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Polyphenol Oxidase Activity and Differential Accumulation of Polyphenolics in Seed Coats of Pinto Bean (*Phaseolus vulgaris* L.) Characterize Postharvest Color Changes

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Postharvest darkening of pinto bean (*Phaseolus vulgaris* L.) was evaluated in a population of recombinant inbred lines derived from a cross between CDC Pintium (a regular-darkening line) and 1533-15 (a slow-darkening line). Flavonoid metabolite concentrations, polyphenol oxidase activity, lignin concentration, and seed coat anatomy characteristics were assessed for cosegregation with the darkening phenotype. Significantly lower kaempferol concentrations ($p = 0.00001$) together with differences in polyphenol oxidase activity ($p = 0.0045$) were two of the key findings associated with these recombinant inbred lines. In addition, two different assays (thioglycolic acid and Klason lignin) to quantify lignin together with an assessment of extractable condensed tannin were used to estimate the contribution of these polymers to changes in the seed coat tissue. This is the first report of precise biochemical characterization of polyphenolics that associate with postharvest darkening in legumes.

KEYWORDS: ***Phaseolus vulgaris***; **postharvest darkening**; **condensed tannin**; **proanthocyanidin**; **kaempferol**; **lignin**; **polyphenol oxidase**; **quinone**

INTRODUCTION

Postharvest color changes in crops are responsible for major economic losses in fresh fruits, vegetables (*1, 2*), and products manufactured from wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (*3–5*). In legumes, similar visible changes have been associated with deterioration of cooking quality (*6–8*). Patterned common bean (*Phaseolus vulgaris* L.) cultivars, such as pinto (mottled) and carioca (striped), that can retain the bright background color during storage are appealing to the consumer for a longer period and command a higher market value.

A pinto bean line, 1533-15, was identified in the CDC breeding program, University of Saskatchewan, that darkens slowly (SD) thus retaining its bright background color much longer than regular, rapid-darkening (RD) cultivars such as CDC Pintium (*9*). Preliminary studies of the two parental lines indicated that there were significant differences in the polyphenolic content (*10*). However, the biochemical basis for the postharvest-darkening trait could not be established, and other associated biochemical phenotypes, such as lignin, were not explored. Without examining the genetic basis of the trait by means of an inbred population derived from a controlled cross, it was impossible to know whether the polyphenolic differences were a reflection of a trait due to an environmental variable in a given growing season or whether differences were randomly associated with the darkening trait.

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The primary research objective for the work presented here was to identify polyphenolic characteristics associated with the postharvest darkening trait in RILs developed from a cross between CDC Pintium and 1533-15, therefore segregating for the SD and RD phenotypes. Characterization included an evaluation of condensed tannin (CT), kaempferol, and lignification. Since a study of mung bean implied that a thicker seed coat with structurally different palisade cells was associated with beans exhibiting more pigment and having a hard-seeded character (*11*), the RIL characterization was augmented with measurements of the seed coat anatomy.

Secondly, a polyphenol oxidase (PPO, E.C. 1.14.18.1 *syn.* tyrosinase [monophenolase activity] and E.C. 1.10.3.2 [diphenolase activity]) screening assay was explored to examine as to whether the difference in postharvest darkening among the RILs was related to differences in PPO activity. Enzymatic oxidation activities were reported as a possible mechanism in seed coat darkening of selected Brazilian bean cultivars (*12, 13*); however, differences in PPO activity have not yet been associated with the SD trait.

MATERIALS AND METHODS

Analytical Instrumentation. *Liquid Chromatography.* Seed coat extracts and authenticated chemical standards were separated using a high pressure liquid chromatograph (HPLC, Model 1100 Series, Agilent Technologies Canada Inc., Mississauga, ON) equipped with an autosampler, quaternary pump and solvent degasser, UV-photodiode array (UV-PDA) detector, and Zorbax XDB reversed-phase (RP) C_{18} column

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(3.0 mm × 150 mm, 5 μm particle size). Instrument separations and data analyses were controlled using ChemStation computer software for LC 3-D systems (Rev. B.02.01-SR1[260]).

Absorbance Readings (AU). Butanol-HCl (BuOH-HCl) [A_{550}] and PPO [A_{475}] assays were measured with a scanning spectrophotometer (Agilent, Model 8453) controlled by ChemStation computer software (ver. Rev A. 08.03[71]).

Reagents. HPLC-grade chemicals were obtained from Sigma, unless otherwise noted. Organic solvents were supplied by OmniSolv (Charlotte, NC), and acids and bases were ACS quality. Filtered water was obtained from a Milli-Q Ultrapure Water Purification system (Millipore, Billerica, MA). Chemical standards and reference compounds (e.g. ±-catechin) were weighed to 0.1 mg, and stock solutions were stored at −20 °C. Working solutions were freshly prepared for each assay or HPLC analysis.

Plant Material. *Mature Seed Coat Assays.* (i) Pinto bean parental lines, CDC Pintium (RD) and 1533-15 (SD), were harvested in the field at Outlook, SK in 2004. Mature seed was stored at 10 °C until manually decorticated, air-dried, and ground to pass through a 1 mm sieve (#18, USA-standard ASTME-11-61). (ii) RILs developed from a cross between these parental lines were grown at Saskatoon, SK (Preston plots) in 2005 and south of Saskatoon (SPG plots) in 2006. Lines were classified as RD or SD based on whether they darkened upon exposure to 72 h of UV light, a quick method for distinguishing between these two phenotypes (*9*).

The parental lines grown in 2004, nine individuals from the SD group, and 12 individuals from the RD group grown in 2005 were used for TGA lignin and KL assays of mature seed coat. Seed coat for CT assays was collected from two locations, Preston plots (2005) and SPG plots (2006). For plant anatomy specimens, only seed coat tissue from decorticated, parental lines (harvested in 2004) was embedded and sectioned.

Immature Seed Coat Assays. Seed was collected from the parental lines and RILs grown in the field in Saskatoon in 2006. Plants were tagged at flowering, and immature seed was collected 13 and 20 days after flowering (DAF) from the same progeny lines tested in 2005, with the addition of two more SD lines, and frozen (−20 °C) until testing. The developing seeds were classified into five groups, by length (≤6.5 mm, 7 to 8 mm, >8 to 10 mm, >10.5 mm to ≤12 mm, and ≥12.5 mm), just prior to manual decortication. Decorticated seed coat tissue was processed and assayed as described for 2005 tissue.

Seed Coat Anatomy. Hand-cut tissue (~2 mm × 10 mm) from decorticated seed coats of field-grown CDC Pintium and 1533-15 (2004 harvest) was fixed in either 2% glutaraldehyde (pH 6.8 at 4 °C, rinsed with 50 mM sodium phosphate buffer [pH 6.8] followed by three rinses with 50% aq ethanol [EtOH]) (*14*) or in FAA (formaldehyde/acetic acid/50% aq ethanol, 5:5:90 v/v/v) (*15*). Fixed tissue was dehydrated in a water/ethanol/*n*-butanol series and embedded in Paraplast Plus using a standard infiltration protocol (*14, 15*). Transverse sections were cut at 10−15 μm using a rotary microtome and mounted on glass slides with Haupt's fixative (1% gelatin in 15% aq glycerol preserved with 0.03% sodium azide) (*15*). Heated and dried mounts were dewaxed in xylene, rehydrated in an EtOH/water series followed by staining with Toluidine Blue O (TBO, Merck CI 52040, 0.5% w/v, in 2.5% sodium carbonate, pH 11), 15−30 s, and destained in deionized water (*14, 15*). Additional histochemical staining reactions for phenolics were used on hand-sectioned fresh mounts and embedded tissue sections to investigate the localization of CT polymers. These tests included EtOH/HCl (2:1 v/v) (*16*), phloroglucinol in 25% HCl (v/v) (*17*), and 1% vanillin/HCl (stained for 30 min to allow for color development) (*16*). Photomicrographs were obtained at 100× magnification using a binocular Axioskop 40 microscope and brightfield illumination (Carl Zeiss Canada Inc., Toronto, ON) equipped with a Pixelink camera (model PL-A686C) controlled by Pixelink Capture SE software (standard edition).

Biochemical Analyses. Unless otherwise stated, 100−300 mg (±0.1 mg) of ground seed coat (three to four replicates) was used in all biochemical assays.

Lignin. (i) Gravimetric assay: Acid-insoluble residues (KL) were based on the AOAC Official Method (*18*) (973.18, rev. 2000) as modified by Huntley et al. (*19*) to generate both an acid-soluble and -insoluble lignin fraction from sequential sulfuric acid digests (72% H_2SO_4 followed by dilution to 4% and autoclaving for 1 h before filtering). (ii) Spectrophotometric: TGA (*syn* 2-thiolethanoic acid) lignin was obtained using a modified method (*20, 21*). Sample tissue, as specified under Plant Material, was assayed from 2004 grown parents and 2005 grown RILs only.

A standard curve was constructed with a TGA lignin standard from a bulked preparation of mature *P. vulgaris* stem tissue. The bulked stem preparation was obtained by processing 25 g of oven-dried (60 ± 2 °C) tissue from mature *P. vulgaris* plants using a scaled TGA assay adjusted for this sample size. The final pellet in the bulked stem preparation was precipitated twice by redissolving the pellet (1.0 M NaOH) and precipitating in concentrated HCl (1:5 acid/base). In the last precipitation, the acidified pellet was washed with six changes of deionized water (3× ratio w/v) to reduce HCl carryover before drying (60 ± 2 °C). The resulting TGA lignin standard was stored in a desiccator at 4 °C. This standard was prepared twice to confirm the reproducibility of the extraction. Absorbance (280 nm) readings of the resulting preparations were compared, based on masses dissolved in 1.0 M NaOH and determined to vary by ≤0.005 AU (data not shown). Samples were diluted before absorbance readings to ensure that absorbance fell between 0.100 and 0.900 AU.

Condensed Tannin. Seed coat tissue was subjected to hydrolysis in BuOH/HCl (70:30 v/v) to form monomeric anthocyanidins by cleavage of the CT polymer (*22*) and quantified at the maximal absorbance nearest to 550 nm using a scanning spectrophotometer and converted to milligrams per gram of seed coat using the equation for the linear portion of the standard curve (*23*). Purified CT polymer from big trefoil (*Lotus uliginosus* Hoff.) (*24*) was used initially as a primary standard to construct the standard curve for CT concentration calculations. Partially purified CT polymer from sainfoin (*Onobrychis viciifolia* Scop.) (70% aq acetone extract of LH-20 Sephadex fractionated sainfoin leaf tissue) was used subsequently. A set of diluted standards was processed at the same time as the samples. BuOH/HCl was used as the reference blank and for diluting samples having an absorbance >0.990 AU.

Control Samples for Lignin and CT Assays. Decorticated seed coat of *Lens culinaris* L. cv CDC Gold (CT-free lentil) was used as a sample to provide a matrix blank in both lignin and CT protocols. Leaf tissue with no CT, *Lotus japonicus* Regel., alfalfa (*Medicago sativa* L.) (*25*), and big trefoil (high concentrations of CT) were used as control samples for high and zero CT extracts. *L. japonicus*, alfalfa, big trefoil, and sainfoin tissue and samples of CT polymers were obtained from the Forage Testing Program, a gift of Dr. M. Y. Gruber, Saskatoon Research Centre, Agriculture and Agri-Food Canada.

Flavonoid Profiling by UV-PDA Detection. Samples (2.0 and 10.0 μL, filtered, 0.2 μm) from the combined MeOH/acetone extracts and authentic standards (dissolved in 80% aq MeOH, filtered at 0.2 μm) were separated by HPLC (described in Analytical Instrumentation) using an aq acetonitrile gradient modified with 0.05% trifluoroacetic acid (v/v) (Aldrich) with a flow rate of 0.4 mL min^{-1} as follows: 5% acetonitrile (6 min), 5− 60% acetonitrile (14 min), hold 60% acetonitrile (6 min), return to 5% acetonitrile (4 min), hold 5% acetonitrile (1 min), and post-time 5% acetonitrile (2 min). Chromatograms were generated for 280 and 330 nm profiles. UV spectral data were collected between 200 and 400 nm for characterization of compounds. Blank injections (10 μL, 50% MeOH) were executed within each sample set to ensure that there were no late eluting compounds affecting the next run. Selected HPLC-grade standards dissolved in 100% MeOH [(±)-catechin ([2*S*,3*R*]-2-[3,4-dihydroxyphenyl]-3,4 dihydro-1[2*H*]-benzopyran-3,5,7-triol) and kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one)] were injected as a check of RT reproducibility during each sample set. Kaempferol was monitored at 330 nm, and the aglycone in seed coat extracts was quantified based on peak area at 20.3 min. Although partially purified CT reference standards were available to provide retention time and UV spectral data, an accurate concentration could not be calculated to quantify CT in seed coat extracts due to the presence of small amounts of other unknown UV-absorbing compounds. Therefore, CT was monitored at 280 nm and identified by the UV-spectrum eluting at 3.1 min, coincident with the elution of the CT

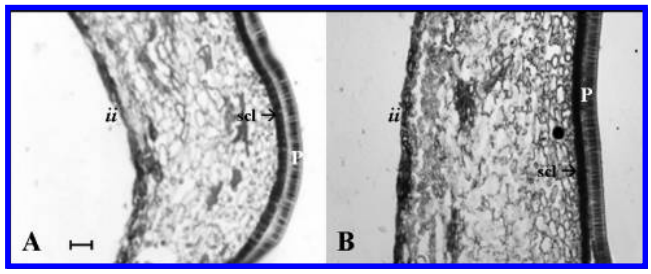

Figure 1. Transverse sections of seed coat fixed in glutaraldehyde (**A**) CDC Pintium and (**B**) 1533-15 (ii, inner integument; scl, sclerid cells with TBO-stained pigment; P, palisade layer; bar, 50 μm; and 100× magnification).

reference standard. To quantify CT in the seed coat extracts, (±)-catechin equivalents were calculated to measure CT concentration.

Polyphenol Oxidase Assay. A modified PPO screening assay from wheat protocols (*4*) was used to measure activity in whole-seed assays. To prevent pinto bean discoloration, MOPS buffer was substituted with MES, pH 6.5. Four seeds, in two replicates, for each RIL tested were soaked overnight (minimum of 10 h) in 3.0 mL of sterile 50 mM MES in 50 mL capped tubes (polypropylene centrifuge Falcon tubes, BDH) at 10 °C. Imbibing the seed for at least 10 h and including the solution in the activity test was critical because a very low activity was obtained unless the assay included the imbibing solution. Immediately prior to the PPO assay, 3 mL of 50 mM MES with 10 mM L-DOPA was added to the preimbibed bean sample. Final volumes (6 mL, each sample) were corrected by using sterile 50 mM MES where imbibition had reduced the original 3 mL volume. Samples were continuously rocked on a reciprocating shaker (~40 rpm, Model #Z-R, Fred Stein Laboratories, Inc.), and absorbance readings (475 nm) were taken at 5 min intervals for 90 min. Control assays included (i) buffer and L-DOPA with no bean samples, (ii) buffered beans with no L-DOPA, and (iii) positive controls, using wheat cultivars with known high (AC Karma) and low (AC Melita) PPO activity. PPO data for figures were calculated from the initial velocity of the reaction by plotting the slope versus time.

Statistical Analysis. Significant differences in the biochemical assays and quantification by HPLC were tested using Student's *t* test and are reported as probability (*p*) values at the 95% level. Data for individual samples were averaged from three or more replicates, and standard errors of the means (SE) were used to determine error bars on graphs for individual samples. Data for averages reported for each phenotype were from all the replicates in each phenotype, and error bars for the averaged data represent the SE over all replicates (*n*). Multivariate analysis to examine the influence of factors that separated the two phenotypes was determined by principal components analysis (PCA) based on a correlation cross-products matrix using PC-ORD software (*26*).

RESULTS AND DISCUSSION

Integument Morphology in Pinto Bean and Histochemical Detection of Polyphenolics. There were no anatomical or histochemical differences evident between the parental lines (CDC Pintium and 1533-15) based on microscopic screening of the 10−15 μm seed coat sections. Measurements taken over many sequential sections from both lines determined that there were no significant differences in the thickness of the epidermal layers or within the cells of the palisade layer (**Figure 1**). Ruzin (*14*) cautioned that although tissue dimensions are affected by dehydration and compression during fixing and sectioning (which may obscure accurate "real life" measurements), samples fixed and sectioned under the same conditions should represent any differences that may be present. Flask-shaped (sclerid) cells that comprise part of the palisade layer, stained deeply with TBO, are reflective of CT deposition and secondary lignification in the cell walls (**Figure 1**, labeled P and scl). The hilum and vascular bundles also are stained deeply with TBO, indicative of secondary lignification elsewhere in the inner seed coat.

Unlike in black beans, anthocyanins are not produced in the seed coat of pinto beans (*27*). Pinto seed coat tissue from mature seed was treated initially with acidified MeOH to distinguish between anthocyanins and CT. There was a lapse of over 30 min before a red color developed, typical for confirming the presence of CT-type polymers and eliminating anthocyanins as a source of pigmentation (*16*). Fresh-mount analyses with histochemicals that detect CT (phloroglucinol-HCl and vanillin-HCl) in both mature and developing seed coat (tissue from 13 and 20 DAF) did not reveal any differences (data not shown). Color development within 30−40 min in immature seed coat tissue was indicative that CT had already accumulated throughout the tissue in both lines, although in untreated samples, the background was colorless.

The seed coat tissue closest to the hilum reacted the most quickly, particularly in immature specimens, suggesting that CT was more concentrated in this area. Developmentally, the youngest seed available (collected at 13 DAF and ≤6.0 mm in length) had already accumulated CT and slowly was stained uniformly throughout. Since it is not feasible to quantify unextractable CT in mature seed coat by histochemical stains (*21*), only its occurrence was established by histochemical diagnosis in fresh mounts.

Lignin Concentration in Parental and Recombinant Inbred Lines of Pinto Bean. 'Klason lignin' (KL) represents an acid-insoluble residue after the aggressive removal of polysaccharides and protein by sulfuric acid digest and heat. It appeared that a significantly lower acid-insoluble KL concentration was associated with the SD trait in pinto bean RILs as compared to the RD population ($p < 0.0001$) (**Figure 2**). However, control assays with low lignin tissue of sainfoin and big trefoil (developing leaves containing high concentrations of CT) also produced high KL values (data not shown). This result indicated that something other than lignin had contributed to the KL determination. In research with forage legumes, low lignin-control leaf samples of alfalfa and *L. japonicus*, where CT is absent, returned low KL values, and CT-rich leaf samples of sainfoin and big trefoil returned high KL values (*28*). This control experiment confirmed that KL values could be adversely affected, most likely by a carry-over of CT. Since uncharacterized polyphenolics and phenolic-related esters have been reported to interfere in acid-insoluble types of lignin determinations (*28*), the KL data bring into question the validity of the statistical difference in lignin content between RD and SD phenotypes.

With an alternative assay, TGA-lignin, we demonstrated that there were no significant differences in lignification associated with the SD trait (**Figure 2**). Control assays from earlier experiments (as conducted for the KL determination with high and low CT concentration in developing leaves) established that CT did not interfere with the TGA-lignin assay (*29*). The TGA method was known to resolubilize the lignin polymer and precipitate interfering compounds such as CT and hydroxycinnamates (*30*). In view of data from the TGA-lignin assay, and the results of seed coat anatomy research, it is unlikely that lignin concentration contributes to postharvest color changes in the seed coat. However, the KL assay may have the potential to reveal other differences that cosegregate with the postharvest darkening trait.

Whether KL values reflect only components of the lignin polymer is open to argument. Several researchers have suggested that KL measurements could also include other plant polymers such as CT or that cell wall cross-linked phenolic acids can interfere in lignification estimates (*28, 31*). The acid-soluble

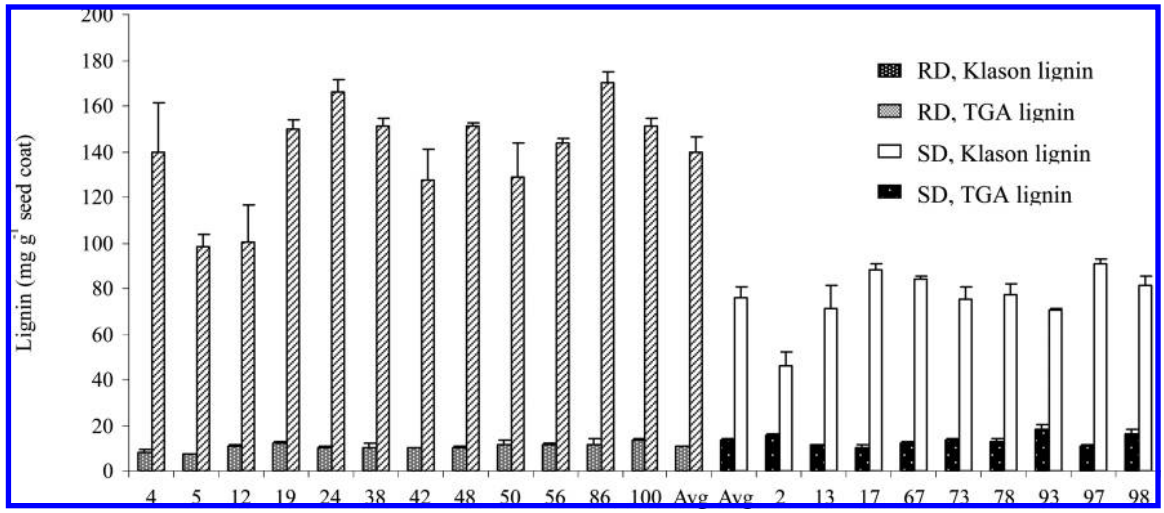

Figure 2. Lignin concentration (mg g^{-1} seed coat) in parental lines and RILs of mature pinto bean based on KL and TGA assays. Error bars represent a standard error of the means for individual RILs ($n = 4$). Avg: average for each phenotype (RD, $n = 48$ and SD, $n = 36$).

component of the KL method produced extracts from the RD RILs that were unquestionably more darkly pigmented than the acid-soluble extracts from the SD RILs. We suggest that the aggressive sulfuric acid digest, which included autoclaving (60 min, 103 421.3 Pa), solublized otherwise unextractable CT in the seed coat tissue. Using the semipurified CT polymer from big trefoil (a control assay with no plant tissue), the KL assay produced both a soluble highly pigmented extract and an insoluble residue, evidence that phenolic-derived polymers can interfere in the gravimetric portion (acid-insoluble residue) of the KL assay. The interpretation of our data is supported by reports that this type of extraction can sequester other polymers besides lignin (e.g., hemicellulose *28, 32*), as well as quinones or other oxidized polyphenolics (*30, 33–38*). On the basis of these reports, it is feasible that not only was it an otherwise unextractable CT polymer solubulized in the pinto bean KL assays that darkened the soluble portion of the extract, but also quinone-type oxidation products became part of an acid-insoluble residue, thereby contributing to gravimetric measurements.

Kaempferol Concentration in Seed Coat of Mature Pinto Bean. Kaempferol occurred as a UV-detectable aglycone in significantly different concentrations ($p \leq 0.00001$) between the two RIL phenotypes. Averaged data for the kaempferol concentration in the RD RILs was 1.31 mg g^{-1} seed coat as compared to 0.403 mg g^{-1} seed coat in the SD RILs (**Figure 3**). Both aglycone and trace amounts of kaempferol glycoside appeared in the mature seed coat tissue of both the parental lines and the RILs. This result agrees with the kaempferol concentration reported in significantly higher amounts in CDC Pintium (RD) as compared to 1533-15 (SD) from examination of mature seed, harvested in 2003, and analyzed by HPLC and mass spectrometry (*10*). In addition, the kaempferol concentration decreased during storage in both parental RD and SD pinto bean lines (*10*). This phenomenon is typical of oxidation reactions and conceivably accounts for continued postharvest darkening.

Interestingly, in our study of developing seed coat from the 2006 harvest, kaempferol was not detectable as an aglycone in the developing seed coat tissues of any of the RILs or parental lines at any of the immature stages. According to UV-spectral analysis, there were only trace concentrations of flavonol-like compounds in the extracts of developing seed coat tissue. Some of these trace spectra may have been kaempferol glycosides, similar to those reported in the earlier study of mature seed (*10*).

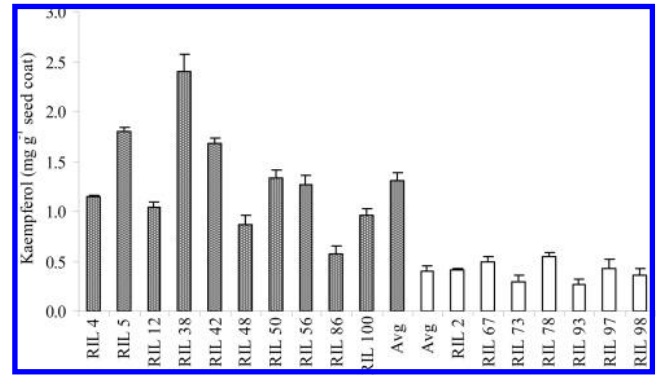

Figure 3. Concentration (separated on a RP-C_{18} Zorbax column by an acetonitrile gradient, detected at 330 nm by UV-PDA) of kaempferol (aglycone, mg g^{-1} seed coat) in mature seed coat of pinto bean RILs. Error bars indicate SE for individual RILs ($n = 3$). Filled bars, RD lines; open bars, SD lines. Avg: averaged values for each phenotype (RD, $n = 30$ and SD, $n = 21$).

In terms of seed-darkening phenomena, kaempferol is relevant to color changes during storage because flavonols oxidize to form colored byproducts (*39, 40*). This type of conversion would explain the observed decrease of postharvest kaempferol concentration in aged parental pinto bean lines (*10*). Since the accumulation of kaempferol did not occur until very late in the seed filling, flavonols may contribute to postharvest color changes by way of quinone formation or similar PPO-mediated reactions, only after a period of storage time. Since the mature RD RILs contained significantly higher amounts of kaempferol, as compared to the mature SD RILs, a greater pool of PPO substrate was available in RD lines for conversion to darkened pigmentation during storage. It was not determined whether kaempferol participated to the same or greater extent than CT in postharvest discoloration.

Extractable CT Concentration in Developing and Mature Seed Coat of Pinto Bean. *BuOH/HCl Assays.* On the basis of histochemical analyses, it was determined that there would be no interference by anthocyanins because these compounds were absent in the pinto bean seed coat. Thus, in the oxidative extraction of CT, the tissue was not preincubated in MeOH with PVPP. In mature seed coat tissue, the extractable CT concentration did not statistically correlate with the postharvest darkening trait in the pinto bean RILs ($p = 0.6772$) grown in 2005, nor in

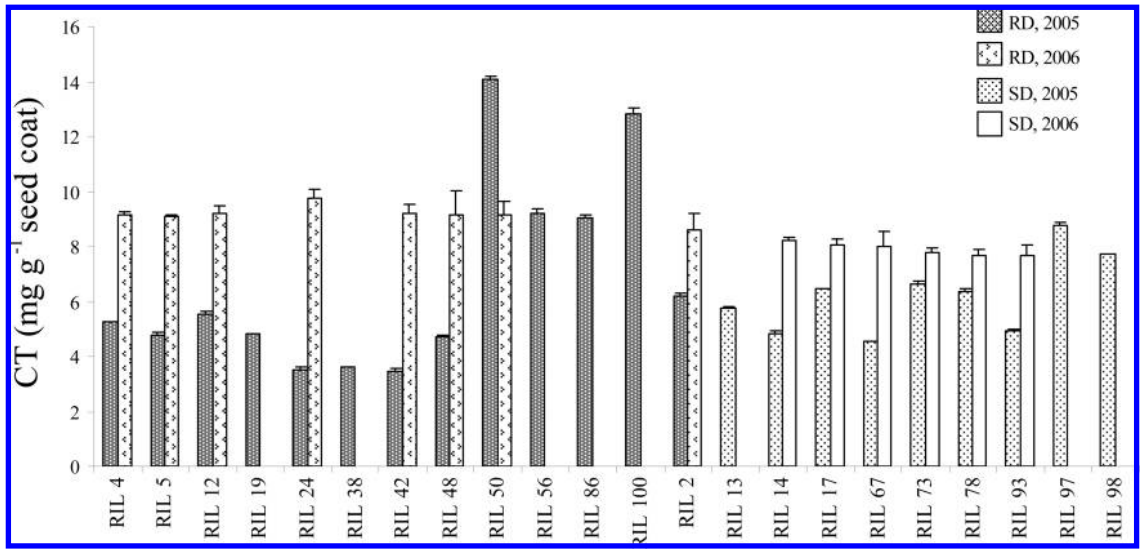

Figure 4. Concentration of CT (quantified by BuOH/HCl colormetric assay) in mature seed coat of pinto bean RILs. Error bars for individual samples are SE ($n = 3$). Within-year averages of the CT concentration between RD and SD phenotypes were not significantly different (6.7 and 6.2 [2005] and 9.2 and 8.0 [2006] mg g^{-1} seed coat, respectively).

2006, even when compared between two growing locations and different years (**Figure 4**).

In 2004 harvested parental material, there was a significant difference ($p \leq 0.0046$) in extractable CT concentration in the mature seed coat of CDC Pintium (RD) (17.8 mg g^{-1} seed coat) as compared to 1533-15, the SD parental line (8.3 mg g^{-1} seed coat). This difference could reflect a genetic variable unrelated to the darkening phenotype or environmental differences during seed development and storage. There was no significant difference in extractable CT between the parental lines in 2005 or 2006 (CDC Pintium, 9.7 and 9.5 mg g^{-1} seed coat and 1533-15, 11.2 and 8.6 mg g^{-1} seed coat, respectively).

UV-Spectral Quantification by HPLC. The UV-spectral characteristics were compared from extracts of developing seed coat (13 and 20 DAF, harvested in 2006) of both the parental lines and the RILs. When the CT concentration was monitored, there were no statistical differences between the two parental lines ($p \geq 0.8633$) or between the two RIL phenotypes ($p \geq 0.7241$). The averaged CT concentration was 98.97 and 101.81 mg g^{-1} seed coat in the developing seed of the RD and SD lines, respectively. The variability between replicate samples of the developing seed was very pronounced in both lines: SE ranged from 5.8 to 31.9, with a median SE of 19.2. By contrast, replicate samples in the extractable CT assays (BuOH/HCl) of mature seed coat consistently had a low range of SE (0.011 to 0.85). This range of variability in CT from developing seed implied that there could be other factors masking the relationship of CT to postharvest darkening that was consistent with seed filling stages. Simple averages across the population might not show phenotypic differences. Therefore, developing seed was reclassified according to size (mm) to address a possible source of variability that reflected seed development.

This subclassification also did not reveal a significant difference in CT concentration in immature seed coat between

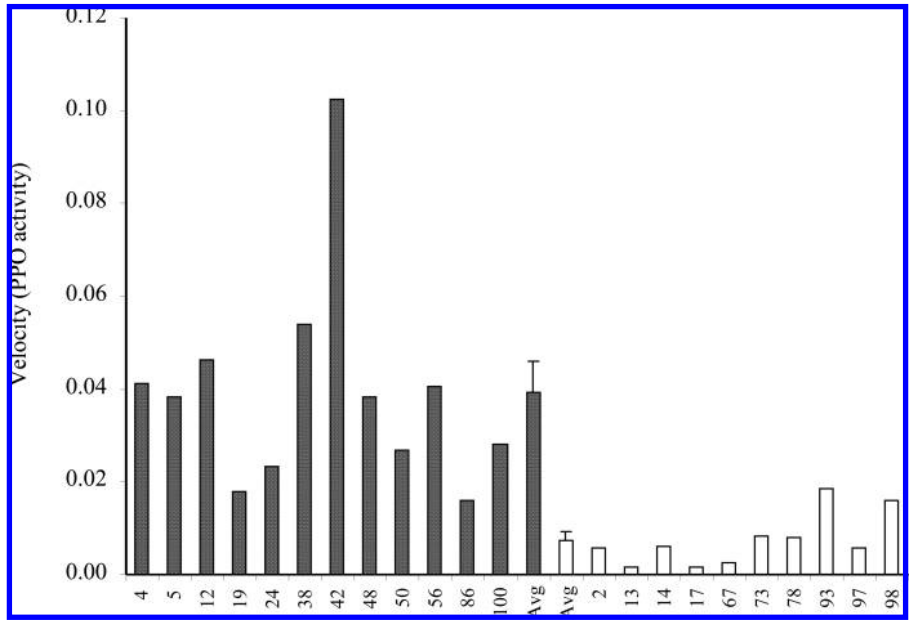

Figure 5. PPO activity in pinto bean. Enzyme activity plots (from initial velocities) of each pinto bean line (RD, shaded bars and SD, open bars) were used to calculate the slope from the linear regression equation for each PPO activity assay, monitored at 475 nm. Avg: averaged values of each phenotype ($n = 36$, RD and $n = 13$, SD).

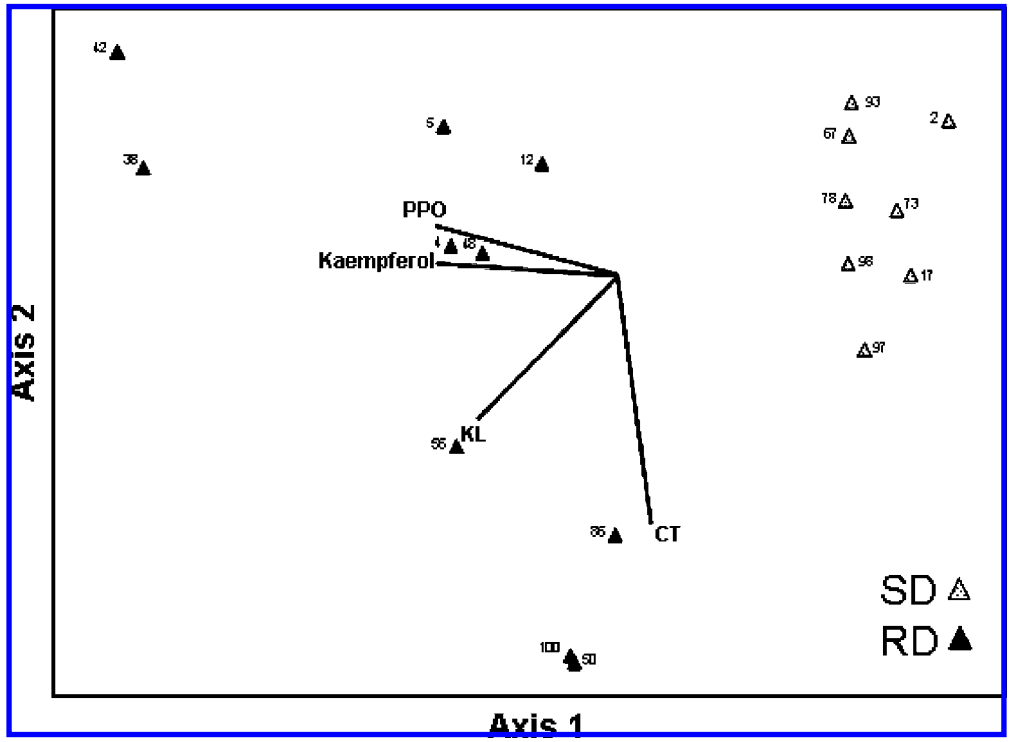

Figure 6. PCA of the four factors (kaempferol, PPO, KL, and CT) that influence the clustering of the two phenotypes (RD and SD). The first and second axes account for significant variance between the two groups (55.5 and 30.5%, respectively). Numbered symbols in the scatterplot correspond to individual RILs.

either parental lines or RIL phenotypes. It was observed, however, that extractable CT decreased by at least 1 order of magnitude between 20 DAF and harvest (~3 weeks later), with the average range of CT dropping from 99.0 to 102.0 mg g^{-1} seed coat to 8.6 to 11.2 mg g^{-1} seed coat. Evidently, a very active mechanism was converting CT and changing CT extraction and detection. The lack of difference in CT concentration between phenotypes therefore would be reflective only of the extractable, unbound CT and would not represent the immobilized, cross-linked, or insoluble oxidation products. On the basis of seed coat histochemical observations, CT quantification, and UV-spectral analysis, there appeared to be a rapid conversion of CT to an unextractable product over the seed filling period, as well as during storage.

The complexity of quantifying total CT (extractable, bound, enzymatically converted, and auto-oxidized) complicated phenotypic evaluation of plant populations because the measurement is so dependent upon extracting soluble compounds. Insoluble quinone formation, or other cross-linked bioproducts, are equally important in the estimation of CT concentration. We suggest using the aggressively oxidative KL assays for acid-insoluble lignin to evaluate unextractable pigment-related compounds as a means to estimate bound CT and CT-related quinones. Entirely insoluble forms of oxidized CT or flavonols are likely present as quinone moieties that can be complexed with other cellular constituents (*41*). Hence, an absolute quantification that accounts for all the polyphenolic products in the pinto bean seed coat is unlikely to be achieved. However, differences such as these are reflective of a dissimilar expression of CT biosynthesis and likely account for the quantitative distributions of the Hunter Laboratory values L^* and a^* that were reported for this pinto bean population (*42*).

Polyphenol Oxidase Screening Assays in Seed Coat of Mature Pinto Bean. The mechanisms of enzymatic browning are poorly understood because this is a complex reaction that involves both enzymatic catalysis (monophenolase and diphenolase activities) and auto-oxidation of the ensuing products. Many forms of polyphenol oxidase protein (bound, soluble, and latent) have been described, but purification of the native metalloenzyme has challenged enzymologists because the dinuclear copper center is very sensitive to molecular oxygen (*36, 41*).

The major catalytic function of PPO enzymes is to insert oxygen in a position ortho to an existing hydroxyl group in an aromatic ring, followed by oxidation of the ensuing diphenol to the corresponding quinone. While the oxidation of monophenolic compounds to *o*-diphenols occurs enzymatically, complexity arises because this activity is rarely apparent, the product being colorless and utilized subsequently by diphenolase for *o*-quinone formation, which is a visible product (*37, 43*). In addition, *o*-diphenols are unstable and prone to either form dimers that react with other cell constituents or undergo auto-oxidation reactions during tissue disruption and thence to precipitate (*35, 43*).

Despite the complexity of this cascade of reactions, whole-seed PPO assays of mature pinto bean contributed valuable insight into the biochemical parameters related to the postharvest-darkening trait in the RILs. The RD trait strongly associated with a higher PPO activity as compared to the activity in the RILs expressing the SD trait ($p = 0.0045$) (**Figure 5**). The whole seed assay method reported here, with buffered MES, was an efficient method to assess PPO activity in mature pinto bean populations. The inclusion of the imbibing solution as a requirement for complete activity measurement confirmed recent reports that there are bound forms of PPO that have a greater activity than soluble (leached) enzyme (*44*). Moreover, there are reports of latent forms with unknown activation mechanisms that may come into play once the reaction is initiated (*43*).

Link between Polyphenolics and PPO-Mediated Postharvest Color Changes. Although quinone products appear to be the final enzymatic outcome of PPO-mediated reactions, these compounds can further modify protein moieties and additional flavonoid polymers, such as CT (*35, 36, 45*). Through subsequent auto-oxidation reactions, complex, cross-linked colored

products form that are difficult to analyze (*1, 41*). As discussed in the results from histochemical staining and analytical chromatography, the young seed coat (≤6.0 mm) with no apparent characteristic pinto spots displayed ample CT concentration in an extractable form. On the basis of these histochemical reactions in the pinto seed coat, there was evidence that rapid conversion of CT to unextractable pigment, likely an oxidized or cross-linked quinone (*46*), occurred very early in development. By the time seed had reached stage 3 (>8.0 mm), histochemically unreactive colored pinto spots had appeared that were indicative of PPO activity. Similar developments in seed coat discoloration were documented in *Arabidopsis* seed coat mutants, and the changes were attributed to enzymatic oxidation over time (*47, 48*). For example, seeds of a transparent testa mutant, TT10, that were yellow at harvest, turned brown during postharvest storage and eventually resembled the dark brown wild-type (*49*). Without a doubt, enzyme-mediated oxidation formed brown polymeric compounds in intact, previously light-colored seed during storage, similar to the changes we observed in developing seed.

A multivariate PCA test was used to ascertain the relative influence of the differences in the polyphenolic traits. The separation of RD and SD phenotypes was significant on the first and second axes (55.5 and 30.5% variance, with eigenvalues of 2.22 and 1.218, respectively) (**Figure 6**). The data for kaempferol, PPO, and KL influenced the clustering most strongly and were highly correlated with the first axis. The results from these tests clearly separated the RD and SD groups along this axis. The wide scatter of the RD RILs on the ordination indicated that there was greater variability of the four traits among individuals with this phenotype as compared to the SD group (**Figure 6**).

Inclusion of the CT data in the matrix was responsible for further separation of phenotypes in PCA ordination on the second axis (30.5% of variance, **Figure 6**). The positions of KL and CT in the ordination demonstrated that these traits associated more strongly with each other than with kaempferol and PPO. Considering the surmised interference of oxidized CT residues in KL determination, the ordination along the second axis supports the putative association of KL and CT. This graphical representation underscores the disappearance of CT as a function of PPO activity that reduced the CT concentration in mature seed by an order of magnitude. Since CT contributes to the formation of postharvest darkening, this additionally accounts for the separation of the two phenotypes. The roles of CT and kaempferol accumulation, together with PPO catalysis and quinone formation, appear to be key parameters in evaluating bioactive compounds present in legume crops.

In future work, monitoring PPO and extractable CT through very early seed stages until maturity could provide important insight into the disappearance of CT over time. If CT could be measured in all its forms, we assert that there would be significant differences in CT concentration between RD and SD phenotypes. Improved biochemical phenotyping would then provide an accurate basis for genetically mapping the population.

SAFETY

Routine safety measures (gloves and fume hood) are required for organic solvents and acidic and basic solutions. There are no unusual toxic or explosive properties associated with the materials in these methods. A fume hood is necessary to control the vapors from thioglycolic acid lignin assays and acetone-based extractions.

ABBREVIATIONS USED

AU, absorbance units; aq, aqueous; BuOH, *n*-butanol; CDC, Crop Development Centre; CT, condensed tannin; DAF, days after flowering; EtOH, ethanol; L-DOPA, 3,4-dihydroxy-L-phenylalanine; KL, Klason lignin; mAU, milli-absorbance units; MeOH, methanol; MES, 2-(*N*-morpholino)ethane sulfonic acid; MOPS, 3-(*N*-morpholino)propane sulfonic acid; PPO, polyphenol oxidase; PVPP, polyvinylpolypyrrolidone; PCA, principal components analysis; RD, rapid darkening; RIL, recombinant inbred line; RT, retention time; RP, reversed-phase; SD, slow darkening; TBO, toluidine blue O; TGA, thioglycolic acid; UV-PDA, ultraviolet photodiode array.

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