Flavonol Glycosides from Montcalm Dark Red Kidney Bean: Implications for the Genetics of Seed Coat Color in *Phaseolus vulgaris* L.

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Three flavonol glycosides were isolated and identified from the commercial dark red kidney bean (*Phaseolus vulgaris* L.) cultivar Montcalm. In order of highest to lowest concentration these compounds were 3′,4′,5,7-tetrahydroxyflavonol 3-*O*-*β*-D-glucopyranosyl (2→1) *O*-*β*-D-xylopyranoside (compound **1**), quercetin 3-*O*-*β*-D-glucopyranoside (compound **2**), and kaempferol 3-*O*-*β*-D-glucopyranoside (compound **3**). Compound **1** is a flavonol glycoside that has not been reported before in *P. vulgaris* L. These three flavonol glycosides were yellow compounds that do not contribute to the garnet red color of Montcalm seed coats. Red-colored compounds which tested positive for proanthocyanidins are most likely responsible for the red seed coat color of Montcalm. Previous work on the chemistry of the compounds produced from the multi-allelic seed coat gene series *C*-*C*^{r}-*c*^{u} indicated that neither anthocyanins nor flavonol glycosides were detected from seed coat extracts in the presence of the *c*^{u} locus. However, the seed coat color genotype of Montcalm is *c*^{u} *J g B v rk*^{d} and three flavonol glycosides were found. Technological advances such as modern HPLC analysis of seed coat extracts may allow for detection of small amounts of compounds which previously could not be seen using paper chromatography. Alternatively, the change of the *Rk* allele to *rk*^{d} may allow for the synthesis of flavonol glycosides in the presence of *c*^{u}.

Keywords: *Dry bean; high-performance liquid chromatography; flavonoids*

INTRODUCTION

Dry seeds of common bean (*Phaseolus vulgaris* L.) exhibit considerable variation in seed coat color. Understanding the genetics of seed coat color in dry bean is important because the major market classes are identified by their seed coat color, and consumers exercise color preferences when buying beans (Adams and Bedford, 1973). Seed coat color in *P. vulgaris* is determined by the amount and presence of flavonol glycosides, tannins, and anthocyanins (Beninger et al., 1998, 1999; Takeoka et al., 1997). Poor digestibility of cooked bean colored seeds have also been directly related to the tannin content of these beans (Elias et al., 1979; Aw and Swanson, 1985).

A reconciliation of the gene symbols for the genes that control the wide array of seed coat colors in *P. vulgaris* was achieved by Prakken (1970, 1972). Feenstra (1960) identified flavonoid compounds in a limited number of genotypes and found associations between genes and their associated flavonoid compounds. However, Feenstra (1960) was unable to determine the function of single alleles that control seed coat color. Leakey (1988) presented a hypothetical scheme that outlined the genes and gene products that might account for the different colors observed. However, no attempts had been made until recently (Bassett, 1992, 1994a,b, 1996a,b) to breed genetic stocks with the wide array of colors critical to identify the genes responsible for producing specific flavonoid compounds.

Our laboratory has undertaken work to indentify the flavonoid compounds that impart color in seed coats of *P. vulgaris* and relate these to the action of the genes. The objectives of the present research were to characterize the flavonoid compounds present in dark red kidney bean cultivar cv. Montcalm and determine their contributions to seed coat color.

MATERIALS AND METHODS

Plant Material and Extraction Procedure. Seed of Montcalm dark red kidney bean (1997 stock) was obtained from the Michigan Crop Improvement Association, P. O. Box 21008, Lansing, MI 48909. Montcalm is a popular and widely grown dark red kidney bean cultivar that is favored for its superb processing quality. Preliminary high-pressure liquid chromatography analyses indicated the presence of flavonol compounds in low concentration relative to tannins and other phenolics. A total of 3.3 kg fresh weight (in 100−200 g allotments) of Montcalm beans were washed with distilled water and then placed in enough distilled water to soak them. After the beans soaked for 1 h or more, seed coats were separated from cotyledons and then freeze-dried. The water exudate from the soaked beans was also freeze-dried and stored at −20 °C. After lyophilization the seed coats were placed in a small blender and ground to a fine powder. Dried, ground bean seed coat was then packed into a glass column (5.0 × 30.0 cm) and extracted sequentially with hexane, ethyl acetate, and methanol. This procedure was repeated a number of times until a total of 0.33 kg of dried seed coat had been extracted. Extracts were dried under reduced pressure in a rotary evaporator and immediately processed for flavonoid extraction.

Isolation of Flavonoids. To remove polyphenolics such as tannins, which constituted a large part of the methanol extracts, these were dissolved in 100% MeOH and loaded onto a column (3.0 × 20 cm) with Sephadex LH-20 gel as the stationary phase. The Sephadex gel had been allowed to equilibrate overnight in 100% MeOH prior to loading the

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column. The extract was then added to the top of the column and eluted with 100% MeOH, and the separation was monitored with a hand-held UV light at 366 nm. The flavonoid band was collected as it eluted off the column, and much of the red color from the MeOH extract was retained on the Sephadex column. The column was then washed with acetone:water 1:1 to remove most of the adsorbed polyphenolics. However, the polyphenolics were not completely removed after washing and the column could be used, at most, three times before it had to be repacked with fresh Sephadex. The flavonoid extract from this Sephadex column was then rechromatographed on another Sephadex LH-20 column (1.25 × 30 cm) as above to further remove tannins. The flavonoid-containing fractions from this second column were then combined and dried under reduced pressure in a rotary evaporator.

Preparative HPLC. Purification of flavonoid compounds from the fractions obtained from the Sephadex gel was achieved on a Shiseido Capcell Pak reverse-phase C18 column (10 × 250 mm, 5 μm) using a Waters 600 multisolvent pump, 996 photodiode array detector, and a 717 autosampler. The solvent system was ACN:H_2O 30:70 isocratic with a flow rate of 2.0 mL/min. If the peaks collected were not pure, they were rechromatographed under the above conditions and the compounds collected pure after one or more purification runs. All chromatograms were analyzed using Waters Millenium 32.0 software.

Analytical HPLC. Three Montcalm seeds were soaked, and the seed coats were removed and placed in a mortar along with a small amount of Celite as abrasive. Methanol (100%) was then added, and the seed coats were ground with a pestle. After grinding for 15 min, the methanol extract was centrifuged at 20 000 g and filtered through a 0.2 μm filter. Methanol:water 1:1 was then added to the seed coats, and these were again ground; the extract was centrifuged and filtered as above. The MeOH and MeOH:H_2O extracts were then combined and rotary evaporated to dryness. Extracts were then dissolved in 1.0 mL of ACN:H_2O 25:75 for HPLC analysis. The HPLC system is the same as that described above except that the column used was a RP C18 analytical column (AG120, 5 μm, 4.6 × 250 mm). A linear gradient system was employed where solvents ACN:H_2O 1:9 were altered to ACN:H_2O 9:1 over 20 min, followed by a return to starting conditions. Total analysis time was 30 min, and the flow rate was 1.0 mL/min.

NMR and Chemical Analyses. The three purified compounds were dissolved in 1 mL of DMSO-d_6, and spectra were obtained with a VXR 500 MHZ NMR with Varian software at the Max T. Rogers NMR facility in the Department of Chemistry, Michigan State University. UV−vis spectra were obtained with chemical shift reagents $AlCl_3$ and HCl to confirm B-ring hydroxylation patterns.

Compound 1. 3′,4′,5,7-Tetrahydroxyflavonol 3-*O*-β-D-glucopyranosyl (2→1) *O*-β-D-xylopyranoside was obtained as a light yellow film: ^{1}H NMR (ppm) δ 12.5 [1H, s, H-5], 6.09 [1H, s, H-6], 6.30 [1H, s, H-8], 7.64 [1H, d, J = 2.21, H-2′], 7.53 [1H, d, J = 1.98, H-6′], 6.81 [1H, d, J = 8.4 Hz, H-5′], 5.71 [1H, d, J = 7.29, Hz H-1″], 3.31 [1H, m, H-2″], 3.09 [1H, m, H-3″], 3.24 [1H, m, H-4″], 3.45 [1H, m, H-5″], 3.53 [1H, d, J = 10.38, H-6A″], 3.29 [1H, m, H-6B″], 4.58 [1H, d J = 7.06, H-1‴], 3.04, [1H, m, H-2‴], 3.14 [1H, m, H-3‴], 3.24 [1H, m, H-4‴], 3.72 [1H, dd, J = 16.34, 6.18, H-5A‴], 3.20 [1H, m, H-5B‴]; ^{13}C NMR (ppm) 154.96 [C-2], 132.71 [C-3], 177.01 [C-4], 161.14 [C-5], 99.14 [C-6], 164.5 [C-7], 93.65 [C-8], 156.37 [C-9], 103.04 [C-10], 121.79 [C-1′], 115.20 [C-2′], 145.03 [C-3′], 148.88 [C-4′], 115.74 [C5′], 120.92 [C-6′], 97.88 [C-1″], 81.73 [C-2″], 76.04 [C-3″], 69.53 [C-4″], 76.78 [C-5″], 60.48 [C-6″], 104.44 [C-1‴], 73.78 [C-2‴], 76.04 [C-3‴], 69.37 [C-4‴], 65.58 [C-5‴].

Compound 2. Quercetin 3-*O*-β-D-glucopyranoside was obtained as a yellow film: ^{1}H NMR δ 12.5 [1H, s, H-5], 6.01 [1H, s, H-6], 6.21 [1H, s, H-8], 7.54 [2H, d, J = 9.0 Hz, H-2′6′], 6.74 [1H, s, H-5′], 5.39 [1H, d, J = 7.51 Hz, H-1″], 3.32 [1H, m, H-2″], 3.09 [1H, m H-3″], 3.16 [1H, m, H-4″], 3.22 [1H, m, H-5″], 3.57 [1H, d, J = 10.61, H-6A″], 3.30 [1H, m, H-6B″]; ^{13}C NMR (ppm) 155.38 [C-2], 132.93 [C-3], 176.47 [C-4], 160.93 [C-5], 99.70 [C-6], 171.31 [C-7], 94.0 [C-8], 156.58 [C-9], 102.0 [C-10], 121.41 [C-1′], 115.10 [C-2′], 145.01 [C-3′], 149.23 [C-4′], 115.73 [C-5′], 120.56 [C-6′], 101.38 [C1″], 74.09 [C-2″], 77.34 [C-3″], 69.88 [C-4″], 76.52 [C-5″], 60.94 [C-6″].

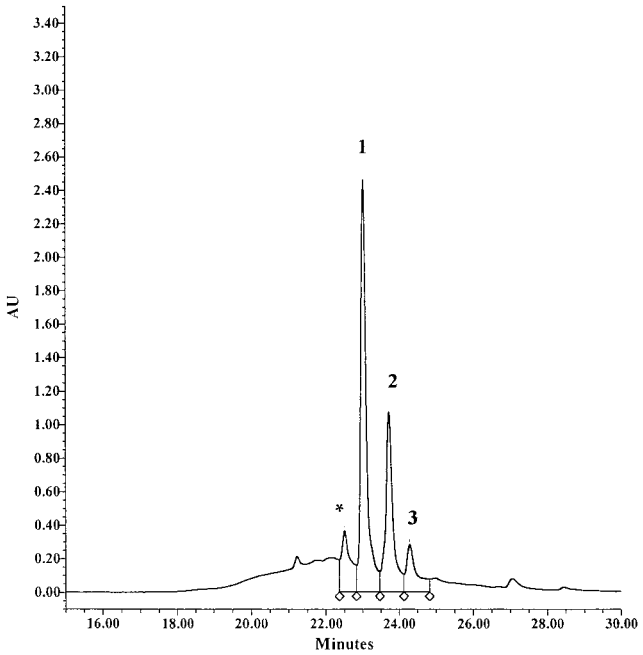


Figure 1. HPLC profile (λ = 355 nm) of the methanol extract from Montcalm seed coats: * = unidentified, **1** = quercetin 3-*O*-glucose-xylose, **2** = quercetin 3-*O*-glucoside, **3** = kaempferol 3-*O*-glucoside.

Compound 3. Kaempferol 3-*O*-β-D-glucopyranoside was obtained as a yellow film: ^{1}H NMR δ 12.25 [1H, s, H-5 (OH)], 6.08 [1H, s, H-6], 6.31 [1H, s, H-8], 7.96 [2H, d, J = 8.84 Hz, H-2′6′], 6.82 [2H, d, J = 9.06 Hz, H-3′5′], 5.21 [1H, d, J = 7.29 Hz, H-1″], 3.55 [1H, m, H-2″], 3.08 [1H, m H-3″], 3.22 [1H, m, H-4″], 3.24 [1H, m, H-5″], 3.50 [1H, d, J = 11.5, H-6A″], 3.30 [1H, m, H-6B″].

RESULTS AND DISCUSSION

Identification of Flavonol Glycosides. HPLC analysis of the methanol extract from Montcalm red kidney seed coats showed two main flavonoid compounds with small amounts of two other compounds (Figure 1). Compounds **1** and **2** were purified from the crude extract and subjected to ^{1}H and ^{13}C NMR analyses, whereas only the ^{1}H NMR spectrum was obtained for compound **3**.

The ^{1}H and ^{13}C NMR spectra for **1** were compared to published spectra (Agrawal, 1989; Beninger et al., 1998; Markham and Geiger, 1994), and the ^{13}C NMR spectrum was found to be identical for that of kaempferol 3-*O*-glucosyl-xyloside except for the downfield shift of the C3′ carbon to145.03 ppm due to hydroxylation at this position. The ^{1}HNMR also differed from the aglycon kaempferol in that three B-ring protons at 7.64, 7.53, and 6.81 were found that represented the H2′, H6′, and H5′ protons, respectively. Since no OCH_3 peaks or other unassigned peaks were present in the spectra, this indicated that OH substitutions were present at the 3′ and 4′ positions of the B-ring, which, along with the rest of the spectral data, is indicative of a quercetin flavonoid aglycon. To be certain of the ortho 3′,4′ configuration of the B-ring OH groups the methanol spectrum of a small quantity of **1** was scanned (200−700 nm), and several drops of $AlCl_3$ solution were added (5% w/v, Markham 1982). The band I absorption (356 nm) was increased

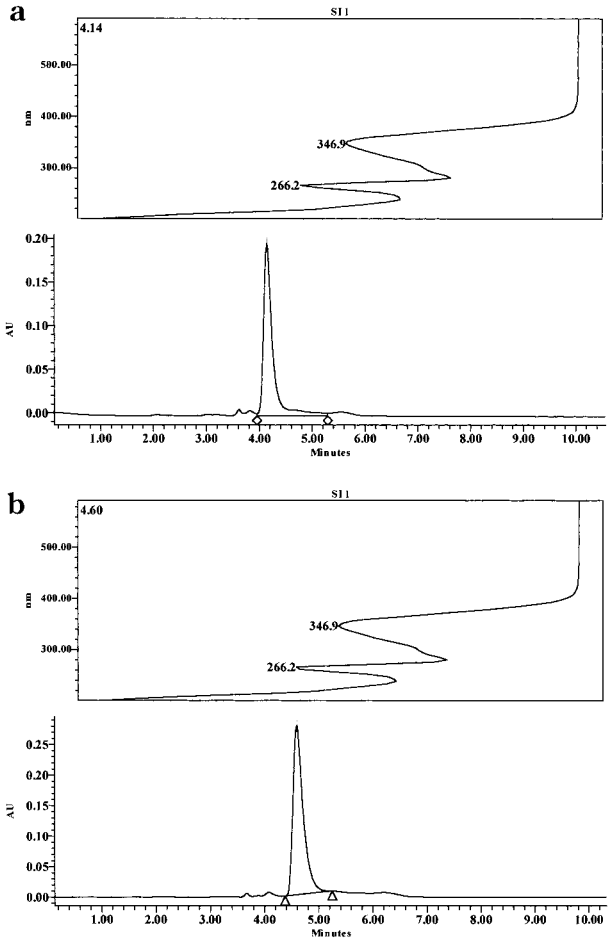
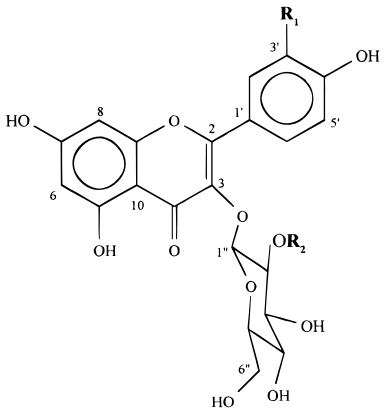


Figure 2. (a) Unknown compound **3** from Montcalm. (b) Unknown compound **3** from Montcalm co-injected with 200 μL of astragalin at 1 mg/mL. Note increase in peak height and area when compared to that of **2a**.

by 71 nm to 425 nm, indicating chelation by Al^{2+} at the 4,5 and 3′,4′ positions. On addition of a small amount of HCl:H_2O 1:2, the band I absorption decreased to 404 nm, which indicated the replacement of the Al^{2+} with H^+ at the 3′,4′ positions (Markham, 1982). The sugar region of the ^1H and ^{13}C NMR spectra corresponded to that of a 3-*O*-β-D-glucopyranosyl (2→1) xylopyranoside sugar moiety previously identified by Beninger et al. (1998).

The ^1H and ^{13}C NMR spectra of **2** agreed closely with the published spectra for this flavonoid (Agrawal, 1989; Markham and Geiger, 1994). Addition of $AlCl_3$ and HCl produced results similar to that of **1** confirming the ortho dihydroxy orientation at the 3′ and 4′ positions.

Compound **3** was present at a low concentration and enough isolated compound was available only for ^1H NMR analysis, the spectrum of which closely matched that of kaempferol 3-*O*-β-D-glucoside (astragalin). As additional confirmation, astragalin which had been identified previously from the Manteca-type bean genotype (Beninger et al., 1998) was made up in methanol solution to 1.0 mg/mL. Compound **3**, which had been purified from Montcalm beans, was then analyzed by HPLC (Figure 2a). Two-hundred microliters of the 1.0 mg/mL astragalin solution was added to this vial and then reanalyzed by HPLC. The peak for compound **3** showed an increase in area, and the spectrum was unaltered, indicating that compound **3** and astragalin have identical retention times and spectra (Figure 2b).



Compound 1 R_1 = OH, R_2 = xylose
Compound 2 R_1 = OH, R_2 = H
Compound 3 R_1 = R_2 = H

Figure 3. Structure of compounds **1**−**3**.

Considering the ^{1}HNMR and HPLC data, compound **3** was, therefore, identified as astragalin. The structure for the three compounds above is given in Figure 3.

Implications for Genetics of Seed Coat Color. Black or purple seed coat color in *P. vulgaris* is due to the presence in high concentration of three main anthocyanins: delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside (Takeoka et al., 1997). Anthocyanins are water-soluble phenolic glycosides that impart cyanic colors in higher plants and typically have absorption maxima in the 450−600 nm wavelength range (Strack and Wray, 1994). Anthocyanins were not detected in any of the seed coat extracts from Montcalm and therefore do not give the garnet red color [same as "garnet brown" of Smith and Madson (1948)] to these seed coats. The seed coat color of Montcalm is likely due to compounds that behave chemically as proanthocyanidins (tannins). During the flavonoid isolation procedure, red compounds adhered to Sephadex LH-20 gel packed in a glass separation column. The passing of plant extracts through a column packed with Sephadex LH-20 has long been a procedure used to remove tannins from preparations (Markham, 1982). The red compounds, which were retained on the column with MeOH as the eluting solvent, were washed off with acetone:water 1:1, after which they tested positive for the presence of proanthocyanidins with a vanillin assay (Markham, 1982).

It is unclear how polmerized proanthocyanidins give color since unaltered proanthocyanidins are colorless (Stafford, 1990). In sorghum [*Sorghum bicolor* (L.) Moench] secondary changes and complexes of proanthocyanidins with other phenolics in the seed coat may result in brown seed coat colors that darken on development (Stafford, 1990). The flavonol glycosides that were isolated from the seed coat extracts of Montcalm were yellow in color. Therefore it is doubtful that these flavonols had much influence on the red seed coat color given their low concentration compared to the quantity and intensity of the red-colored tannins.

Bassett (1998 and personal communication) indicated that the color genotype for Montcalm is c^u *J g B v* rk^d. According to Feenstra (1960) the multiple allelic series *Rk* - *rk* - rk^d with *J* (which is the same gene as *Sh* in his paper) give creamish, testaceous, and red-brown seed coats, respectively. Feenstra (1960) chemically analyzed seed coat genotypes that were all dominant

Rk and found that the combination of c^u and *J* with *Rk* did not allow for the production of anthocyanins or flavonol glycosides. We found that for Montcalm (c^u *J g B v* rk^d) there are three flavonol glycosides present and the majority of red color came from the fraction containing proanthocyanidins. One of the reasons for the discrepancy between our and Feenstra's findings may be due to advances in the sensitivity of instruments such as HPLC systems equipped with photodiode arrays. Feenstra conducted his seed color studies over 30 years before the advent of modern HPLC instrumentation, and he therefore may not have detected these flavonols which are present at very low concentration in kidney beans. Alternatively, the change from *Rk* in Feenstra's genotypes to rk^d may allow for the formation of flavonol glycosides.

The apparent absence of anthocyanins in Montcalm seed coat extracts disagrees with Leakey's (1988) synthesis of the literature on genetic control of red color in dry bean. Goldsborough (1984, as cited by Leakey, 1988) also failed to find anthocyanins in his acetic acid extracts from red beans carrying the *rk* allele. Leakey (1988) proposed that although anthocyanins had not been detected by Goldsborough, they may have been present, but in some way not understood, were immobilized in the seed coat. However, we are now discovering that polyphenols and their oxidation/polymerization products, rather than flavonoid monomers (Leakey, 1988), play a more important role in determining the red color of kidney bean seed coats than was originally thought.

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