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Pigmented Soybean (*Glycine max*) Seed Coats Accumulate Proanthocyanidins during Development[1]

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The dominant *I* gene inhibits accumulation of anthocyanin pigments in the epidermal layer of soybean (*Glycine max*) seed coats. Seed-coat color is also influenced by the *R* locus and by the pubescence color alleles (*T*, tawny; *t*, gray). Protein and RNA from cultivars with black (*i,R,T*) and brown (*i,r,T*) seed coats are difficult to extract. To determine the nature of the interfering plant products, we examined seed-coat extracts from Clark isogenic lines for flavonoids, anthocyanins, and possible proanthocyanidins by thin-layer chromatography. We show that yellow seed-coat varieties (*I*) do not accumulate anthocyanins (anthocyanidin glycosides) or proanthocyanidins (polymeric anthocyanidins). Mature, black (*i,R,T*) and imperfect-black (*i,R,t*) seed coats contained anthocyanins, whereas mature, brown (*i,r,T*) and buff (*i,r,t*) seed coats did not contain anthocyanins. In contrast, all colored (*i*) genotypes tested positive for the presence of proanthocyanidins by butanol/HCl and 0.5% vanillin assays. Immature, black (*i,R,T*) and brown (*i,r,T*) seed coats contained significant amounts of procyanidin, a 3′,4′-hydroxylated proanthocyanidin. Immature, black (*i,R,T*) or brown (*i,r,T*) seed-coat extracts also tested positive for the ability to precipitate proteins in a radial diffusion assay and to bind RNA in vitro. Imperfect-black (*i,R,t*) or buff (*i,r,t*) seed coats contained lesser amounts of propelargonidin, a 4′-hydroxylated proanthocyanidin. Seed-coat extracts from these genotypes did not have the ability to precipitate protein or bind to RNA. In summary, the dominant *I* gene controls inhibition of not only anthocyanins but also proanthocyanidins in soybean seed coats. In homozygous recessive *i* genotypes, the *T-t* gene pair determines the types of proanthocyanidins present, which is consistent with the hypothesis that the *T* locus encodes a microsomal 3′-flavonoid hydroxylase.

Commercially grown soybean (*Glycine max*) varieties have yellow seed. However, some soybean varieties are self-colored and accumulate anthocyanins within the epidermal layer of the seed coat. The *I* locus and the *R* locus along with the pleiotropic *T* locus have major effects on seed-coat color (reviewed by Bernard and Weiss, 1973; Palmer and Kilen, 1987). The *I* locus (inhibitor) has four alleles that determine the absence or presence of pigment along with the spatial distribution of the pigments. Unlike other species in which the presence of pigment is usually the dominant phenotype, the absence of anthocyanin pigments in soybean seed coats is dominant because of the *I* allele, which inhibits pigment accumulation, resulting in a yellow seed-coat color. Cultivars that are homozygous recessive (*i*) are self-colored and have pigment distributed across the entire seed coat. Cultivars with the i^i and i^k alleles have pigment restricted to the hilum and saddle region, respectively. The *R* locus controls the color of the pigment in self-colored (*i*) seed coats. In the presence of the dominant *R* allele, pigmented seed coats are black. In the homozygous recessive condition (*r*), pigmented seed coats contain a brown pigment. The pubescence color locus, *T*, exhibits a pleiotropic effect on seed-coat color. Cultivars with self-colored seed and tawny pubescence have black (*i,R,T*) or brown (*i,r,T*) seed. Cultivars with gray pubescence have imperfect-black (*i,R,t*) or buff (*i,r,t*) seed-coat color.

The anthocyanins (anthocyanidin glycosides) of the mature, black seed coat (*i,R,T*) have been identified as cyanidin-3-monoglucoside and delphinidin-3-monoglucoside (Buzzell et al., 1987), which have 3′,4′ and 3′,4′,5′ B-ring hydroxylation patterns, respectively. In mature, imperfect-black seed coats (*i,R,t*), delphinidin is the primary anthocyanin, whereas cyanidin-3-monoglucoside is drastically reduced in quantity (Buzzell et al., 1987). Thus, the *T* locus is thought to encode a 3′-flavonoid hydroxylase that results in conversion of pelargonidin or its dihydroflavonol precursors to cyanidin. The pleiotropic purple flower color allele *W1* is thought to encode a 3′,5′-flavonoid hydroxylase responsible for converting pelargonidin to delphinidin (Buzzell et al., 1987).

Another product of the flavonoid pathway, the proanthocyanidins, exist as oligomers of leucoanthocyanidins (Fig. 1), which are unstable intermediates in the synthesis of anthocyanins. Proanthocyanidins are polymers of flavan-3-ols and get their name from the formation of their respective anthocyanidins when heated in hot acid. Functionally, proanthocyanidins are believed to be defense chemicals because of their toxicity (reviewed by Scalbert, 1991).

Although the presence of proanthocyanidins has not been shown in *G. max*, proanthocyanidins have been shown to be present in several other species of the Leguminosea. Proanthocyanidins have been identified in the vegetative parts of *Vicia faba* (Crofts et al., 1980), *Phaseolus vulgaris* (Ma and Bliss, 1978), and *Lotus corniculatus* (Sakar and Howarth, 1976). Proanthocyanidins have been identified in the seed coat of black bean (*P. vulgaris* var Cubagua) (Carmona et al., 1991). Moreover, other species such as sorghum and barley contain proanthocyanidins that are restricted to the seed coat (Haslam, 1981; Butler, 1989; Stafford, 1990). Genetically, the presence of proanthocyanidins has been shown to be controlled by one incompletely dominant gene in common bean

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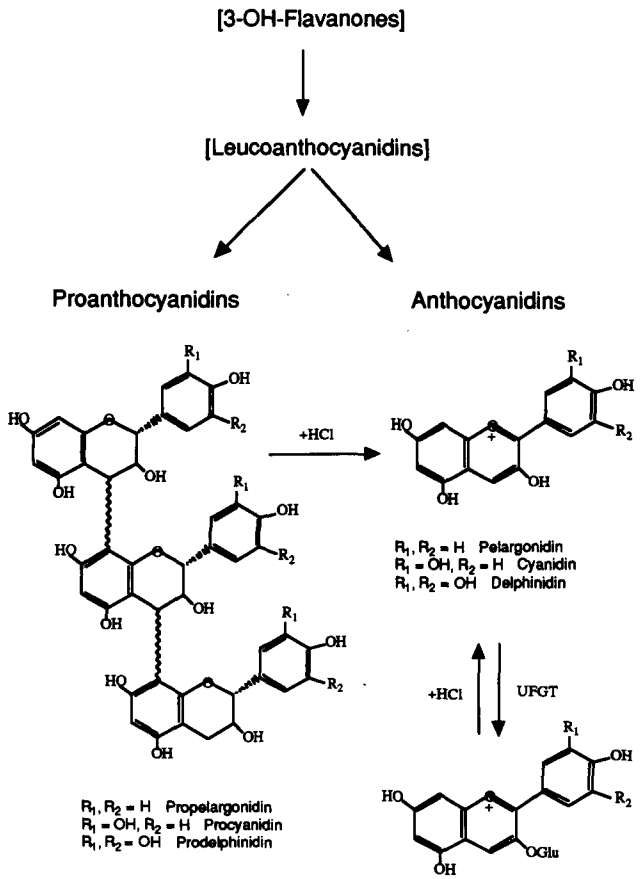

Figure 1. The biosynthetic pathways leading to the anthocyanins and the proanthocyanidins. In vitro hydrolysis treatment of anthocyanin glycoside produces the respective anthocyanin aglycone (anthocyanidin). Likewise, hydrolysis of proanthocyanidin produces the respective anthocyanidin. The common names for the compounds representing the three patterns of hydroxylation of the flavonoid B-ring are indicated. UFGT, UDP-3-*O*-glucosyltransferase.

(Ma and Bliss, 1978) and by a single dominant gene in faba bean (Crofts et al., 1980). A single dominant gene is also responsible for the production of tannins in birdsfoot trefoil (Dalrymple et al., 1984).

Few investigators have studied self-colored soybean varieties because of their absence in commercially common soybean varieties. However, virtually all wild soybeans and 9% of the soybean germplasm collection have self-colored seed coats (Bernard and Weiss, 1973). Some of the self-colored lines display variegated seed-coat colors reminiscent of transposable element-induced somatic mutations (Chandlee and Vodkin, 1989), whereas others have defects in the seed-coat structure (Bernard and Weiss, 1973). Not only does the recessive *i* allele affect the anthocyanin pathway, it also affects the abundance of a Pro-rich cell-wall protein (Lindstrom and Vodkin, 1991). We have recently shown that proteins and RNA are difficult to extract from black (*i,R,T*) and brown (*i,r,T*) seed coats but are easily extracted from imperfect-black (*i,R,t*) and buff (*i,r,t*) seed coats (Nicholas et al., 1993).

Because of our difficulties obtaining soluble proteins and RNA from seed coats of certain genotypes, we sought to determine what, if any, secondary plant products in the pigmented seed coats might be interfering with standard extraction procedures. We show that the proanthocyanidin, procyanidin, is present in black and brown seed coats (*i,T*) at a very early developmental stage. Additionally, the flavonoid extract from these genotypes not only has the ability to precipitate proteins but also has the ability to affect RNA in vitro. Imperfect-black and buff (*i,t*) seed-coat extracts did not have protein- and RNA-binding abilities, although they tested positive for the presence of small amounts of propelargonidin. The *I* locus controls the absence or presence of proanthocyanidins, and the *T* locus determines the types of proanthocyanidins present. Thus, the combination of the homozygous recessive *i* allele in combination with the dominant *T* allele allows the formation of secondary products with tannin-like abilities.

MATERIALS AND METHODS

Plant Material and Genetic Nomenclature

Clark isolines of *Glycine max* (L.) Merr. were obtained from the U.S. Department of Agriculture Soybean Germplasm Collections (U.S. Department of Agriculture/Agricultural Research Service, University of Illinois at Champaign-Urbana). All soybean lines used were homozygous at the *I, R, T* loci; thus, only one allele is indicated in the text and figures. The Clark isolines used were also homozygous *W1* for purple flower color. Materials were collected from plants grown in the greenhouse with additional light extending the day length to 14 h. Additional materials were obtained from the field. For developmental studies, seed from Clark isolines was divided into the following categories by fresh weight of the entire seed: 25 to 50, 50 to 75, 75 to 100, 100 to 200, 200 to 300, 300 to 400, 400 to 500, and >500 mg (fully expanded and mature). Developing seed of 75 to 100 mg fresh weight corresponds to approximately 27 DAF. Seed coats were dissected from seed, frozen in liquid nitrogen, freeze dried, and stored at −20°C.

Preparation of Extracts and TLC Analysis

Freeze-dried materials were ground with a mortar and pestle with sand to a fine powder. Generally, proanthocyanidins were extracted from 50 mg of freeze-dried seed coats with 0.5 mL of 90% methanol for 1 h to overnight in a microtube with constant agitation from a Vortex mixer. The supernatant was saved, and the pellet was washed with 0.5 mL of 50% methanol. The supernatants were combined, and the volume was reduced by evaporation in a SpeedVac concentrator (Savant) at room temperature. The extract was treated with chloroform to partition fats and Chl into the chloroform layer. The aqueous methanol fraction containing unhydrolyzed flavonoids was used for TLC analysis, butanol-HCl test, 0.5% vanillin test, protein precipitation, and RNA binding as described below. Alternatively, the aqueous extract after chloroform partitioning was hydrolyzed in 1 M HCl final concentration in a boiling water bath for 1 h, followed by ethyl acetate partitioning of the anthocyanidins into the

ethyl acetate fraction. The ethyl acetate fraction was then spotted onto TLC plates.

Extracts were analyzed by TLC performed on SigmaCell Type 100 cellulose plates. Samples of extracts were spotted and run in one or two dimensions using one of the following solvents: chloroform:acetic acid:water, 10:9:1; *n*-butanol: acetic acid:water, 4:1:5. This solvent was mixed in a separating funnel, and the upper phase was used (following overnight separation); 30% (v/v) acetic acid: acetic acid:water; and forestal: water:acetic acid:HCl, 10:30:3.

Chromatograms were thoroughly dried and then analyzed under UV light. Following initial analysis, the chromatogram was fumed with ammonium hydroxide and viewed under UV light. Color changes were noted and R_F values were calculated. Spots of interest were scraped and eluted in methanol. A Shimadzu UV-visible recording spectrophotometer (UV-160) was used for UV-visible spectroscopy.

Proanthocyanidin Tests

Analyses of proanthocyanidins were carried out on the unhydrolyzed aqueous methanol fraction of seed-coat extracts following chloroform partitioning. Standard methods were used for a butanol-HCl assay (Bate-Smith, 1975; Watterson and Butler, 1983), 0.5% vanillin test (Price et al., 1978), vanillin TLC detection, and a radial diffusion assay of protein precipitation (Hagerman, 1987) using tannic acid as a standard.

An in vitro RNA-binding assay was developed to assess polyphenolic binding to RNA. For determination of proanthocyanidin-nucleic acid binding, total RNA from yellow seed coats (*I*) was isolated from Richland seed. Seed coats were ground in liquid nitrogen, and RNA was extracted with buffer and then phenol:chloroform, followed by lithium chloride precipitation (McCarty, 1986; Lindstrom and Vodkin, 1991). Proanthocyanidins were extracted from 20 mg (dry weight) of yellow (*I,R,T*), black (*i,R,T*), brown (*i,r,T*), imperfect-black (*i,R,t*), and buff (*i,r,t*) seed coats by the usual procedures. A 5- or 10-μg aliquot of RNA was incubated with 2, 5, or 10% final volume of the extracted polyphenols from each genotype. All reactions were brought to a final volume of 50 μL and allowed to incubate on ice for 5 min. Following incubation, RNA was precipitated overnight in ethanol. The RNA pellet was washed with 80% ethanol before analysis. Pellets were dissolved in water, and absorbance spectra were taken. Following reprecipitation, electrophoresis was carried out on a 1.2% formaldehyde agarose gel (Maniatis et al., 1982). Following electrophoresis, gels were photographed and then transferred overnight to nitrocellulose. Blots were cross-linked and hybridized to a PRP1 sequence-specific probe representing an abundant cell-wall protein in the seed coat. The 98-bp *Bst*NI/*Eco*RI fragment from pB1–3 (Lindstrom and Vodkin, 1991) was purified by Geneclean II (Bio 101, Inc., La Jolla, CA) and labeled with [^{32}P]dATP using random primers.

RESULTS

Anthocyanidins (Derived from Proanthocyanidins) Are Detected in Immature, Self-Colored Seed Coats

Seed-coat extracts from immature, green seed of <200 mg fresh weight with homozygous recessive *i* genotypes contained pink/red compounds after hydrolysis in 1 M HCl that did not migrate from the origin following development of TLC plates in chloroform:acetic acid:water (10:9:1) solvent (data not shown). These compounds had properties of anthocyanidins, although visible anthocyanins were not present in the immature, green seed coats at this stage of development.

We then examined the seed-coat flavonoid extracts using other solvent systems to resolve the anthocyanidins. Figure 2 shows the relative positions when separated by one-dimensional TLC of compounds obtained from 1 M HCl-treated flavonoid extracts of immature and mature seed-coat extracts of self-colored Clark isolines. The anthocyanin glycosides of mature seed coats without HCl treatment are also shown for comparison. Immature, green seed coats from black (*i,R,T*) and brown (*i,r,T*) genotypes contained a single spot at R_F 55. The compound was bright pink in visible light but displayed orange fluorescence under UV light. It turned blue under visible light after fuming with ammonium hydroxide but remained orange under UV light. The area was recovered from the TLC plate, and the compound was eluted in methanol. We identified this hydrolysis product as cyanidin based on its maximum A_{550} and comigration with the aglycone form of cyanidin in the mature seed-coat extract. Cyanidin in the immature, green seed coat from extracts of black (*i,R,T*) and brown (*i,r,T*) genotypes is likely derived from hydrolysis of oligomeric procyanidins (Fig. 1), because no free anthocyanidins are found in unhydrolyzed extracts of immature seed (data not shown).

Seed-coat extracts from immature seed from imperfect-

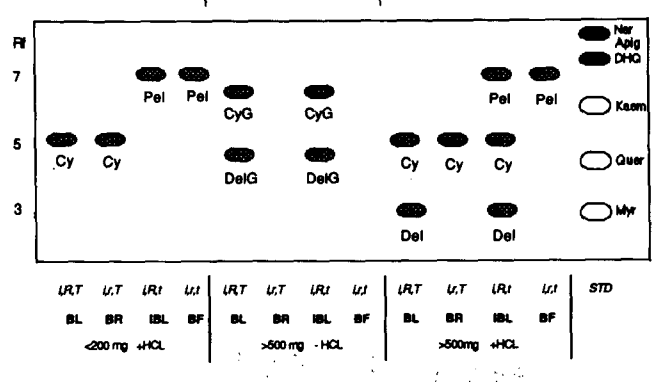

Figure 2. TLC of flavonoids before and after 1 M HCl treatment using Clark isoline extracts. Seed coats from seed of <200 mg and seed of >500 (fresh weight) were extracted by standard procedures (see "Materials and Methods"). TLC was performed on cellulose with development in forestal solvent. All <200-mg extracts were hydrolyzed in 1 M HCl. One set of >500-mg extracts was spotted without hydrolysis and the other was hydrolyzed in 1 M HCl. The shaded spots represent the typical red color of the anthocyanidins. The figure is not meant to be quantitative but only to indicate position and presence of spots. The following R_F values were obtained for each compound: pelargonidin (Pel), R_F = 75; cyanidin (Cy), R_F = 55; delphinidin (Del), R_F = 38; cyanidin-3-glucoside (CyG), R_F = 68; delphinidin-3-glucoside (DelG), R_F = 50. Nar, Naringenin; Apig, apigenin; DHQ, dihydroquercetin; Kaem, kaempferol; Quer, quercetin; Myr, myricetin; BL, black; BR, brown; IBL, imperfect-black; BF, buff; STD, standard.

black (*i,R,t*) or buff (*i,r,t*) genotypes had a single anthocyanidin at R_F 75. The spot was pink in visible light but displayed orange fluorescence under UV light. It turned blue in visible light following ammonium hydroxide fuming but remained orange under UV light. The spot was recovered, and the compound was eluted in methanol. It had maximum A_{530} and comigrated with the purified pelargonidin standard obtained from seed-coat extracts from mature, red buff seed coat (Buzzell et al., 1987). Thus, we identified pelargonidin (from propelargonidin) to be present after acid hydrolysis of immature seeds coats with *i,t* genotypes.

Mature, black seed-coat extracts (*i,R,T*) without the hydrolysis treatment contained cyanidin-3-glucoside and delphinidin-3-glucoside, and mature, imperfect-black seed-coat extracts (*i,R,t*) contained primarily delphinidin-3-glucoside with only trace amounts of cyanidin-3-glucoside as expected (Buzzell et al., 1987). Extracts from mature, brown (*i,r,T*) and buff (*i,r,T*) seed coats lacked anthocyanin glycosides on TLC in the absence of acid hydrolysis, indicating that *r* prevents the accumulation of anthocyanins.

Because Buzzell et al. (1987) described only the anthocyanin glycosides of mature seed coats and did not assay for aglycone forms, we subjected the mature seed-coat extracts to acid hydrolysis. Following acid hydrolysis, mature, black (*i,R,T*) seed coats contained aglycones of cyanidin (derived from both procyanidin and cyanidin-3-glucoside) and delphinidin (derived from delphinidin-3-glucoside). Analysis of TLC patterns from mature, imperfect-black seed coats (*i,R,t*) revealed both cyanidin and delphinidin, although both were were much reduced, as expected from the reduction of total anthocyanin glycosides in the mature seed (Buzzell et al., 1987). Additionally, a compound comigrated with the pelargonidin found in immature seed-coat extracts from this same genotype. Thus, mature, imperfect, black seed coats treated with HCl contained trace amounts of cyanidin (from small amounts of cyanidin-3-glucoside), delphinidin (from delphinidin-3-glucoside), and pelargonidin (from propelargonidin).

Seed-coat extracts from the mature, brown seed coats (*i,r,T*) treated with HCl contained cyanidin, although no cyanidin-3-glucoside is found in unhydrolyzed extracts of this genotype at any stage of development. Likewise, extracts from mature, buff (*i,r,t*) seed treated with HCl contained a single anthocyanidin that comigrated with pelargonidin found in immature seed-coat extract from this same genotype. Thus, the anthocyanidins found in brown and buff appear to be derived solely from proanthocyanidins and not from anthocyanin glucosides, because no anthocyanin glucosides or free anthocyanidins are found in unhydrolyzed extracts.

Chemical Tests Confirm the Presence of Proanthocyanidins

Because of the presence of cyanidin and pelargonidin following acid hydrolysis of immature seed-coat extracts, we speculated that these anthocyanidins may be derived from proanthocyanidin precursors because no corresponding anthocyanin glycosides or free anthocyanidins are present in the immature seed coats. Proanthocyanins are not precursors of anthocyanins in vivo; rather, they are oligomers of leucoanthocyanin, an intermediate in the synthesis of anthocyanins (Fig. 1).

The assay results obtained from the chemical tests used to confirm the presence of proanthocyanidins in soybean seed coats are listed in Table I. Proanthocyanidins from seed-coat aqueous methanol extracts were adsorbed to polyvinylpolypyrolydone and hydrolyzed with butanol-HCl to elute free anthocyanidins. Immature, black (*i,R,T*) or brown seed coats (*i,r,T*) gave a bright red coloration (A_{550}) following this assay. Immature, imperfect-black (*i,R,t*) or buff seed coats (*i,r,t*) gave a red-orange coloration (A_{530}). Anthocyanidin products resulting from the acid hydrolysis could be only partially removed from the polyvinylpolypyrolydone using butanol (Watterson and Butler, 1983). By the 0.5% vanillin assay, all self-colored extracts were positive for the presence of proanthocyanidins. Immature, black (*i,R,T*) and brown (*i,r,T*) seed-coat extracts had approximately the same amounts of proanthocyanidins. Imperfect-black (*i,R,t*) or buff (*i,r,t*) seed-coat extracts had lesser amounts, with buff having significantly less than all genotypes. The same pattern was found using

Table I. *Tests for proanthocyanidin in soybean seed coats*

Isoline	Genotype	Phenotype	Proanthocyanidin Tests					
			Butanol-HCl assay[a]	Vanillin assay[b]	Vanillin TLC detection[c]	Chemicals detected by TLC	In vitro RNA spectral shift[d]	Protein precipitation[e]
Clark 1	*I,R,T*	Yellow					No	No
Clark 9	*i,R,T*	Black	550 nm	+++	+++	Procyanidin	Yes	Yes
Clark 14	*i,r,T*	Brown	550 nm	+++	+++	Procyanidin	Yes	Yes
Clark 30	*i,R,t*	Imperfect-black	535 nm	++	++	Propelargonidin	No	No
Clark 33	*i,r,t*	Buff	535 nm	+	+	Propelargonidin	No	No

[a] Interflavonoid bonds are broken within the proanthocyanidins giving rise to their respective anthocyanidins. The color of the extract becomes red to orange with an *A* between 530 and 550 nm. [b] A 0.5% acidic vanillin solution is added to the extract; the vanillin, an aromatic aldehyde, condenses with the flavan-3-ols and oligomers to form soluble pigments with an A_{550}. [c] Extracted proanthocyanidins are spoted and run on cellulose TLC plates. Acidic vanillin is sprayed directly onto the plate and condenses with the proanthocyanidin and flavan-3-ol spots after heating. [d] RNA is incubated with proanthocyanidin extracts and binding is detected by UV spectrophotometry and formaldehyde gel electrophoresis followed by northern blot analysis. [e] Protein precipitation is measured by a radial diffusion assay based upon the preferential ability of certain proanthocyanidins to bind to proteins, forming insoluble complexes.

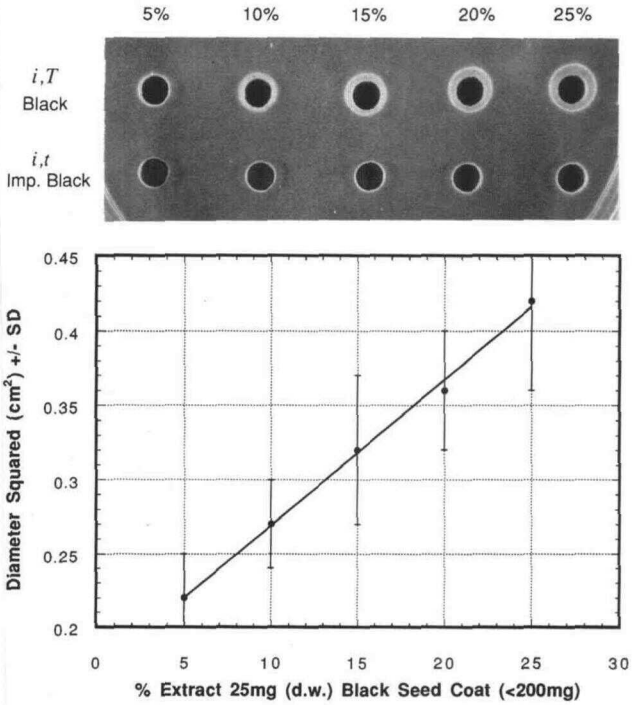

Figure 3. Protein precipitation by seed-coat flavonoid extracts. Upper, Black (*i,T*) and imperfect-black (*i,t*) seed coats (25 mg) were extracted by standard procedures (see "Materials and Methods"), and different percentages of extract were brought to a common volume (45 μL). The extracts were then successively spotted into the wells of an agarose plate containing BSA (Hagerman, 1987). After the material was incubated, a precipitation ring was visualized and measured for each black (*i,T*) seed-coat extract. None of the imperfect (Imp.)-black (*i,t*) seed-coat extracts had the ability to precipitate proteins. Lower, Black seed-coat extract elicits a linear radial diffusion response. Black seed coat (*i,T*) (25 mg dry weight [d.w.]) were extracted in 90% methanol, followed by a 50% methanol wash. After the extract was partitioned with chloroform, the final extract volume was measured. Percentage volumes were measured, and 15-μL aliquots were spotted in the wells. After the aliquots were brought to diffusional equilibrium, diameters were measured. Each point represents the mean of at least three replicates ±SD. For black seed-coat extract: diameter^2 = 0.010 (percentage of extract) + 0.17 (r^2 = 0.99).

the vanillin reagent sprayed on TLC plates containing these extracts.

Procyanidin, a dihydroxylated proanthocyanidin, was identified in immature, black (*i,R,T*) and brown (*i,r,T*) seed coats based on the data from the 0.5% vanillin test and the butanol-HCl test in combination with TLC separation. Propelargonidin, a 4'-hydroxylated proanthocyanidin, was identified in immature, imperfect-black (*i,R,t*) and buff (*i,r,t*) seed coats based on these same tests in combination with identification on TLC plates.

Proanthocyanidins Are Present in Very Young Seed Coats

Black (*i,R,T*) and brown (*i,r,T*) seed-coat extracts from immature (<200 mg fresh weight) seed were analyzed for protein precipitation ability based on a radial diffusion assay. Tannic acid, a hydrolyzable tannin, was used as a standard. Figure 3 shows the linear precipitation response to increasing percentages of the total flavonoid extract obtained from 25 mg dry weight of black seed coat. Twenty-five percent of the extract obtained from 25 mg of black seed coat precipitated an amount of protein equivalent to approximately 25 μg of tannic acid. Brown seed-coat extracts also precipitated a similar amount. As shown in Figure 3, imperfect-black (*i,R,t*) and buff (*i,r,t*) seed-coat extracts did not precipitate proteins at any of the levels tested (up to 25% of 25 mg of soybean seed coat, or the amount of extract equivalent to the total methanol-soluble flavonoid content of a single seed coat). Figure 4 shows the results from a developmental study used to assess protein precipitation ability of black (*i,R,T*) soybean seed coat throughout development. We found seed-coat extracts from as young as 25 mg fresh weight of seed had the ability to precipitate proteins in a radial diffusion assay. Extracts of both black (*i,R,T*) and brown (*i,r,T*) genotypes precipitated proteins at all developmental stages, whereas imperfect-black (*i,R,t*) and buff (*i,r,t*) did not have the ability to precipitate proteins at any developmental stage (data not shown).

Proanthocyanidins from Black Seed Coat (*i,T*) Have the Ability to Bind RNA in Vitro

Nucleic acid binding was determined by an in vitro RNA assay in which RNA was incubated with seed-coat flavonoid extracts. Following incubation, RNA was ethanol precipitated, washed with 80% ethanol, and resuspended in water. Figure 5 shows the absorbance spectra obtained from RNA incubated with yellow (*I,R,T*) seed-coat extract or with black (*i,R,T*) seed-coat extract. Yellow seed-coat extract had no effect on the spectral absorbance of the RNA pellet. Black seed-coat extract had a profound effect on the spectral absorbance of RNA. The pattern was altered, presumably because of formation of an RNA-procyanidin complex. Imperfect-black (*i,R,t*) polyphenols did not affect the spectral pattern of RNA.

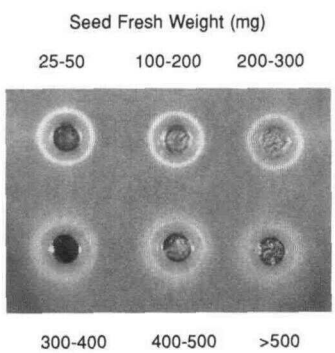

Figure 4. Developmental radial diffusion for black (*i,T*) seed-coat extract. Seed coats (10 mg) form each fresh weight seed category were extracted in 90% methanol, followed by a 50% methanol wash, and were partitioned with chloroform. The entire extract was spotted in successive aliquots. Following incubation, wells were photographed, and well diameters were measured. All seed fresh weight categories had the ability to precipitate proteins.

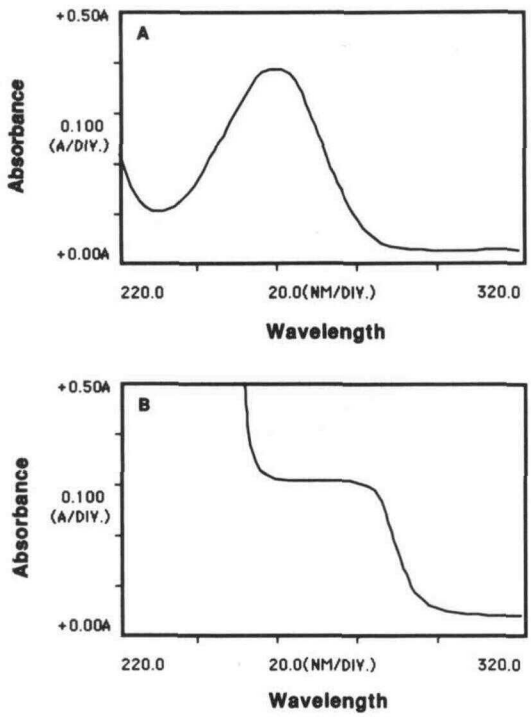

Figure 5. Changes in absorbance pattern after incubation of RNA with black (*i,T*) seed-coat extract. A 10-μg RNA aliquot was incubated with 5% of the total unhydrolyzed flavonoid extract from 25 mg dry weight of seed coats in an in vitro RNA-binding assay. After the RNA was reprecipitated with ethanol, the absorbance spectrum was measured. A, A typical RNA spectrum from an RNA control or that of RNA incubated with yellow (*I*) seed-coat extract or imperfect-black (*i,t*) seed-coat extract. B, The altered spectrum following RNA incubation with black (*i,T*) seed-coat flavonoids.

In vitro RNA binding was also assessed by formaldehyde gel electrophoresis followed by northern blot analysis. RNA treated with the black (*i,R,T*) seed-coat extract migrated faster, presumably due to formation of an RNA-procyanidin complex (data not shown). Imperfect-black seed-coat (*i,R,t*) extract did not affect the migration of RNA. Figure 6 shows the autoradiograph of a northern blot in which increasing amounts of black and imperfect-black seed-coat extracts were added to RNA in the in vitro assay followed by formaldehyde gel electrophoresis and blotting using a cDNA probe for PRP1, an abundant cell-wall protein in the seed coat (Lindstrom and Vodkin, 1991). At all amounts tested, black seed-coat extract affected RNA such that hybridization was not detected. Imperfect, black seed-coat extract (*i,R,t*) did not interfere with the ability of RNA to hybridize to the probe. In a separate experiment, seed-coat extract from yellow (*I,R,T*) seed did not interfere with the ability of RNA to hybridize to the probe (data not shown).

DISCUSSION

Dominant *I* Allele Inhibits Formation of Proanthocyanidins in Soybean Seed Coat

Our data show that soybean varieties containing the dominant *I* gene (and thus having yellow seed coats) do not contain proanthocyanidins (Table I). Thus, the *I* gene, which inhibits anthocyanin production in seed-coat tissue, also inhibits proanthocyanidin accumulation. This result is consistent with the fact that both anthocyanin and proanthocyanidin biosynthetic pathways share a common branch point, the leucoanthocyanidins (flavan-3,4 diols), as shown in Figure 1. Because both require leucoanthocyanidins for their synthesis and share the initial steps of the flavonoid pathway leading to the 3-hydroxy-flavanones (dihydroflavanols), it might be expected that inhibition of anthocyanin biosynthesis at an early step would also inhibit proanthocyanidin formation. This is consistent with the hypothesis that the proanthocyanidin biosynthetic pathway is regulated at the beginning of the 15-carbon flavonoid sequence (Stafford, 1990).

Similarly, soybean varieties that are homozygous recessive (*i*) for the *I* locus have self-colored seed coats and contain anthocyanins at maturity. Our study shows that these varieties also contain proanthocyanidins (Fig. 2, Table I). Proanthocyanidin accumulation was not restricted to mature seed coat but was present in very young seed coat. Thus, the *I* locus is intricately involved in both the inhibition and formation of anthocyanins and proanthocyanidins and must act during the early stages of seed-coat development. The gene product of the *I* locus and its mechanism of anthocyanin inhibition are unknown. We have previously shown that the levels of a seed-coat cell-wall protein, PRP1, and its mRNA are reduced in young seed coats (25–200 mg seed fresh weight) with an imperfect-black genotype (*i,R,t*) (Lindstrom and Vodkin, 1991; Nicholas et al., 1993). Those data indicate that the *I* gene has multiple effects in young seed coats.

T Allele Determines the Hydroxylation Pattern of Proanthocyanidins

The pleiotropic pubescence allele, *T*, also has a major effect on the types of proanthocyanidins present in homozygous recessive self-colored (*i*) seed coats. Both black and brown (*i,T*) hydrolyzed seed-coat extracts from immature seed con-

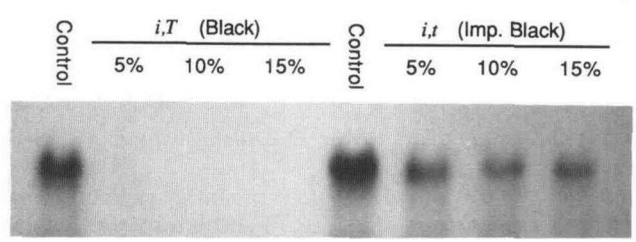

Figure 6. A northern blot of RNA incubated with proanthocyanidins from seed coats. An in vitro RNA-binding assay was performed with black (*i,T*) and imperfect-black (*i,t*) seed-coat extracts from 25 mg of seed-coat material. Each 10 μg of RNA was incubated with increasing percentages of the unhydrolyzed flavonoid extract. Following reprecipitation, the RNAs were separated by electrophoresis, blotted, and probed with a cDNA representing an abundant seed-coat mRNA. Black (*i,T*) seed-coat extract inhibited hybridization at all percentages tested, whereas imperfect (Imp.)-black (*i,t*) seed-coat extract did not inhibit hybridization. In a separate experiment (not shown), yellow seed-coat extract also did not inhibit hybridization.

tained procyanidin (Fig. 2, Table I), which has a 3′,4′-hydroxy B-ring pattern. The 3′-hydroxylation of the B-ring follows naringenin synthesis at the flavanone or 3-hydroxyflavanone level. Immature, imperfect-black seed-coat (*i,R,t*) extracts contained only pelargonidin after acid hydrolysis and also were positive for the presence of proanthocyanidins (Fig. 2, Table I). The presence of propelargonidin, a 4′-hydroxylated proanthocyanidin, within imperfect-black seed-coat extracts is consistent with the absence of 3′,4′-hydroxylated species due to the recessive *t* allele.

The presence of the anthocyanin delphinidin, a trihydroxylated compound, in mature seed coats is associated with the pleiotropic flower color allele, *W1* (Buzzell et al., 1987). Because prodelphinidin was not found in immature seed coats (Fig. 2), our results suggest that the action of the *W1* gene is specific to the anthocyanin pathway and has no parallel function in the proanthocyanidin pathway.

Immature and mature seed coats from brown (*i,r,T*) seed contained procyanidin but did not contain any anthocyanins (Fig. 2). Previously, the brown pigment was speculated to be a polymer of leucoanthocyanidin (Koshimizu and Iizuka, 1963). Our studies confirm this speculation and identify the polymer as procyanidin. We speculate that the *R* gene acts after the formation of leucoanthocyanidin but previous to the formation of anthocyanins, because *R* genotypes contained proanthocyanidins as well as anthocyanins, but recessive *r* genotypes contained only proanthocyanidins. Little is known about the anthocyanin-forming enzymes that act just after leucoanthocyanin production.

Only Black and Brown Seed-Coat Extracts (*i,T*) Have Protein Precipitation Ability

Proanthocyanidins have long been associated with the precipitation of proteins and have been loosely referred to as condensed tannins. Our studies show that only black and brown (*i,T*) seed-coat extracts contain secondary products capable of protein precipitation (Fig. 3, Table I). The procyanidin in these genotypes binds proteins very aggressively during extraction and makes it difficult to solubilize proteins. Surprisingly, seed-coat extracts from 25 mg fresh weight of seed contained the same amount of protein precipitation ability on a per weight basis as did extracts from 200 mg fresh weight of seed (Fig. 4 and data not shown). Seed coats do not begin to accumulate anthocyanins until the seed are greater than 300 mg fresh weight. Thus, the proanthocyanin pathway is active much earlier in development than the anthocyanin pathway.

Although it might be expected that imperfect-black or buff seed-coat extracts (both *i,t*) precipitate proteins if they contain any proanthocyanidins, they did not (Fig. 3). This agrees well with our previous data showing that protein and RNA are easily extracted from *i,t* genotypes but are nearly inextractable from *i,T* genotypes (Nicholas et al., 1993). Lack of protein precipitation by propelargonidin may not be surprising considering that hydroxylation patterns of proanthocyanidins drastically affect their association with proteins (Scalbert, 1991). Propelargonidin is monohydroxylated and may not contain the reactive groups necessary for effective precipitation.

Seed-Coat Extracts with an *i,T* Genotype Have an Effect on RNA in Vitro

There have been few studies of the interaction of proanthocyanidins on nucleic acids. Our studies indicate that seed-coat extracts with procyanidin present (black and brown seed with *i,t* genotypes) have an effect on RNA by forming a complex that alters the absorbance spectra, RNA migration, and ability to hybridize to DNA (Figs. 5 and 6). Substantiating this hypothesis is the fact that a red color results following hydrolysis of the poor quality RNA isolated from immature, black seed coats. The spectrum of the solution is characteristic of anthocyanidins. Our data clearly show that black and brown seed-coat extracts have an effect on RNA by forming a complex. What relevance, if any, this RNA-proanthocyanidin interaction has in vivo is not known. It has been hypothesized that the spatial relationship between phenolic groups in tannins optimizes a shape that could interact with nucleic acids due to hydrogen bonding to the phosphate backbone (Haslam, 1977).

In summary, like other angiosperm species, *G. max* contains proanthocyanidins that are restricted to the seed-coat tissue. Studies indicate that proanthocyanidins are toxic to bacteria and fungi and act as feeding deterrents to herbivores and insects. For these reasons primarily, proanthocyanidins have been considered defense chemicals. In evolutionary terms the presence of proanthocyanidin in the black-seeded wild soybean stocks may represent a primitive defense chemical that has been replaced by the simple, less metabolically expensive isoflavonoids (Gornall and Bohm, 1978). Recently, it was suggested that tannin-containing plants have had an evolutionary advantage over their competitors (Scalbert, 1991). In cultivated crops like soybean, this advantage is outweighed by other selective actions imposed by breeding to improve appearance and acceptability in food processing. Cultivars with yellow seed coats (*I* or *i*^{i}) that lack proanthocyanidins and anthocyanins are preferred.

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LITERATURE CITED

Bate-Smith EC (1975) Phytochemistry of proanthocyanidins. Phytochemistry **14:** 1107–1113

Bernard RL, Weiss MG (1973) Qualitative genetics. *In* BE Caldwell, ed, Soybeans: Improvement, Production, and Uses, Ed 1. American Society of Agronomy, Madison, WI, pp 117–149

Butler LG (1989) Sorghum polyphenols. *In* PR Cheeke, ed, Toxicants of Plant Origin, Vol IV. CRC Press, Boca Raton, FL, pp 96–109

Buttery RI, Buzzell RI (1973) Varietal differences in leaf flavonoids of soybeans. Crop Sci **13:** 103–106

Buzzell RI, Buttery BR, MacTavish DC (1987) Biochemical genetics of black pigmentation of soybean seed. J Hered **78:** 53–54

Carmona A, Seidl DS, Jaffe WG (1991) Comparison of extraction methods and assay procedures for the determination of the apparent tannin content of common beans. J Sci Food Agric **56:** 291–301

Chandlee JC, Vodkin LO (1989) Unstable expression of a soybean gene during seed coat development. Theor Appl Genet **77:** 587–594

Crofts HJ, Evans LE, McVetty PBE (1980) Inheritance, characterization, and selection of tannin-free fababeans (*Vicia faba* L.). Can J Plant Sci **60:** 1135–1140

Dalrymple EJ, Goplen BP, Howarth RE (1984) Inheritance of tannins in birdsfoot trefoil. Crop Sci **24:** 921–922

Gornall RJ, Bohm BA (1978) Angiosperm flavonoid evolution: a reappraisal. Syst Bot **3:** 353

Hagerman A (1987) Radial diffusion method for determining tannin in plant extracts. J Chem Ecol **13:** 437–448

Haslam E (1977) Symmetry and promiscuity in procyanidin biochemistry. Phytochemistry **16:** 1625–1640

Haslam E (1981) Vegetable tannins. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 7: Secondary Plant Products. Academic Press, New York, pp 527–555

Koshimizu Y, Iizuka N (1963) Studies on Soybean Virus Diseases in Japan (in Japanese), English summary translated by H Inagaki in 1967. Tohoku National Agricultural Experiment Station Bulletin No. 27, pp 1–107

Lindstrom JT, Vodkin LO (1991) A soybean cell wall protein is affected by seed color genotype. Plant Cell **3:** 560–571

Ma Y, Bliss FA (1978) Tannin content and inheritance in common bean. Crop Sci **18:** 201–204

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

McCarty DR (1986) A simple method for the extraction of RNA from Maize tissue. Maize Genet Coop News Lett **60:** 61

Nicholas CD, Lindstrom JT, Vodkin LO (1993) Variation in proline rich cell wall protein in soybean lines with anthocyanin mutations. Plant Mol Biol **21:** 145–156

Palmer RG, Kilen TC (1987) Qualitative genetics. *In* JR Wilcox, ed, Soybeans: Improvement, Production, and Uses, Ed 2. American Society of Agronomy, Madison, WI, pp 135–209

Price ML, Van Scoyoc S, Butler LG (1978) A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J Agric Food Chem **26:** 1214–1218

Sakar SK, Howarth RE (1976) Specificity of the vanillin test for flavanols. J Agric Food Chem **24:** 317–320

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry **30:** 3875–3883

Stafford H (1990) Pathway to proanthocyanidins (condensed tannins), flavan-3-ols, and unsubstituted flavans. *In* Flavonoid Metabolism. CRC Press, Boca Raton, FL, pp 63–99

Watterson JJ, Butler LG (1983) Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. J Agric Food Chem **31:** 41–45