoid enzymes can be distinguished from those of flavonoid enzymes for parsley cultures. Flavonoid enzymes have a longer lag period and reach maximal activity later than phenylpropanoid enzymes. In juvenile ivy leaf discs, a similar pattern was observed for PAL (a general phenylpropanoid enzyme) and DFR (a flavonoid enzyme), however, the response was slower than in the parsley cells.

In conclusion, the lack of competence to accumulate anthocyanin in shoot tissue formed during the mature phase of development of ivy was the result of a lack of activity of the anthocyanin biosynthetic enzyme DFR. In contrast, there was an induction of DFR activity and an accumulation of anthocyanin in response to carbohydrates and light by competent juvenile phase tissue. The induction of PAL activity and accumulation of phenylpropanoids and flavonols in mature phase tissue demonstrates that the mature phase was responsive to carbohydrates and light. Therefore, the lack of induction of DFR activity was apparently specific and did not represent a general inactivity of the biosynthetic pathway. The greater activity of PAL and accumulation of phenylpropanoids in mature than juvenile phase discs represents another phase-dependent difference in expression. This may be related to differences in other aspects of phenylpropanoid metabolism in juvenile and mature phase tissue, such as differences in lignin biosynthesis or the accumulation of other phenylpropanoid products. Future research will test hypotheses regarding mechanisms by which DFR is inducible in juvenile phase tissue, but not inducible in tissue of mature phase ivy.

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