Combined analysis of transcriptome and metabolite data reveals extensive differences between black and brown nearly-isogenic soybean \((Glycine\ max)\) seed coats enabling the identification of pigment isogenes

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Abstract

**Background:** The \(R\) locus controls the color of pigmented soybean \((Glycine\ max)\) seeds. However information about its control over seed coat biochemistry and gene expressions remains limited. The seed coats of nearly-isogenic black \((iRT)\) and brown \((irT)\) soybean \((Glycine\ max)\) were known to differ by the presence or absence of anthocyanins, respectively, with genes for only a single enzyme (anthocyanidin synthase) found to be differentially expressed between isolines. We recently identified and characterized a UDP-glycoseflavonoid-3-O-glycosyltransferase \((UGT78K1)\) from the seed coat of black \((iRT)\) soybean with the aim to engineer seed coat color by suppression of an anthocyanin-specific gene. However, it remained to be investigated whether \(UGT78K1\) was overexpressed with anthocyanin biosynthesis in the black \((iRT)\) seed coat compared to the nearly-isogenic brown \((irT)\) tissue.

In this study, we performed a combined analysis of transcriptome and metabolite data to elucidate the control of the \(R\) locus over seed coat biochemistry and to identify pigment biosynthesis genes. Two differentially expressed late-stage anthocyanin biosynthesis isogenes were further characterized, as they may serve as useful targets for the manipulation of soybean grain color while minimizing the potential for unintended effects on the plant system.

**Results:** Metabolite composition differences were found to not be limited to anthocyanins, with specific proanthocyanidins, isoflavones, and phenylpropanoids present exclusively in the black \((iRT)\) or the brown \((irT)\) seed coat. A global analysis of gene expressions identified \(UGT78K1\) and 19 other anthocyanin, \((iso)flavonoid,\) and phenylpropanoid isogenes to be differentially expressed between isolines. A combined analysis of metabolite and gene expression data enabled the assignment of putative functions to biosynthesis and transport isogenes. The recombinant enzymes of two genes were validated to catalyze late-stage steps in anthocyanin biosynthesis \(in\ vitrō\) and expression profiles of the corresponding genes were shown to parallel anthocyanin biosynthesis during black \((iRT)\) seed coat development.

**Conclusion:** Metabolite composition and gene expression differences between black \((iRT)\) and brown \((irT)\) seed coats are far more extensive than previously thought. Putative anthocyanin, proanthocyanidin, \((iso)flavonoid,\) and phenylpropanoid isogenes were differentially-expressed between black \((iRT)\) and brown \((irT)\) seed coats, and \(UGT78K2\) and \(OMT5\) were validated to code UDP-glycoseflavonoid-3-O-glycosyltransferase and anthocyanin 3’-O-methyltransferase proteins \(in\ vitrō\), respectively. Duplicate gene copies for several enzymes were overexpressed in
Background

Flavonoids represent a class of plant secondary metabolites that have evolved a variety of physiological functions including pigmentation, pathogen defense, and UV protection [1]. Additionally, metabolic engineering of flavonoids has become an important target for plant biotechnology, as flavonoids provide health benefits to foods, favorable agronomic traits to crops, and may be used in the future to color commercial transgenic materials such as grains to facilitate their identification and monitoring [2-4].

Commercial soybean (Glycine max (L.) Merr.) has a yellow grain. However rare spontaneous mutants exist that have black (iRT) or brown (iRT) seed coat (testa) color phenotypes. Black (iRT) and brown (iRT) soybean seed coats contain proanthocyanidins (PAs, a.k.a. condensed tannins) but differ in the presence/absence of anthocyanins [5]. A goal for biosafety is to engineer a novel red seed coat color as a marker for transgenic soybean grains to facilitate their identification [4], and could potentially be achieved by the suppression of anthocyanin-specific genes that are overexpressed in the black soybean seed coat. However the genes have not yet been identified.

Six genetic loci (I, R, T, Wp, W1, and O) [6] identified by classical genetics control flavonoid-based seed coat color in soybean. The I locus controls the presence or absence and spatial distribution of flavonoid pigments and has four alleles (i, I, iR, I), I gives completely non-pigmented seed coat, I restricts pigment to the hilum and I to a saddle-shaped region, whereas the i allele results in a fully pigmented seed coat [6]. The recessive i allele results from spontaneous deletion of CHS4 or CHSI promoter sequences and results in the abolishment of a posttranscriptional RNA silencing mechanism that results in the increased accumulation of chalcone synthase (CHS) transcripts in the seed coat [7,8].

The T locus is a pleiotropic locus that controls the type and abundance of flavonoid pigments in the seed coat in addition to other traits such as seed coat cracking and trichome pigmentation [5,9,10]. Genetic polymorphisms that affect the expression or function of the flavonoid 3'-hydroxylase gene (F3'H) have been shown to co-segregate with recessive t alleles [11,12].

The W1 locus controls flower color and affects seed color only in an iRT background; where W1 and w1 alleles give imperfect black and buff seed coat colors, respectively [6]. The W1 allele for purple flower color was shown to code flavonoid 3',5'-hydroxylase (F3'S'H), as a 65-bp insertion in the gene (F3'S'H) co-segregated with white flower color (w1) [13].

The Wp locus was suggested to code the flavonone 3'-hydroxylase gene (F3'H1) by microarray analysis as high levels of F3'H1 transcripts co-segregated with purple flower color (Wp), and low levels with pink (wp) flowers [14]. In the seed coat, recessive wp resulted in a change from black (iRTWp) to a lighter grayish (iRTwp) color [14].

The O locus affects the color of brown (iRTO) seed coats, with the recessive o allele giving a red-brown (iTO) phenotype [6]. The O locus has been suggested to code the proanthocyanidin (PA, a.k.a. condensed tannin) biosynthesis gene anthocyanidin reductase (ANR), as the gene was located between markers that flank the O locus on the soybean physical map [15]. However molecular genetics analyses have not yet demonstrated the identity of the O locus gene.

Finally, the R locus controls the presence (R) or absence (r) of anthocyanins in black (iRT) or brown (iRT) seed coats, respectively [16]. Despite the identification of several pigment genes from soybean, only transcripts for anthocyanidin synthase (ANS) genes (ANS23-1, ANS27-1 and ANS100) have been shown to be overexpressed in the black (iRT) seed coat compared to the brown (iRT) nearly-isogenic line by northern blot [17]. The upregulation of several ANS genes suggests the R locus to code a regulatory factor, and raises the possibility that other isogenes for anthocyanin biosynthesis may be upregulated.

Recently, a cDNA coding the UDP-glycose:flavonoid-3-O-glycosyltransferase (UF3GT) gene (UGT78K1) was isolated from the black (iRT) seed coat and shown to function in anthocyanin biosynthesis in vitro and by complementation of a gene mutation in Arabidopsis [18]. However UGT78K1 expressions have not been investigated in relation to seed coat color.

The soybean genome sequence Glyma1 was predicted to code 46,430 protein-coding genes with nearly 75% of the genes present in multiple copies [19]. This may suggest a relatively high frequency of functional redundancy and increased difficulty in identifying soybean genes by traditional approaches. However, using transcriptome analysis tools such as the Soybean GeneChip equipped with 37,500 probe sets in combination with broad-coverage metabolite analysis methodologies such as LC-MS/MS, gene functions could potentially be efficiently predicted. The combined analysis of transcriptome and metabolite data has been shown to be a powerful
approach for the functional identification of unknown genes [20-22]. Metabolite differences caused by the overexpression of a transcription factor, the exposure to a nutritional stress, and by species differences have been correlated with differences in transcriptome profiles to successfully predict the functions of unknown genes in flavonoid, glucosinolate, and alkaloid biosyntheses [21-23].

In the present study we have employed targeted and non-targeted metabolite analysis methodologies and have demonstrated that black (iRT) and brown (irT) nearly-isogenic soybean seed coats do not just differ in the presence/absence of anthocyanins, and have extensive differences in procyanidin, (iso)flavonoid and phenylpropanoid compositions. The underlying differences in gene expressions were then identified by microarray analysis, and the putative functions of 20 unknown genes were assigned by comparison to metabolite data. From the set of differentially regulated genes, two putative late-stage anthocyanin genes were selected and the functions of their coded enzymes were validated in vitro. In addition, a set of gene candidates potentially coded by the R locus have been provided.

**Results**

qRT-PCR indicates differential expressions of anthocyanin and proanthocyanidin genes in nearly-isogenic black (iRT) and brown (irT) soybean seed coats

Among the flavonoid genes identified from soybean seed coats, only ANS transcripts were found to be overexpressed in the seed coat of black (iRT) soybean compared to the brown (irT) isolate, however no RFLP difference was observed when probing ANS genes [17]. This suggests that ANS overexpression is not the only molecular difference between black (iRT) and brown (irT) seed coats. To examine whether other anthocyanin genes are upregulated, transcript profiles of the recently identified UGT78K1 [18] and the DFR1 gene [GenBank: AF167556] were measured from seed coat cDNA by quantitative RT-PCR (qRT-PCR) using ANS2/ANS3 transcripts [GenBank:AY382829/GenBank: AY382830] as a positive control. Phosphoenol pyruvate carboxylase (PEPC) [GenBank: D10717] was used as an endogenous reference for normalization of the qRT-PCR measurements of target genes across seed coat samples, as PEPC is expressed at similar levels in a wide range of soybean tissues and has been used previously as a reference for gene expressions during soybean seed coat development [8,24].

qRT-PCR demonstrated UGT78K1 to be significantly overexpressed in the black (iRT) seed coat at the transition stage (300 mg - 400 mg FSW) and early maturation stage (400 - 500 mg FSW) of seed development compared to the brown (irT) isolate, and to be almost undetectable at earlier stages (Figure 1). Stereomicroscopy and photospectroscopic measurements confirmed these developmental stages to coincide with the stages of anthocyanin biosynthesis in the black (irT) seed coat.

![Figure 1](image_url)
In conclusion, these results show that the recently identified *UGT78K1* is upregulated in the black *(iRT)* seed coat, in addition to *ANS2*/*ANS3*, and that there is a simultaneous downregulation of the PA gene *LAR1* at the stages of anthocyanin biosynthesis. These results demonstrate that expression differences between black *(iRT)* and brown *(iRT)* seed coats are not limited to anthocyanin genes, and may suggest extensive differences in gene expressions and phenolic compositions.

**Combined analysis of targeted and non-targeted methodologies indicate overaccumulation of anthocyanins, altered procyanidin, and reduced flavonol, benzoic acid, and isoflavone compositions in the seed coat of black *(iRT)* soybean**

The structures of anthocyanins from only a few (Korean) black soybean varieties have been described [27,28] and information regarding other phenolic compositions may be limited to proanthocyanidins (PAs), where acidic-catalyzed hydrolysis of black *(iRT)* and brown *(iRT)* seed coats showed the PAs to be 3’,4’-hydroxylated [5]. A more extensive analysis of phenolic composition differences between black *(iRT)* and brown *(iRT)* seed coats could greatly improve our understanding of seed pigmentation, and may suggest gene activities that function in pigment biosynthesis. For these reasons, black *(iRT)* and brown *(iRT)* Clark seed coat metabolites were extracted at the transition stage (300 mg - 400 mg FSW) of seed development with multiple solvents and analyzed by targeted high performance liquid chromatography-diode array detection (HPLC-DAD) and non-targeted HPLC-tandem mass spectrometry (HPLC-MS/MS). To ensure seed coats were at the same stage of development, the days post anthesis, pod length, pod color, embryo morphology, and transcript levels of the developmental marker gene Gm-r1083-1191, a putative cutin biosynthesis gene [29], and the metabolism gene *DFR1* were ensured to be equivalent between black *(iRT)* and brown *(iRT)* tissues (Additional file 1: Table S1).

For a targeted analysis of anthocyanins, black *(iRT)* and brown *(iRT)* Clark seed coats were extracted with acidified aqueous methanol and metabolites were identified using HPLC-DAD by measuring the absorbance at 520 nm in comparison to authentic standards. Ten cyanidin pigments were identified, the most abundant were the anthocyanins cyanidin-3-O-glucoside (peak 5, 43.6% total peak area), cyanidin-3-O-galactoside (peak 4, 17.4% peak area) and petunidin-3-O-glucoside (peak 6, 14.6% total peak area) with lesser amounts of delphinidin-, pelargonidin-, and peonidin-3-O-glucosides (peaks 3, 7; 8; 8.9%, 1.2%, and 1.1% peak areas, respectively), the aglycone cyanidin (peak 9, 3.9% peak area), the 3-deoxyanthocyanidin diosmetinidin (peak 10, 2.0% peak area),
and the two unidentified anthocyanin-like compounds (peaks 1 and 2, 1.6% and 0.8% peak areas, respectively) (Table 1). To our knowledge this is the first report of a 3-deoxyanthocyanidin from the soybean seed coat. The structures of all identified anthocyanins are represented in Figure 3. By contrast, cyanic pigments were not detected from the brown (iRT) seed coat at any stage of seed development (not shown).

To compare the structural features of PAs, black (iRT) and brown (iRT) seed coats were extracted with 70% acetone and subjected to phloroglucinol cleavage analysis [30,31]. PA free monomers, polymer subunit compositions, and mean degree of polymerization (mDP) did not differ significantly between nearly-isogenic black (iRT) and brown (iRT) seed coats. The soluble PA mDP was 4, and the subunit and monomer compositions consisted mainly of epicatechin with minor amounts of unidentified compounds (Additional file 2: Figure S1A – S1C). Reaction with DMACA reagent demonstrated similar amounts of soluble PAs from both black (iRT) and brown (iRT) seed coats (P = 0.08) (Additional file 2: Figure S1F). Further extraction of seed coat residues at 95°C in the presence of acid and phloroglucinol yielded only low levels of epicatechin-phloroglucinol adduct, epicatechin terminal units, and several unidentified compounds (Additional file 2: Figure S1B), and acid catalyzed cleavage of seed coat residues at 95°C identified relatively high, but equivalent amounts of 3',4'-hydroxylated solvent insoluble PAs (Additional file 2: Figure S1E).

For a non-targeted analysis of soluble phenolic metabolites, nearly isogenic black (iRT) and brown (iRT) Clark seed coats were extracted with acidified aqueous methanol and analyzed by HPLC-MS/MS over a mass range of 100 amu to 1000 amu. Identities were assigned to peaks by comparison of retention times and mass fragmentation patterns to authentic standards. Peaks with no corresponding authentic standards were identified by mass fragmentation patterns in comparison to the literature. A total of 177 peaks were detected, of which 78 peaks were assigned to 37 compounds (Table 2). Twenty-eight compounds were detected from the black (iRT) seed coat (Table 2); consisting mainly of unidentified compounds (35.7%) and anthocyanins (32.1%), followed by procyanidins (25.0%). By contrast, 23 compounds were detected from the brown (iRT) seed coat (Table 2); consisting mainly of unidentified compounds (56.5%), followed by flavonols (13.0%) and procyanidins (13.0%). The majority of compounds (62.2% of all compounds identified by HPLC-MS/MS) were exclusive to the black (iRT) or brown (iRT) seed coat, suggesting extensively different biochemical specializations of these tissues. The distribution of compound classes from black (iRT) and brown (iRT) Clark seed coats is illustrated in Figure 4.

The 14 compounds exclusive to the black (iRT) seed coat were anthocyanins (8 compounds), procyanidins (4 compounds), and unknown compounds (2 compounds) (Table 2). HPLC-MS/MS (Table 2) confirmed the identity of anthocyanins found by HPLC-DAD (Table 1). Procyanidin tetramers and larger oligomers were not detected as they exceeded the mass range (1000 amu) analyzed by the HPLC-MS/MS experiments, however 3 PA trimers (Procyanidin trimers 1, 2, and 4) and the A-type procyanidin dimer were exclusive to the black (iRT) seed coat (Table 2).

The 9 compounds exclusive to the brown (iRT) seed coat consisted of 4 unknown compounds (Unknown compound 2, 10, 12, and 13), 2 flavonols (Unknown flavonol 1 and 2), 2 benzoic acids (Orcinol O-hexoside and Unknown benzoic acid 1), and the isoflavone daidzein O-hexoside malonylated (Table 2).

Taken together, our metabolite analysis experiments show that nearly-isogenic black (iRT) and brown (iRT) Clark seed coats have compositional differences not limited to the presence or absence of anthocyanins; with specific flavonol, isoflavone, and benzoic acids exclusive to the brown (iRT) seed coat and anthocyanins and unique procyanidin oligomers exclusive to the black (iRT) seed coat. These results may suggest extensive differences in the underlying gene expressions.

Table 1 HPLC-DAD analysis of anthocyanins from the seed coat of black (iRT) soybean variety Clark

| Peak | Rt (min) | Area (%) at (520 nm) | Identity* |
|------|---------|---------------------|-----------|
| 1    | 1.7     | 1.6                 | unidentified compound 1 |
| 2    | 2.6     | 0.8                 | unidentified compound 2 |
| 3    | 5.5     | 8.9                 | delphinidin-3-O-glucoside |
| 4    | 6.3     | 17.4                | cyanidin-3-O-galactoside |
| 5    | 6.7     | 43.6                | cyanidin-3-O-glucoside |
| 6    | 7.1     | 14.6                | petunidin-3-O-glucoside |
| 7    | 7.4     | 1.2                 | pelargonidin-3-O-glucoside |
| 8    | 7.7     | 1.1                 | peonidin-3-O-glucoside |
| 9    | 9.5     | 3.9                 | Cyaniding |
| 10   | 9.7     | 2.0                 | diosmetinidin |

*For chemical structures see Fig. 3.
Microarray Analysis Indicates Concomitant Upregulation of Specific Phenylpropanoid, Flavonoid, and Anthocyanin Isogenes and Downregulation of Other Flavonoid, Benzoic Acid, and Isoflavone Isogenes in the Black (iRT) Seed Coat Transcriptome

Nearly 75% of genes in the soybean genome are present in multiple copies [19], and previously only transcripts for ANS isogenes were found to be overexpressed in the black (iRT) seed coat compared to the nearly-isogenic brown (irT) tissue [17]. For a global analysis of isogene expressions, black (iRT) and brown (irT) Clark seed coats were dissected at the transition stage of seed development (300 - 400 mg FSW) for microarray analysis using the Affymetrix Soybean GeneChip. Seed coats were ensured to be at the same stage of development as described above for the metabolite analyses (Additional file 1: Table S1).

Of the 37,500 probe sets present on the Soybean GeneChip, 80 were found to be upregulated more than 2-fold in black (iRT) soybean (Additional file 3: Table S2).

### Table 2 HPLC-MS/MS analysis of seed coat extracts of black (iRT) and brown (irT) soybean

| Compound | Identity | [M+] (m/z) | Major fragment(s) | Rt (min) | Genotypea |
|----------|----------|------------|-------------------|---------|-----------|
| 1        | Unknown 1| 413        | 183, 115          | 1.4     | B         |
| 2        | Unknown 2| 381        | 184, 125          | 1.6     | irT       |
| 3        | Unknown 3| 183        | -                 | 1.7     | irT       |
| 4        | Unknown 4| 162        | -                 | 1.9     | B         |
| 5        | Unknown 5| 409        | 303               | 1.9     | B         |
| 6        | Unknown 6| 437        | 273               | 2.3     | B         |
| 7        | Unknown 7| 416        | -                 | 2.3     | B         |
| 8        | Unknown 8| 580        | 182, 265, 437     | 2.5     | B         |
| 9        | Unknown 9| 507        | 485, 323, 132     | 2.9     | B         |
| 10       | Orcinol O-hexoside | 305 | 287         | 4.0     | irT       |
| 11       | Unknown 10| 331   | 248             | 4.2     | irT       |
| 12       | Procyanidin trimer 1| 867  | 579, 309, 299   | 4.7     | irT       |
| 13       | Procyanidin B2* | 579  | 291             | 5.5     | B         |
| 14       | Unknown 11| 607   | 409, 455        | 6.0     | B         |
| 15       | Procyanidin trimer 2| 867  | 577, 407        | 6.4     | B         |
| 16       | Delphinidin-3-O-glucoside* | 465  | 303             | 6.6     | irT       |
| 17       | Procyanidin trimer 3| 867  | 579             | 6.6     | irT       |
| 18       | Epicatechin* | 291  | -               | 6.9     | B         |
| 19       | Cyanidin-3-O-galactoside* | 449  | 287             | 7.1     | irT       |
| 20       | Daidzein O-hexoside malonylated | 503  | 417             | 7.4     | irT       |
| 21       | Unknown 12| 481   | 319             | 7.4     | irT       |
| 22       | Cyanidin-3-O-glucoside* | 449  | 287             | 7.5     | irT       |
| 23       | Petunidin-3-O-glucoside* | 479  | 319, 303        | 8.0     | irT       |
| 24       | Unknown 13| 341   | 278, 111        | 8.1     | irT       |
| 25       | Pelargonidin-3-O-glucoside* | 433  | -               | 8.3     | irT       |
| 26       | Peonidin-3-O-glucoside* | 463  | 301, 286        | 8.7     | irT       |
| 27       | Procyanidin dimer 2| 579  | 409, 291        | 9.2     | B         |
| 28       | Unknown flavonol 1| 331  | -               | 9.0     | irT       |
| 29       | Procyanidin trimer 4| 867  | 579, 699        | 9.2     | irT       |
| 30       | A-type procyanidin dimer | 593  | -               | 9.7     | irT       |
| 31       | Unknown anthocyanin 1| 317  | -               | 9.6     | irT       |
| 32       | Unknown flavonol 2| 615  | -               | 9.6     | irT       |
| 33       | Cyanidin* | 287  | -               | 10.0    | irT       |
| 34       | Unknown 14| 487   | -               | 10.8    | B         |
| 35       | Diosmetinidin* | 285  | -               | 10.3    | irT       |
| 36       | Unknown benzoic acid 1| 633  | 331, 315        | 11.0    | irT       |
| 37       | Quercetin-3-O-glucoside* | 465  | 303             | 11.0    | B         |

*Based on co-chromatographic and MS fragmentation comparison to an authentic standard.

*Black soybean (iRT), brown soybean (irT), both (B).

*bTrace amounts in irT.

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Classification using the Gene-Bins ontology tool (http://bioinfoserver.rsbs.anu.edu.au/utils/GeneBins/index.php) demonstrated the majority of upregulated genes to be “unclassified without homology” (28.7%) or “unclassified with homology” (24.8%), followed by “biosynthesis of secondary metabolites” (7.9%) (Additional file 4: Figure S2). This latter class consisted exclusively of putative (and some characterized) phenylpropanoid, flavonoid, and anthocyanin isogenes. Interestingly, putative isogenes of the phenylpropanoid pathway (4CL-like and 4CL2), the flavonoid pathway (CHS4/5, F3H-like, DFR2, ANS2/ANS3), and the late-stage anthocyanin pathway (UGT78K1, UGT78K2, OMT-like, OMT5, GST21 and GST26) were all upregulated more than 2-fold in the black (iRT) seed coat, more than 234-fold in the case of the GST26 probe set (Table 3). A BLASTP search of the soybean genome sequence Glyma1 (http://www.phytozome.net/soybean) using the conserved plant secondary product glycosyltransferase signature sequence (PSPG box) [32] identified 214 annotated Family 1 glycosyltransferases (UGTs) (Additional file 5: Table S3). Of the 214 UGTs, only 2 were found to be upregulated more than 2-fold from the black (iRT) seed coat; the recently identified UF3GT gene.

Table 3 Putative phenylpropanoid/flavonoid gene sets upregulated more than 2-fold in the seed coats of black (iRT) soybean compared to the brown (irT) isolinea,b

| Gene Family                  | Probe Sets       | Name                               | Target Description                                      | Black/brown fold change | P-value   | d-score |
|------------------------------|------------------|------------------------------------|--------------------------------------------------------|-------------------------|-----------|---------|
| Phenyl-propanoid/Flavonoid pathway | GmaAffx.80720.1. | DFR2*                              | Dihydroflavonol-4-reductase                             | 62.45                   | 0.000002  | 24.70   |
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | GmaAffx.42116.1. | CHS4*/CHS5*                         | Chalcone synthase (LOC732575)                           | 10.99                   | 0.000020  | 8.45    |
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | GmaAffx.42116.1. | S1_x_at                            | Anthocyanidin synthase                                  | 8.06                    | 0.000026  | 7.124398|
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | Gma.1163.1.      | ANS2*/ANS3*                         | Anthocyanidin synthase                                  | 6.77                    | 0.000118  | 8.77    |
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | Gma.17141.1.     | F3H-like                           | Moderately similar to A. thaliana putative flavonone 3-β-hydroxylase clone U20744 (74%) | 6.43                    | 0.004849  | 203     |
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | Gma.7423.2.      | 4CL-like                           | 4-coumarate-CoA ligase-like protein                     | 2.80                    | 0.005815  | 1.96    |
|                              | S1_a_at          |                                    |                                                        |                         |           |         |
|                              | Gma.5621.1.      | G4DT*                              | Pterocarpan 4-dimethylallyltransferase                  | 2.36                    | 0.009232  | 1.80    |
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | Gma.8472.1.      | 4CL2*                              | 4-Coumarate-CoA ligase 2                                 | 2.27                    | 0.000108  | 4.60    |
| Glycosyl-transferase         | GmaAffx.71999.1. | UGT78K2                            | Glycosyltransferase                                     | 3.09                    | 0.000124  | 4.50    |
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | Gma.1002.2.      | UGT78K1*                           | UDP-glucose:flavonoid 3-O-glucosyltransferase           | 2.81                    | 0.000229  | 4.03    |
|                              | S1_at            |                                    |                                                        |                         |           |         |
| O-methyl-transferase         | Gma.9647.1.      | OMT-like                           | O-methyltransferase, putative                           | 2.58                    | 0.000098  | 4.74    |
|                              | S1_at            |                                    |                                                        |                         |           |         |
|                              | GmaAffx.57777.1. | OMT5                               | Caffeoyl-CoA O-methyltransferase 5                       | 2.48                    | 0.000157  | 4.33    |
|                              | S1_at            |                                    |                                                        |                         |           |         |
| Glutathione S-transferase    | GmaAffx.71212.1. | GST26                              | Glutathione S-transferase                               | 234.11                  | 0.000003  | 21.22   |
|                              | A1_at            |                                    |                                                        |                         |           |         |
|                              | Gma.5139.1.      | GST21                              | Glutathione S-transferase                               | 2.05                    | 0.000656  | 3.09    |

*aExpression values were obtained using RMA (Irizarry et al., 2003).
*bSee Table S2 for the complete list of upregulated genes.
*Previously characterized flavonoid biosynthesis genes.
UGT78K1 [18] was upregulated more than 2.8-fold and a second uncharacterized glycosyltransferase, which we named UGT78K2 in accordance with UGT nomenclature [33], was upregulated more than 3-fold (Table 3).

A search of the soybean genome sequence with the conserved OMT-domain sequence (PLN02589; NCBI) and the ontology ‘O-methyltransferase’, identified 84 annotated OMTs (Additional file 6: Table S4). Of the 84 OMT genes, only probe sets for OMT-like and OMT5 were upregulated in the black (iRT) seed coat, more than 2.4-fold and 2.5-fold, respectively (Table 3).

A total of 52 probe sets were downregulated more than 2-fold in the black (iRT) seed coat (i.e. black/brown < 0.5 fold); among these, 2 probe sets by more than 10-fold, 12 probe sets by more than 3-fold, with the remainder between 2- and 3-fold (Table 4; Additional file 7: Table S5). Classification of down-regulated genes using the Gene-Bins ontology tool demonstrated the majority to be “unclassified without homology” (45.5%) or “unclassified with homology” (12.1%), followed by “biosynthesis of secondary metabolites” (9.1%) (Additional file 8: Figure S3). The latter class consisted primarily of characterized and putative (iso)flavonoid isogenes. Isogenes for common flavonoid biosynthesis (CHS1, CHS9, F3’H), PA biosynthesis (LAR1), and isoflavone biosynthesis (IFR1-like) were all down-regulated more than 2-fold in the black (iRT) seed coat, more than 35-fold in the case of the CHS1 (Table 4). The flavonoid genes CHS9 and F3’H were down-regulated more than 21-fold and 2.5-fold, respectively, the putative isoflavone gene IFR1-like by more than 3.6-fold, and the putative PA gene LAR1 by more than 2.2-fold (Table 4).

Of the differentially regulated probe sets, 12 represented transcription factor genes, 8 were upregulated and 4 downregulated (Additional file 3: Table S2 and Additional file 7 Table S5, respectively), however none of these genes clustered with known flavonoid transcription factor orthologs by phylogenetic analysis (not shown).

A previous study mapped the R locus between markers A668_1 and K387_1 on MLG K (Chromosome 9) [34]. Nineteen differentially-regulated probe sets were associated with genes located on chromosome 9 of the soybean genome sequence Glyma1 (Additional file 9: Table S6). Interestingly, all genes were located in a 5.16 Mb region of the distal arm of chromosome 9, and only the downregulated AP2/ERF (Glyma09g36840) and serine carboxypeptidase-like protein (Glyma09g36080) and the upregulated polyamine oxidase (Glyma09g36150) were located between markers (BARC-025669-04989 and Sat_293) that flank the putative position of the R locus (http://soybeanphysicalmap.org) (Figure 5).

To validate the microarray results, 28 differentially-regulated genes were selected and their relative expression

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**Table 4 Putative phenylpropanoid/flavonoid gene probe sets downregulated more than 2-fold in the black (iRT) compared to the brown (iRT) soybean seed coat**

| Gene Family | Probe Sets | Name | Target Description | Black/brown fold change | P-value | d-score |
|-------------|------------|------|---------------------|-------------------------|---------|---------|
| Phenylpropanoid/Flavonoid pathway | Gma17605.1. S1_at | CHS1* | Chalcone synthase | 0.028 | 0.000007 | -16.45 |
| | GmaAffx.92491.1. S1_s_at | CHS9* | Chalcone synthase | 0.047 | 0.000005 | -19.36 |
| | GmaAffx.89828.1. S1_s_at | IFR1-like | Highly similar to isoflavone reductase homolog 1 (IFR1) (93%) | 0.272 | 0.000132 | -4.46 |
| | GmaAffx.8455.1. S1_at | sf3’H1* | Flavonoid 3’-hydroxylase | 0.400 | 0.000646 | -3.10 |
| | GmaAffx.93221.1. S1_s_at | OOMT-like | Moderately similar to Rosa rugosa orcinol O-methyltransferase (OOMT-A) (72%) | 0.410 | 0.003428 | -2.17 |
| | GmaAffx.34868.1. A1_at | LAR1 | Highly similar to Phaseolus coccineus leucoanthocyanidin reductase (LAR) (88%) | 0.443 | 0.000016 | -9.02 |
| | Gma4340.2. S1_a_at | Moderately similar to P. trichocarpa flavonoid O-methyltransferase-related (73.9%) | 0.462 | 0.004180 | -2.08 |
| | Gma10949.1. S1_s_at | Moderately similar to A. thaliana 3-chloroallyl aldehyde dehydrogenase/aldheyde dehydrogenase (ALD42C4) (75.5%) | 0.495 | 0.000273 | -3.92 |
| Transport | GmaAffx.39265.1. S1_at | MATE1 | Weakly similar to A. thaliana antiporter/drug transporter MATE family protein (69.8%) | 0.414 | 0.000265 | -3.95 |

*aExpression values were obtained using RMA (Irizarry et al., 2003).
*bSee Table S5 for the complete list of up-regulated genes.
*Previously characterized flavonoid biosynthesis genes.
profiles were measured by semi-quantitative RT-PCR (semi-qRT-PCR) using black (iRT) and brown (irT) seed coat cDNA as templates. Phosphoenol pyruvate carboxylase (PEPC) was used as the endogenous control to ensure equal loading and the flavonoid genes CHS7/CHS8 and the putative PA gene ANR1 were used as negative controls as they were not found to be differentially expressed in the microarray analysis. Semi-qRT-PCR confirmed the differential-expressions of all 28 genes and demonstrated similar expressions for CHS7/CHS8 and ANR1 (Additional file 10: Figure S4). Furthermore, as the upregulated probe set GmaAffx.42116.1.S1 did not distinguish between CHS4 and CHS5 expressions, semi-qRT-PCR was performed with gene specific primers to show the CHS4 gene, and not the CHS5 gene to be upregulated in the black (iRT) seed coat (Additional file 10: Figure S4).

In summary, these results show that numerous specific anthocyanin, flavonoid and phenylpropanoid isogenes are upregulated and other flavonoid, benzoic acid, and isoflavone isogenes are downregulated in the black (iRT) seed coat transcriptome relative to that of the nearly-isogenic brown (irT) tissue.

**Putative Assignment of Function to Genes Differentially-Regulated between Black (iRT) and Brown (irT) Seed Coats**

Gene functions can be successfully predicted based on a combined analysis of transcriptome and metabolite data [21]. We compared differences in gene expressions determined by microarray to differences in metabolite compositions to assign putative functions to genes from the black (iRT) and brown (irT) seed coats (Figure 6). In addition to 7 previously characterized soybean flavonoid genes, several uncharacterized genes belonging to large multi-gene families with possible roles in anthocyanin biosynthesis and transport were upregulated; including one glycosyltransferase (UGT78K2), two O-methyltransferases (OMT5 and OMT-like) and two glutathione S-transferases (GST21 and GST26) (Table 3). Considering the accumulation of metabolites with specific molecular structures in the black (iRT) seed coat, putative functions of the differentially-expressed genes were assigned to specific structural modifications and transport functions (Figure 6).

Anthocyanins in the seed coat of black (iRT) soybean are glycosylated at the 3-position of the C-ring (Figure 3). The glycosyltransferase UGT78K2 is assigned to code a UDP-glycosyl:flavonoid 3-O-glycosyltransferase (UF3GT) protein (Figure 6) and may have functional redundancies in anthocyanin biosynthesis with the previously characterized UGT78K1 [18], which was also upregulated in the black (iRT) seed coat (Table 3). Two anthocyanins (petunidin-3-O-glucoside and peonidin-3-O-glucoside) and one deoxyanthocyanin (diosmetinidin) from the black (iRT) seed coat are methylated at the 3-position of the B-ring (the 3’-position) (Figure 3). The two upregulated O-methyltransferases were assigned to code anthocyanin 3’-O-methyltransferase (AOMT) proteins (Figure 6) and may have redundant function in anthocyanin biosynthesis this tissue.

**UGT78K2 and OMT5 Code UDP-Glycosyl:Flavonoid 3-O-Glycosyltransferase and Anthocyanin 3’-O-Methyltransferase Proteins, Respectively**

Reducing the expressions of late-stage anthocyanin genes by RNA interference (RNAi) in the seed coat of black soybean may be an effective strategy to engineer seed coat color while minimizing unintended effects on other flavonoid subpathways. However, functional redundancies may require that multiple genes be silenced in order to reduce enzyme activity enough to reduce pigment levels. Two glycosyltransferases (UGT78K1 and UGT78K2) were upregulated in the black (iRT) seed coat relative to the nearly-isogenic brown (irT) tissue (Table 3) and the upregulation paralleled the accumulation of anthocyanins with 3-O-glycosylated structures (Figure 2), suggesting the function of these genes as UDP-glycosyl:flavonoid 3-O-glycosyltransferases (UF3GTs). We have characterized UGT78K1 previously and shown it to possess UF3GT activity towards anthocyanidins in vitro and in vivo [18]. To
determine the catalytic function of UGT78K2 [GenBank: HM591298], the ORF was cloned into an N-terminal hexahistidine fusion tag vector and expressed in E. coli for analysis of the coded recombinant protein in vitro. The recombinant protein (rUGT78K2) was purified by ion-metal-affinity chromatography (IMAC) had an apparent mass of 50.9 kDa that matched well with the calculated mass of the native UGT78K2 protein (49.04 kDa) in addition to the 2.04 kDa N-terminal hexahistidine fusion tag encoded by the pET-14b vector (Figure 7A). In assays containing UDP-glucose as the sugar donor and cyanidin as the acceptor, the recombinant enzyme transferred glucose to the 3-position of cyanidin to form cyanidin-3-O-glucoside (Figure 7B, C), whereas the boiled enzyme and bacteria expressing the corresponding empty vector could not catalyze this reaction (not shown). Similarly, in assays containing UDP-galactose as the sugar donor and cyanidin as the acceptor, the recombinant enzyme transferred galactose to the 3-position of cyanidin to form cyanidin-3-O-galactoside (not shown). Cyanidin-3-O-glucoside and cyanidin-3-O-galactoside from recombinant enzyme assays were identified by HPLC-DAD in comparison to the authentic standards, and confirmed by HPLC-MS/MS with the characteristic parent ions 449.0 [cyanidin-3-O-glucoside or cyanidin-3-O-galactoside] and the MS/MS fragment
As we have performed the kinetics analysis for recombinant UGT78K1, we were able to employ the same experimental conditions to test the kinetics of UGT78K2. Interestingly, UGT78K2 converted cyanidin-3-O-glucoside with greater velocity than UGT78K1 and higher concentrations of cyanidin were required to inhibit its activity (Figure 7D) despite the enzymes having 93% amino acid similarity (Additional file 11: Figure S5).

Methylation of the B-ring of anthocyanins has a reddening effect on flower color (reviewed by [35]) and thus engineering the expression of 3’-O-methyltransferases could change the redness of seed color. However, despite the identification of several flavonoid OMTs from soybean, none have been shown to accept anthocyanins as substrates. Our microarray analysis identified two O-methyltransferases (OMT5 and OMT-like) to be upregulated in the black (iRT) seed coat (Table 3) and the upregulation paralleled the accumulation of 3’-O-methylated anthocyanins in this tissue (Figure 2), suggesting the function of these genes as anthocyanin 3’-O-methyltransferases (AOMTs). Following the approach described above, the ORF of OMT5 [GenBank: HQ856048] was cloned for expression and functional characterization of the corresponding recombinant protein in vitro. The purified recombinant protein had an apparent mass of 30.0 kDa that matched well with the calculated mass (27.78 kDa) in addition to the 2.04 kDa N-terminal hexahistidine fusion tag encoded by the pET-14b vector (Figure 8A). In assays containing S-adenosyl-L-methionine (SAM) as the methyl donor and cyanidin-3-O-glucoside as the acceptor, the recombinant enzyme transferred a methyl group to the 3’-position of the acceptor to form peonidin-3-O-glucoside (Figure 8B, C), whereas the boiled enzyme and bacteria expressing the corresponding empty vector could not catalyze this reaction (not shown). Peonidin-3-O-glucoside from recombinant enzyme assays was identified by HPLC-DAD in comparison to the authentic standard, and confirmed by HPLC-MS/MS with the characteristic parent ion 463.1 [peonidin-3-O-glucoside]+1 and the MS/MS fragment 301.0 [peonidin]+1 corresponding to the loss of glucose (-162 amu).

To further investigate the function these genes, UGT78K2 and OMT5 expression profiles were measured from seed coat cDNA throughout black (iRT) and brown (irT) soybean seed development by quantitative RT-PCR (qRT-PCR) (Figure 9) and expressions were compared to anthocyanin accumulation profiles throughout seed development (Figure 2). Similar to UGT78K1, UGT78K2 and OMT5 expressions peaked at the transition and early maturation stages of seed development (300 mg - 400 mg FSW, 400 mg - 500 mg
FSW) in the black (iRT) seed coat (Figure 9), the stages at which anthocyanins accumulate (Figure 2), and were low or almost undetectable at early stages of seed development in the black (iRT) seed coat.

Together the qRT-PCR and recombinant enzyme assays suggest that UGT78K2 and OMT5 code UF3GT and AOMT proteins, respectively, that function in anthocyanin pigment biosynthesis in the seed of black (iRT) soybean.

**Discussion**

The seed coat compositions of black (iRT) and brown (irT) soybean (*Glycine max*) were known to differ only by the presence/absence of anthocyanins, however our metabolite analysis demonstrated these nearly-isogenic tissues to have extensive differences in anthocyanin, proanthocyanidin (PA), (iso)flavonoid, and phenylpropa-noid content (Table 2). Of the 37 compounds detected, 24 compounds (62.2% of all compounds identified by HPLC-MS/MS) were exclusive to either the black (iRT) or the brown (irT) seed coat. Analysis of the distributions of compound classes between seed coats demonstrated the black (iRT) to a have a greater proportion of anthocyanins and PAs, and the brown (irT) to have a greater proportion of flavonol, benzoic acid, isoflavone, and unknown metabolite compositions (Figure 4). Metabolites exclusive to the black (iRT) seed coat included PA trimers (Procyanidin trimer 1, 2, and 4) and an A-
type procyanidin dimer in addition to various anthocyanins (Table 2).

The anthocyanins identified from variety Clark were mainly similar to those reported previously from various Korean varieties [27,28] however Clark was the first shown to contain a 3-deoxyanthocyanidin (diosmetinidin) in the seed coat (Table 2). 3-deoxyanthocyanidins are relatively rare intensely coloured pigments found in Sorghum bicolor in response to fungal attack [36], and from the cotyledons of yellow soybean in response to UV-C treatment [37]. The biosynthesis of 3-deoxyanthocyanidins from flavonones has been shown to be a minor activity of a DFR isogene in Sorghum [38]. It would be interesting to determine whether the DFR gene that was highly overexpressed in the black Clark seed coat (DFR2) had similar catalytic activity, and whether the Korean varieties contain genetic alterations related to DFR2.

Analysis of the black (iRT) seed coat transcriptome compared to that of the nearly-isogenic brown (iRT) tissue identified 80 probe sets to be upregulated (Table 3). This is almost double the number of probe sets upregulated by overexpression of the MYB transcription factor PAP1 in Arabidopsis (38 probe sets) [21], similar to the number upregulated by overexpression of the anthocyanin factor LAP1 in M. truncatula (61 probe sets), and less than half the number upregulated by overexpression of LAP1 in M. sativa (270 probe sets) [39]. The higher numbers of genes upregulated in anthocyanin-overaccumulating tissues of black soybean, M. truncatula and M. sativa likely reflect the fact that legumes have undergone genome duplication and that genes with multiple copies are frequently retained [19]. Our data supported this hypothesis as duplicate copies of several genes were upregulated in the black (iRT) seed coat (e.g. 4CL2 and 4CL-like, UGGT78K2 and UGGT78K1, OMT5 and OMT-like, and GST21 and GST26).

Functional redundancies can complicate gene identification by traditional approaches. Previous to the present study, genes for only 5 flavonoid enzymes (CHS, F3H, F3′H, F3′5′H, and UF3GT) were identified to be involved in pigment biosynthesis in the soybean seed coat [7,11-14,18]. In the present study, 20 isogenes for 14 proteins were identified to have putative roles in flavonoid biosynthesis in black (iRT) or brown (iRT) seed coats using a combined analysis of microarray and metabolite data (Figure 6).

Transcriptome analysis has previously demonstrated a critical role of CHS7 and CHS8 genes for isoflavonoid biosynthesis in developing embryos of soybean [40]. However, our microarray and semi-quantitative RT-PCR data demonstrated that CHS7 and CHS8 were not differentially regulated in black (iRT) and brown (iRT) seed coats (Table 3). Interestingly, it was CHS4 that was upregulated and CHS1 and CHS9 that were downregulated in the black (iRT) seed coat (Table 3 and Table 4 respectively). As CHS4 is upregulated with anthocyanin genes, it likely has a role in anthocyanin biosynthesis in this tissue. Consistent with this, spontaneous mutations of the dominant i allele to the recessive i allele have been shown to affect the promoter region of the CHS4 gene [7]. By contrast, the downregulation of CHS1 and CHS9 may suggest that they have a role in the parallel downregulated biochemical pathways, such as flavonol and isoflavonoid biosynthesis, as these metabolites, and transcript levels of genes for their biosynthesis, were reduced or not present in the black (iRT) seed coat (Figure 6). These results emphasize the complexity of isogene expressions that exist to achieve a single enzyme function in soybean.

Microarray analysis identified differentially regulated probe sets for only 3 gene functions (LAR, GST, and MATE) that have been previously shown to be involved in PA biosynthesis and transport. Recombinant LAR enzymes from several legume species have been shown to convert leucoanthocyanidin to (+)-catechin in vitro [25,26,41,42]. However, the in vivo role of LAR in PA biosynthesis remains questionable as endogenous expression has been shown not to correlate with PA levels in M. truncatula and heterologous expression has failed to increase PA accumulations [41]. Similarly, our metabolite data showed that soybean PAs lack catechin subunits (Additional file 2: Figure S1) and that LAR1 expressions (Figure 1) do not parallel PA accumulations during seed development (not shown). Interestingly, 4 PAs were found to be exclusive to the black (iRT) seed coat (Table 2) however the total subunit compositions, the mean degree of polymerization (mDP), and the total amounts of soluble and solvent-insoluble PAs were not different compared to the brown (iRT) seed coat (Additional file 2: Figure S1). These results suggest the PA oligomers of black (iRT) and brown (iRT) seed coats may differ only in their subunit linkages. However, the potential influence of LAR or other PA genes on PA subunit linkages (if any) remains to be determined.

From the set of differentially regulated genes identified by microarray, two putative late-stage anthocyanin genes were selected and the functions of their coded enzymes were determined in vitro. UGT78K2 was found to code a UF3GT enzyme for anthocyanin biosynthesis (Figure 7). A UF3GT cDNA (UGT78K1) has recently been isolated from the black seed coat and functionally characterized [18]. UGT78K2 was 93% similar to UGT78K1 but possessed increased catalytic activity towards cyanidin (Figure 7D). The identification gene redundancy has important implications for seed coat color engineering by RNA interference, as both copies of the gene may have to be silenced to achieve a significant reduction in
UF3GT activity. Similarly, two OMT genes (OMT5 and OMT-like) were found to be overexpressed in the black seed coat (Table 3). OMT5 was cloned and found to code an AOMT enzyme in vitro (Figure 8). UF3GT and OMT genes may be ideal candidates for seed coat color engineering as suppression of UF3GT genes (UGT78K1 and UGT78K2) may result in a reduction in anthocyanin levels similar to that caused by a T-DNA insertion in the Arabidopsis UF3GT [21] and upregulation of an AOMT may result in a more red seed coat, as methylation of the B-ring of anthocyanins has a reddening effect on flower color (reviewed by [35]). However, it remains to be determined whether altering the expressions of these genes could be use to modify seed coat color without causing unintended effects on alternative biochemical pathways.

Prior to the present study, currently known soybean genes did not map between markers A668_1 and K387_1 on chromosome 9 of the soybean physical genomic map, where the R locus is located, leaving no obvious candidates for the R locus gene [15]. In our study, 14 genes identified to be differentially regulated in the black (iRT) soybean seed coat transcriptome were found to be located on chromosome 9 of the soybean genome sequence Glyma1 (Figure 5). Among the gene families represented, only WD40 and MYB have known roles in flavonoid regulation. However the genes demonstrated did not cluster with known flavonoid regulators by phylogenetic analysis (not shown). A Mob1/phocein gene was commonly upregulated in the black (iRT) soybean seed coat and Arabidopsis seedlings overexpressing PAP1 [21]. Other regulatory gene families represented on chromosome 9 were AP2/ERF, serine/threonine protein kinase, calcium/calmodulin-dependent protein kinase, and ethylene-responsive element-binding protein. Interestingly, all upregulated and downregulated genes on chromosome 9 were located in 5.16 Mb region that includes the nearest sequence markers to the R locus. Only the downregulated AP2/ERF transcription factor, the serine carboxypeptidase gene, and the gene moderately similar to the A. thaliana polyamine oxidase ATPAO1 were located between these sequence markers (Figure 5). As a result our study provides a short list of gene candidates that may serve as the focus of future efforts to identify the R locus gene.

Conclusion
Metabolite composition and gene expression differences between black (iRT) and brown (iR) seed coats are far more extensive than previously thought. Putative anthocyanin, proanthocyanidin, (iso)flavonoid, and phenylpropanoid isogenes were differentially-expressed between black (iRT) and brown (iR) seed coats, and UGT78K2 and OMT5 were validated to code UDP-glycosylase-flavonoid-3-O-glycosyltransferase and anthocyanin 3’-O-methytransferase proteins in vitro, respectively. Duplicate gene copies for several enzymes were overexpressed in the black (iRT) seed coat suggesting more than one isogene may have to be silenced to engineer seed coat color using RNA interference.

Methods

Chemicals
Cyanidin was purchased from Indofine (Somerville, NJ, USA) UDP-glucose and SAM from Sigma-Aldrich (Oakville, ON, CA), the 3-O-glucosides of delphinidin and petunidin from Polyphenols (Hanaveien, NO), and all other phytochemicals were purchased from Extrasynthese (Lyon, FR). All solvents for LC-MS/MS and HPLC-DAD analyses were of HPLC grade purchased from Fisher Scientific (Ottawa, ON, Canada).

Plant Materials and Growth Conditions
Black (iRT) soybean (G. max (L.) Merr.) variety Clark (PI547438) and the nearly isogenic brown (iR) soybean (PI 547475) were obtained from the U.S. Department of Agriculture Soybean Germplasm Collection (Agricultural Research Service, University of Illinois at Champaign-Urbana). Seeds were surface-sterilized in 2% sodium hypochlorite, rinsed three times with EtOH, dried, and germinated in autoclaved vermiculite in a Conviron E15 cabinet with a photoperiod of 16 h light (590 μE m⁻² s⁻¹) at 25°C, and 8 h dark at 20°C. Plants were fertilized twice weekly with N:P:K 35-5-10 (1% w/v). After 12 days, seedlings were transplanted to autoclaved soil and 34 days later the photoperiod was changed to 12 h light/12 h dark to encourage reproductive development. For RNA isolation, seed coats were dissected from plants, immediately frozen in liquid N2, lyophilized, and stored at -80°C. Seed coats for comparative analyses were harvested on the same days, 4 h after the initiation of the light cycle to minimize for potential differences in transcript and metabolite accumulations that may be influenced by circadian rhythm.

Seed Coat Metabolite Analyses
Lyophilized seed coats (~40 mg) from each developmental stage were pulverized in HCO2H-MeOH-H2O (400 μL, 15:80:5, v/v) using a FastPrep FP120 Homogenizer (Savant) and incubated at 4°C for 2 h. The slurry was centrifuged at 20,000 g for 10 min, and 5 μl of supernatant from each sample was measured by photospectroscopy using a NANO DROP 2000 (Thermo Scientific) according to the formula A530-0.25A657 to compensate for chlorophyll absorption at 530 nm (Mancinelli, 1990). The quantity of anthocyanins was determined by comparison to a standard curve and expressed as cyanidin.
3-O-glucoside equivalents. For HPLC-DAD and LC-QTRAP analyses samples were extracted for an additional 24 h and 200 μl of supernatant was vortexed with HCO$_2$H-MeOH (1 mL, 15:85, v/v) for 20 sec, centrifuged at 20,000 g for 10 min, and the supernatant was evaporated under a stream of nitrogen gas (to 50 μl), filtered through PVDF (0.45 μm; Millipore), and 20 μl and 5 μl aliquots were analyzed by HPLC-DAD and HPLC-MS/MS respectively, as described below.

For analysis of proanthocyanidins (PAs), lyophilized seed coats were ground to a powder, extracted with 70% acetone (1 mg mL$^{-1}$) and analyzed as described previously [30,31] with (-)-epicatechin, (+)-catechin, and (-)-epicatechin gallate used as standards for the identification of free monomers, and procyanidin B2 used as a standard for the analysis of soluble and insoluble PA oligomer compositions. Quantitation of soluble PAs by reaction with DMACA reagent and insoluble PAs by acid-catalyzed cleavage was performed as described [43] using procyanidin B2 and cyanidin as standards for the respective assays.

**Microarray Analysis, Semi-qRT-PCR, and qRT-PCR**

Total RNA was isolated from pigmented soybean seed coats as described previously (Wang and Vodkin, 1994). The quantity and purity of RNA samples were determined by spectrophotometry using a NANODrop 2000 (Thermo Scientific). RNA integrity was confirmed by microfluidics using a Bioanlyser 2100 equipped with an RNA 6000 Nano Chip (Agilent Technologies Inc., Montreal, QC, Canada).

Microarray was performed on RNA isolated from three plants of each genotype. Briefly, 100 ng of total RNA was in vitro transcribed for 16 h using the GeneChip 3’IVT Express Kit (Affymetrix, http://www.affymetrix.com). Labelled RNA was hybridized to GeneChip Soybean Genome Arrays as described by the manufacturer (Affymetrix). Statistical analyses of data for the identification of differentially regulated genes were performed using FlexArray software (M. Blazejczyk and associates; Genome Quebec, Montreal) that uses R and Bio-Conductor [44]. The raw data was adjusted for background signal, and normalized across all GeneChips using the Robust Multi-array Average (RMA) method [45]. To identify differentially expressed genes, the SAM algorithm, which minimizes variation across arrays and incorporates an estimate of false discovery rate (FDR) [46], was used. Only genes with a fold change $> 2$ or $< 0.5$ and a $P$ value $< 0.01$ were considered to be differentially expressed. All materials and procedures comply with the MIAME standards for array data [47]. The full dataset has been deposited in the Gene Expression Omnibus [GEO: GSE26208].

For semi-quantitative RT-PCR (semi-qRT-PCR), RNA samples (3 μg) were treated with DNase I (Amplification grade, Invitrogen) at 37°C for 15 min to remove contaminating DNA. Reactions (20 μl) were terminated by heating (65°C for 10 min) in the presence of 1 ul of 25 mM EDTA. First-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Parallel reactions were performed in the absence of Superscript III to test for genomic DNA contamination. Gene expressions from each cDNA sample were normalized to the endogenous reference PEPC16. Semi-quantitative RT-PCR (semi-qRT-PCR) reactions (20 μl) consisted of 2 μl of first-strand cDNA (or untreated RNA), 400 nM primers, 200 μM dNTPs, 1.5 mM MgCl$_2$, 1X Taq Buffer, 2.5 units Taq polymerase (Fermentas). PCR cycling was 94°C for 2 min, followed by 23, 25, and 27 cycles for CHS7/CHS8 or 27, 30, 33 cycles for all other genes, 94°C C for 15 sec, 58°C for 1 min, and 72°C 1 min.

For quantitative RT-PCR (qRT-PCR), cDNA template was prepared as described above. Reactions (25 μl) consisted of 2 μl of first-strand cDNA (or untreated RNA), 250 nM of forward and reverse primers, and 12.5 μl of the q SYBR Green Supermix (BioRad). qRT-PCR of each target gene for each seed coat sample was performed in triplicate on cDNA samples or untreated RNA samples using an PTC-200 Peltier thermal cycler equipped with a Chromo4 continuous fluorescence detector (MJ research). PCR cycling was 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 1 min, and 72°C 1 min. qRT-PCR data and PCR efficiencies were analyzed using the Opticon Monitor 3 software (BioRad). To verify the specificity of the RT-PCR reactions, melting curves were performed subsequent to each reaction in addition to fractionation of RT-PCR products on agarose gels. Semi-qRT-PCR and qRT-PCR experiments were performed in triplicate. Primers used in this study can be found in Additional file 12: Table S7.

**Cloning of UGT78K2 and OMT5 cDNAs from the Seed Coat of Black Soybean**

To clone the full-length coding sequences (CDSs) of UGT78K2 and OMT5, PCR primers were designed based on the transcript sequence predicted for Glyma08g07130 (http://www.phytozome.net/soybean) and based on the mRNA sequence of BT098523, respectively. Full-length CDSs for UGT78K2 and OMT5 were amplified from black (iRT) seed coat cDNA by end-to-end PCR using primers UHF/URAR and HOSF/HOSR, respectively (Additional file 12: Table S7) and a high-fidelity DNA polymerase (Invitrogen). The resulting UGT78K2 and OMT5 amplicons (1466 bp and 836 bp, respectively) were cloned into NdeI and BamHI sites,
and NdeI and XhoI sites of the pET-14b vector (Novagen), respectively, and sequenced to confirm their identities.

Expression of Recombinant Proteins in E. coli
The full-length CDSs of UGT78K2 and OMT5 were independently fused in-frame to the N-terminal hexahistididine tag encoded by the pET-14b vector (above). Plasmids were transformed into the expression host E. coli BL21(DE3) pLysS (Novagen) and a single colony for each plasmid was selected for production of recombinant proteins. Soluble recombinant proteins were isolated following growth and induction of E. coli BL21 (DE3) pLysS at 16°C. Recombinant proteins were purified by ion metal-affinity chromatography (IMAC) using a kit (Novagen). To confirm the purity of the recombinant enzyme, the eluted fractions were visualized on 12.5% acrylamide gel stained with 0.25% Coomassie Blue. The amount of purified recombinant enzyme was determined using the BioRad reagent.

Recombinant Enzyme Assays
The UGT78K2 assay (total volume 100 μl) was performed as described previously [18] and consisted of recombinant enzyme (2 μg), cyanidin (20 μM), and UDPG (5 mM) in assay solution. The OMT 5 assay (total volume 200 μl) consisted of recombinant enzyme (3 μg), SAM (100 μM), MgCl2 (150 μM), Tris pH 7.5 (50 mM), and 2-mercaptoethanol (14 mM). UGT78K2 and OMT 5 assays were incubated for 5 min at 30°C and 1 h at 35°C, respectively. Reactions were stopped by vortexing in MeOH (500 μl) for 20 s. Reaction products were identified by comparison to commercial standards dissolved in MeOH. Reactions were prepared for HPLC by centrifugation (21,000 g for 4 min at 4°C) followed by concentration of the supernatant to final volume of 50 μl under a stream of N2 gas. All reactions were filtered through PTFE (0.45 μm; Millipore, MA USA) and 20 μl aliquots were analyzed by HPLC-DAD. Assays were performed in triplicate and experiments repeated thrice.

HPLC-DAD and HPLC-MS/MS Analyses
HPLC-DAD analysis of anthocyanins was performed as described previously [18]. Briefly, separations were achieved at 45°C on a Luna C18(2), 4.6 × 150 mm, particle size 5 μm fitted with a corresponding guard-column (Phenomenex Inc, Santa Ana, CA) using an Agilent 1100 series (Agilent Technologies Inc., Montreal, QC, Canada) equipped with an autosampler with a 100 μL loop, a quaternary pump (maximum pressure, 400 bars), a column thermostat, and a diode array detector (DAD). The mobile phase consisted of 5% HCO3H in H2O (solvent A) and MeOH (solvent B). Optimized elution conditions were a linear gradient of 10-100% B in 25 min with a flow rate of 1 ml min⁻¹, the column was washed for 10 min with 100% B, brought back to starting mobile phase composition in 0.1 min and equilibrated for 5 min prior to the next injection. The HPLC separations were monitored at 520, 476, 350, 280, and 254 nm. The relative quantities of anthocyanins were calculated based on percent area of each peak eluting at specific retention times.

The HPLC-ESI-MS/MS system consisted of an Agilent 1200 series (Agilent Technologies Inc., Montreal, QC, Canada) connected directly to a 3200QTRAP (ABI Sciex, Toronto, Canada). The software used for data acquisition and analysis is Analyst 1.4.2. The chromatographic conditions were the same as described for the HPLC-DAD analysis anthocyanins. The mobile phase was split 50/50 post-column. For enhanced mass scan (EMS), the MS was operated in positive polarity at a scan rate of 4000 amu s⁻¹ within the mass range of 100 - 1000 amu. The optimal source conditions of the mass spectrometer were: collision gas high, curtain gas (N2) 25 L min⁻¹, ion spray voltage 4500 V, source temperature 500°C, source gas 1 (at 50 psig), source gas 2 (at 55 psig). The optimal compound parameters were declustering potential (DP) +20 V, entrance potential (EP) +10 V, collision energy (CE) 10 V, ionization energy 1.0 eV and detector 2800. For enhanced product ion scan the information dependant acquisition (IDA) criteria was as follows: select 1 - 2 most intense peaks which exceeds 10,000 cps, exclude targeted ions after 3 occurrences for 12 s. Product ions were scanned within the mass range of 100 - 1000 amu. All source parameters were the same as for EMS. The optimal compound parameters were DP +70 V, EP +10 V, CE 65 V, and collision energy spread 40 V.

Additional material

Additional file 1: Supplementary Table S1 Developmental properties of black (iRT) and brown (irT) soybean seed coats at the 400 mg fresh seed weight stage.

Additional file 2: Supplementary Figure S1 Proanthocyanidin (PA) subunit compositions, degree of polymerizations, and amounts from the seed coats of black (iRT) and brown (irT) soybean Clark isolines. (A, B, C) iRT top panels, irT bottom panels. (A) Phloroglucinol cleavage products of soluble PA polymers. HPLC retention times: ascorbic acid (1) (Rt: 2.0 min); phloroglucinol (2) (Rt: 3.1 min); epicatechin-phloroglucinol adduct (Rt: 5.5 min); epicatechin (4) (Rt: 9.4 min). (B) Phloroglucinol cleavage products of soluble insoluble PA polymers. HPLC retention times: ascorbic acid (1) (Rt: 2.0 min); phloroglucinol (2) (Rt: 3.1 min); epicatechin-phloroglucinol (Rt: 6.2 min); epicatechin (4) (Rt: 11.0 min). (C) Free monomers. HPLC retention times: epicatechin (1) (Rt: 12.5 min). (D) Mean degree of polymerization (mDP) of soluble PAs. (E) Total soluble PAs. (F) Total soluble PAs. (D, E, F) iRT black bars, irT white bars. (E, D) Amounts are represented as milligrams procyanidin B2 equivalents per gram lyophilized seed coat (LSC).

Additional file 3: Supplementary Table S2 Gene probe sets up-regulated more than 2-fold in the seed coat of black (iRT) soybean compared to the brown (irT) isolate.
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References

1. Winkel-Shirley B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 2001, 126(2):485-493.

2. Butel E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo L, et al.: Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 2008, 26(11):1301-1308.

3. Dixon RA: Engineering of plant natural product pathways. Curr Opin Plant Biol 2005, 8(3):329-336.

4. Kovinich N, Amason JT, DeLuca V, Mik B: Coloring Soybeans with Anthocyanins? In Recent Advances in Phytochemistry. Volume 41. Edited by: Gang DR. Springer; 2011:47-57.

5. Todd JJ, Vodkin LO: Pigmented Soybean (Glycine max) Seed Coats Accumulate Proanthocyanidins during Development. Plant Physiol 1993, 102(2):663-670.

6. Bernard R, Weiss M: Qualitative genetics. In Soybeans: Improvement, Production, and Uses. 1 edition. Edited by: Madison WI. American Society of Agronomy. 1973:117-154.

7. Todd J, Vodkin L: Duplications That Suppress and Deletions That Restore Expression from a Chalcone Synthase Multigene Family. Plant Cell 1996, 16(4):819-835.

8. Buzzell R, Butterv B, MacTavish D: Biochemical genetics of black pigmentation of soybean seed. The Journal of Heredity 1987, 78(1):53-54.

9. Woodworth CM: Inheritance of Cotyledon, Seed-Coat, Hilum and Pubescence Colors in Soy-Beans. Genetics 1921, 6:487-553.

10. Toda K, Yang D, Takahashi R: A single-base deletion in soybean flavonoid 3’-hydroxylase gene is associated with gray pubescence color. Plant Mol Biol 2002, 50(2):187-196.

11. Zabala G, Vodkin LO: Cloning of the pleiotropic T locus in soybean and two recessive alleles that differentially affect structure and expression of the encoded flavonoid 3’-hydroxylase gene. Genetics 2003, 163(1):295-309.

12. Zabala G, Vodkin LO: A rearrangement resulting in small tandem repeats in the F3’S gene of white flower genotypes is associated with the Soybean W1 locus. The Plant Genome 2007, 47(52):S113-S124.

13. Zabala G, Vodkin LO: The wp mutation of Glycine max carries a gene-fragment-rich transposon of the CACTA superfamily. Plant Cell 2008, 17(10):2619-2632.

14. Yang K, Jeong N, Moon JK, Lee YH, Lee SH, Kim HM, Hwang CH, Back K, Palmer RG, Jeong SC: Genetic analysis of genes controlling natural variation of seed coat and flower colors in soybean. The Journal of heredity 2010, 101(6):757-768.

15. Nagai I: A genetico-physiological study of the formation of anthocyanins and brown pigments in plants. Tokyo Univ Coll Agric J 1921, 81-92.

16. Ku HM: Molecular cloning and expression of ANS (anthocyanidin synthase) and GST (glutathione S-transferase) in seed coats of soybean Taihong. National Chung Hsing University; 2009.

17. Kovinich N, Saleem A, Amason JT, Mik B: Functional characterization of a UDP-glucoselflavonoid 3-O-glucosyltransferase from the seed coat of black soybean (Glycine max (L.) Merr). Phytochemistry 2010, 71(12):1253-1263.

18. Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, et al: Genome sequence of the barley genome of the cultivar 'compact'. Nature 2008, 453(7278):78-183.

19. Hiraizumi M, Kato M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, et al: Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. The Journal of biological chemistry 2005, 280(27):25590-25595.

20. Toyoh T, Nishiyama Y, Hiraizumi M, Yano M, Nakajima J, Aizawa M, Inoue E, Takahashi H, Goodenowv DB, Kitayama M, et al: Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. Plant J 2005, 42(2):218-235.

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Authors’ contributions

NK performed the molecular genetics experiments, the targeted analysis of anthocyanins and proanthocyanidins, assisted AM in performing the non-targeted metabolite analysis using LC-MS/MS, and wrote the manuscript. BM and JTA revised the manuscript and acquired the funding to support the research. All authors read and approved of the manuscript.
22. Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhana M, Arita M, Fujiwara T, Saito K: Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* 2004, 101(29):10205-10210.

23. Pienkyn S, Brandt W, Schmidt J, Kranell R, Ziegler J: Functional characterization of a novel benzylosofractinoline 0-methyltransferase suggests its involvement in papaveraine biosynthesis in opium poppy (*Papaver somniferum* L.). *The Plant Journal: for cell and molecular biology* 2009, 60(1):56-67.

24. Sugimoto T, Kawasaki T, Kato T, Whitther RF, Shibata D, Kawayama Y: cDNA sequence and expression of a phosphoprenylpyruvate carboxylase gene from soybean. *Plant Mol Biol* 1992, 20(4):743-747.

25. Tanner GJ, Franchik CT, Abrahams S, Watson JM, Larkin PJ, Ashton AR: Proanthocyanidin biosynthesis in plants. Purification of legume leucanthocyanidin reductase and molecular cloning of its cDNA. *J Biol Chem* 2003, 278(34):31647-31656.

26. Xie DY, Sharma SB, Pavia NL, Ferreira D, Dixon RA: Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis. *Science* 2003, 299(5605):396-399.

27. Choung M, Baek J-Y, Kang S-T, Han W-Y, Shin D-C, Moon H-P, Kang K-H: Isolation and Determination of Anthocyanins in Seed Coats of Black Soybean (*Glycine max* (L.) Merr.). *J Agric Food Chem* 2001, 49:5846-5851.

28. Lee JH, Kang NS, Shin S-O, Shin S-H, Lim S-G, Suh D-Y, Baek I-Y, Park K-Y, Ha TJ: Characterization of anthocyanins in the black soybean (*Glycine max* L.) by HPLC-DAD-ESI/MS analysis. *Food Chemistry* 2009, 112:226-231.

29. Ranathunge K, Shao S, Qutob D, Gijzen M, Peterson CA, Barnard MA: Properties of the soybean seed coat cuticle change during development. *Planta* 2010, 231(1):1171-1188.

30. Kennedy JA, Jones GP: Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J Agric Food Chem* 2003, 49:1740-1746.

31. Downey MO, Harvey JS, Robinson SP: Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Aust J Grape Wine Res* 2003, 9:15-27.

32. Hughes J, Hughes MA: Multiple secondary plant product UDP-glucose glucosyltransferase genes expressed in cassava (*Manihot esculenta Crantz*) cotyledons. *DNA Seq* 1994, 5(1):41-49.

33. Mackenzie P, Owens IS, Burchell B, Bock KW, Bairoch A, Belanger A, Fourmel-Gigleux S, Green M, Hum DW, Ivanjic T, et al: The UDP-glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 1997, 7(4):255-269.

34. Song QJ, Marek LF, Shoemaker RC, Lark KG, Combidco VC, Delanaye X, Spectre JE, Cregan PB: A new integrated genetic linkage map of the soybean. *TAG: Theoretical and applied genetics* 2004, 109(1):122-128.

35. Tanaka Y, Brugliera F, Chandler S: Recent progress of flower colour modification by biotechnology. *Int J Mol Sci* 2009, 10(12):3530-3569.

36. Snyder BA, Nicholson RL: Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. *Science (New York, NY)* 1990, 248(4965):1637-1639.

37. Boiven AD, Galato L, Sambrotta L, Ricco R, Gurni AA, Puntarulo S: Antioxidant capacity of a 3-deoxoyanthocyanidin from soybean. *Phytochemistry* 2001, 58(10):1097-1105.

38. Liu H, Du Y, Chu H, Shi H, Hong Y, Wang W, Wang M, Chu IK, Tao Y, Lo C: Molecular dissection of the pathogen-inducible 3-deoxoyanthocyanidin biosynthesis pathway in sorghum. *Plant & cell physiology* 2010, 51(7):1173-1183.

39. Peel GJ, Pang Y, Modolo LV, Dixon RA: The *LAP1* MYB transcription factor orchestrates anthocyanidin biosynthesis and glycosylation in Medicago. *Plant J* 2009, 59(1):136-149.

40. Dhaubhadel S, Gippen M, Moy P, Farhanghkhoe M: Transcriptome analysis reveals a critical role of CHS7 and CHS8 genes for isoflavonoid synthesis in soybean seeds. *Plant Physiol* 2007, 143(1):326-338.

41. Pang Y, Peel GJ, Wright E, Wang Z, Dixon RA: Early steps in proanthocyanidin biosynthesis in the model legume Medicago truncatula. *Plant Physiol* 2007, 143(3):601-615.

42. Paolocci F, Robbins MP, Madeo L, Arcioni S, Martens S, Damiani F: ectopic expression of a basic helix-loop-helix gene transactivates parallel pathways of proanthocyanidin biosynthesis: structure, expression, and genetic control of leucaanthocyanidin 4-reductase and anthocyanidin reductase genes in Lotus corniculatus. *Plant Physiol* 2007, 143(1):504-516.

43. Li Y-G, Tanner G, Larkin P: The DMACA-HC1 Protocol and the Threshold Proanthocyanidin Content for Bloat Safety in Forage Legumes. *J Agric Food Chem* 1996, 44:893-901.

44. Gentileman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, et al: Bioconductor: open software development for computational biology and bioinformatics. *Genome biology* 2004, 5(10):R80.

45. T. Tanaka Y, Kobayashi H, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Bioscience* 2003, 4(2):249-264.

46. Benjamin Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B* 1995, 57:289-300.

47. Brzazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, et al: Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat Genet* 2001, 29(4):365-371.