**Identification and Characterization of Soybean Pigmentation
Genes and Metabolic Engineering Seed Coat Color to Enable
Visual Identification of Transgenic Grains**

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for Ph.D. degree in
Biology with specialization in Biotechnology.
Ottawa-Carleton Institute of Biology, Carleton University

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ISBN: 978-0-494-83230-1
Our file Notre référence
ISBN: 978-0-494-83230-1

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ABSTRACT

The inability to effectively detect and quantify the levels of adventitious presence of genetically modified (GM) plant grains in non-GM shipments represents a key issue that threatens the adoption of GM commodities in the worldwide market. A potentially safe and economical solution for effective detection is to produce distinct colors in GM grains of natural products by metabolic engineering. Successful attempts to engineer colored GM grains have been limited to the overexpression of plant pigment (anthocyanin) transcription factors in *Arabidopsis*, tobacco, and maize. The question of our research was whether soybean grain color could be manipulated by the suppression of late-stage pigment genes. The potential benefit of this approach would be to impart less unintended effects on the plant system than the induction of numerous genes by overexpression of a transcription factor. Towards testing our hypothesis, I first identified the anthocyanin branchpoint gene *UGT78K1* encoding UDP-glyucose:flavonoid-3-O-glycosyltransferase (UF3GT) from the black soybean seed coat and demonstrated its encoded activity *in vitro*, and by complementation of the *Arabidopsis ugt78d2* knock-out mutation. Subsequently, I identified a second UF3GT gene (*UGT78K2*) and the gene *OMT5* coding anthocyanin-3'-O-methyltransferase (AOMT) from the black seed coat by comparing metabolite and transcriptome data between isogenic black and brown soybean seed coats differing in alleles of the *R* locus. The proanthocyanidin (PA) branchpoint genes *ANR1* and *ANR2* coding anthocyanidin reductase (ANR) enzymes were then isolated from the brown soybean seed coat using RACE and the activities of their

encoded enzymes were demonstrated *in vitro*. In the latter two studies we used liquid chromatography- tandem mass spectrometry (LC-MS/MS) and microarray and/or quantitative (q)RT-PCR for combined analyses of seed coat metabolite and gene expression data from brown, black, and red-brown soybean genotypes. From this data we hypothesized and confirmed that a red-brown soybean grain color could be engineered by the simultaneous suppression of *ANR1* and *ANR2* using RNA interference (RNAi) in brown soybean. The underlying mechanism was identified to involve positive feedback and feedforward mechanisms in the flavonoid pathway to redirect metabolic flux into anthocyanin biosynthesis. These results represent a novel approach to engineering pigmentation in plant tissues.

Dedicated to

My grandmother, Ildiko Kovinich

ACKNOWLEDGEMENTS

I deeply thank my supervisor Brian Miki for providing me a graduate research position in his lab. Brian has been encouraging, supportive, educating, and has provided me ample opportunities to meet and interact with people from the research community and from industry. It is Brian's conviction in me that has led me to succeed where others thought I would fail.

I am also very grateful to John Thor Arnason for being an exceptional mentor and for treating me as if I were one of his own students. The research conducted in this thesis was multi-disciplinary requiring extensive analysis in biochemistry and molecular biology. Thor provided me education, guidance, and full access to his specialized instruments that were essential to conduct the biochemical analyses.

My friend Ammar Saleem provided extensive training on specialized instruments and made himself available at all hours of the day, night, and weekends to help conduct analyses. For this, I am extremely grateful.

I thank Vincenzo De Luca, Ashraff Abdeen, and my PhD advisory committee, Owen Rowland and Tim Xing, for the very interesting meetings and discussions that helped shape my thesis projects.

For technical assistance at the AAFC I thank Tara Rintoul and Dan Brown for conducting soybean transformations, Hlne Labb for constantly keeping me supplied and equipped in the lab, Robert Reid for cultivating soybeans, Ming Hu for assembling some binary vectors for transfer into *Arabidopsis thaliana*, Frances Tran for teaching me SDS-PAGE, and Bill Collins for his helpful advice and criticisms.

I acknowledge the financial support of Ontario Graduate Scholarship, the International Association for Plant Biotechnology – Canadian Scholarship, the Phytochemical Society of North America – Best Presentation Award.

Last but not least, I thank my family. My parents, Vuk and Germaine have provided unparalleled emotional, intellectual, and financial support. My grandmother, Ildiko has encouraged me from the time I was a child to be inquisitive and to value education. I thank my fiancée, Jia Hui (Sally) Lin, for being encouraging, emotionally supportive, and for tolerating me working countless hours in the lab. And I thank the rest of my family for their much needed friendships. I am very fortunate to share this accomplishment with such a wonderful family!

This graduate research experience has been a life-changing experience, and I am tremendously grateful to all of you for making it happen!

PREFACE

Brian Miki, Vince De Luca and John T. Arnason invented the concept of 'coloring GM soybeans with anthocyanins'. Brian Miki and John T. Arnason obtained the funding for the research. Ammar Saleem had a primary role in the development of HPLC-DAD and LC-MS/MS methods for flavonoid analysis. Tara Rintoul and Dan Brown transferred DNA into soybeans. Nikola Kovich invented the approach of suppressing ANR genes to redirect metabolic flux into anthocyanin biosynthesis for coloring soybeans, designed and conducted all experiments in genetics, molecular biology, and biochemistry, analysed data, and wrote the manuscripts for publication.

Each data chapter of this PhD thesis represents a manuscript that has been published, in press, or submitted for publication. The materials have been included in the thesis with kind permissions from Elsevier, Springer Science and Business Media, and BioMed Central publishing companies. We encourage readers to reference these published works directly as indicated in the corresponding chapter.

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List of Abbreviations

Commonly accepted gene and protein nomenclature are used in this thesis.

Genes and their corresponding mRNAs are listed in italics. Proteins are represented by non-italicized uppercase letters. Family 1 glycosyltransferase (UGT) genes have approved names (Mackenzie et al., 1997).

4CL	- 4-coumarate-CoA ligase
Ag	- Red-brown saddle soybean variety Agate
amu	- Atomic mass unit
ANR	- anthocyanidin reductase
ANR1	- ANR isoform 1 from <i>Glycine max</i>
ANR2	- ANR isoform 2 from <i>Glycine max</i>
ANS	- anthocyanidin synthase
ANS2	- anthocyanidin synthase isoform 2 from <i>Glycine max</i>
ANS3	- anthocyanidin synthase isoform 3 from <i>Glycine max</i>
AOMT	- anthocyanin O- methyltransferase
C3G	- Cyanidin-3-O-glucoside
C3Ga	- Cyanidin-3-O-galactoside
CAPS	- N-cyclohexyl-3- aminopropanesulfonic acid
CHI	- chalcone isomerase
CHS	- chalcone synthase
COC	- Coefficient of coincidence
Cy	- cyanidin

DFR	- dihydroflavonol 4-reductase
F3'5'H	- flavonoid 3',5'-hydroxylase
F3'H	- flavonoid 3'-hydroxylase
F3H	- flavanone 3-hydroxylase
FLS	- flavonol synthase
FSW	- Fresh seed weight
G4DT	- pterocarpan 4-dimethylallyltransferase
GFP	- Green fluorescent protein
GST	- glutathione S-transferase
GST26	- GST isoform 1 from <i>Glycine max</i>
GUS	- beta-glucuronidase
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC-DAD	- High performance liquid chromatography - diode array detection
<i>i</i>	- Homozygous recessive <i>i</i> alleles for <i>Glycine max</i> seed coat color
<i>I</i>	- Homozygous dominant <i>I</i> locus alleles for <i>Glycine max</i> seed coat color
I3G	- Isorhamnetin-3-O-glucoside
IFR	- isoflavone reductase
IMAC	- Ion-metal affinity chromatography
ir	- Brown soybean variety Clark
KD	- Red-brown soybean variety Kuro Daizu
LAR	- leucoanthocyanidin reductase
LAR1	LAR isoform 1 from <i>Glycine max</i>

LC-MS/MS	- Liquid chromatography tandem mass spectrometry
LSC	- Lyophilized seed coat
M100	- EMS-mutagenized brown soybean variety Linia N145
M11	- EMS-mutagenized red-brown soybean variety Linia N145
MATE	- multidrug and toxic extrusion protein
MES	- 2-(N-morpholino) ethanesulfonic acid
MRM	- Multiple reaction monitoring
<i>o</i>	- Homozygous recessive <i>o</i> locus alleles for Glycine max seed coat color
<i>O</i>	- Homozygous dominant <i>O</i> locus alleles for Glycine max seed coat color
OMT5	- AOMT from <i>Glycine max</i>
PA	- Proanthocyanidin (a.k.a. condensed tannin)
PEPC16	- Phosphoenol pyruvate carboxylase isoform 16 from <i>Glycine max</i>
PSPG	- plant secondary product glucosyltransferase
Q3G	- Quercetin-3-O-glucoside
<i>r</i>	- Homozygous recessive <i>r</i> locus alleles for Glycine max seed coat color
<i>R</i>	- Homozygous dominant <i>R</i> locus alleles for Glycine max seed coat color
r17	- Brown soybean variety Soysota
RED	- Reductase-epimerase-dehydrogenase

RNAi	- RNA interference
<i>t</i>	- Homozygous recessive <i>t</i> locus alleles for Glycine max seed coat color
<i>T</i>	- Homozygous dominant <i>T</i> locus alleles for Glycine max seed coat color
UF3GT	- UDP-glycose:flavonoid 3-O-glycosyltransferase
UGT78D2	- UF3GT from <i>Arabidopsis thaliana</i>
UGT78K1	- UF3GT isoform 1 from <i>Glycine max</i>
UGT78K2	- UF3GT isoform 2 from <i>Glycine max</i>
wko	- Week-old

CHAPTER 1

General Introduction

Adapted from one publication and one submitted manuscript:

Kovinich N¹, Arnason JT², De Luca V² and Miki B². 2011. Coloring Soybeans with Anthocyanins? *Recent Advances in Phytochemistry*, 41: 47-57.

Kovinich N¹, Saleem A², Rintoul TL³, Brown DCW⁵, Arnason JT^{4,5}, and Miki B^{4,5}. Submitted. Aposomatic GM soybeans: Coloring genetically modified soybean grains with anthocyanins by suppression of the proanthocyanidin genes *ANR1* and *ANR2*. *Transgenic Research*.

¹Designed and conducted research, analyzed data, and wrote the manuscripts for publication

²Invented the concept of 'Coloring soybeans with anthocyanins' and revised the manuscript(s)

³Developed the LC-MS/MS methods

1.1. Thesis rationale

The commercial production of genetically engineered (GE) crops has risen to 250 million acres worldwide (Lemaux, 2008). As the prevalence and diversity of engineered crops increase, contamination of non-modified grain with transgenics is becoming problematic (Doyle, 2008). Distinct colors for the visible identification and quantitation of transgenic materials may provide increased safety to the consumer and freedom to the producer by enabling more effective detection.

1.2. Background

Anthocyanins are flavonoid pigments found in plants that provide red-to-blue color to flowers, leaves, and grains. Proanthocyanidins (PAs) represent another class of flavonoid pigments that if present in plant grains provide a brown color

when oxidized during desiccation. The biosynthesis of anthocyanins and PAs (Figure 1.1) has been well-characterized over the past several decades, with numerous structural, regulatory, and transport genes identified (reviewed by Mol et al., 1998; Winkel-Shirley, 2001; Lepiniec et al., 2006; Zhao and Dixon, 2010). However, the genes for only 4 flavonoid enzymes (CHS, F3H, F3'H, and F3'5'H) have been identified as being involved in pigment biosynthesis in the soybean seed coat (Todd and Vodkin, 1996; Toda et al., 2002; Zabala and Vodkin, 2003; Zabala and Vodkin, 2005; Zabala and Vodkin, 2007; Kavinich et al., 2010). Although often studied separately, the biosynthesis of anthocyanin and PA pigments may be intimately linked in tissues that synthesize both pigments. The anthocyanin branchpoint enzyme UDP-glucose:flavonoid-3-O-glycosyltransferase (UF3GT; EC 2.4.1.115) and the PA branchpoint enzyme anthocyanidin reductase (ANR; EC 1.3.1.77) both utilize the unstable flavonoid precursor, cyanidin, as a substrate (Figure 1.1).

Black soybean, long considered a medicinal food and consumed only occasionally in Japan and Korea, is similar in composition to cultivated yellow soybean, except that it accumulates high levels of anthocyanin and PA pigments in the seed coat (Todd and Vodkin 1993). Interestingly, the black seed coat has a red color during mid-stages of seed development due to the presence of only moderate amounts of anthocyanins. By contrast, the seed coat of brown soybean accumulates only PA pigments and browns during desiccation (Todd and Vodkin 1993).

leucoanthocyanidin reductase; ANR, anthocyanidin reductase; MATE, multidrug and toxic extrusion protein.

Since the first reported engineering of a “novel” flower color by the expression of a maize enzyme in petunia (Meyer et al., 1987), numerous experiments have successfully modified flower color in various plant species by manipulating flavonoid gene expressions (Tanaka et al., 2005). For example, a blue-hued rose was produced by the expression of a viola gene for flavonoid-3'-5'-hydroxylase (*F3'5'H*), as increased hydroxylation of the anthocyanin B-ring imparts bluer color (Katsumoto et al., 2007). Another interesting example was the modification of flower color by suppression of the flavonol synthase (*FLS*) gene in petunia resulting in the redirection of metabolic flux into the anthocyanin pathway (Holton et al., 1993). However, successful reports of engineering novel grain color may be limited to two studies that have overexpressed anthocyanin transcription factors in Maize (Shen and Petolino, 2006), Arabidopsis, and Tobacco (Geekiyanage, 2009). The suppression of late-stage pigment biosynthesis genes for the purposes of engineering grain color may result in more predictable changes in the seed coat metabolome, and thus have lower potential for unintended effects on the biosafety of food and feed.

1.3. Hypotheses

- i. The suppression of the UF3GT gene(s) in the seed coat of black soybean would result in reduced anthocyanin accumulations (Figure 1.1) and produce a red-brown grain color;
- ii. The suppression of the ANR gene(s) in the seed coat of brown soybean would redirect metabolic flux into the anthocyanin pathway (Figure 1.1) and produce a red grain color.

1.4. Objectives

- i. Identify the UF3GT gene(s) from black seed coat cDNA;
- ii. Determine whether the UF3GT gene(s) (or other pigment genes) have a key role in anthocyanin biosynthesis (and thus color) in the black soybean seed coat;
- iii. Identify the ANR gene(s) from brown seed coat cDNA;
- iv. Determine whether the ANR gene(s) (or other pigment genes such as UF3GT) control metabolic flux between anthocyanin and PA pathways (and thus color) in pigmented soybean seed coats.
- v. Engineer colored soybeans by suppression anthocyanin and/or PA genes

CHAPTER 2

Methods Overview

2.1. Molecular biology methods

Standard molecular biology methods were used in the research presented in this thesis with one exception. The extraction of RNA from soybean seed coats required a specific protocol (Wang et al., 1994) to remove proanthocyanidins (PAs), as these compounds bind and precipitate RNA.

2.2. Biochemistry methods

Standard biochemistry practices were used for metabolite extractions with one detail that could use clarification. The isolation of flavonoids from enzyme reactions (described in the data chapters) involved evaporating methanol or ethyl acetate solvents under a stream of nitrogen gas to concentrate the extract. This practice was achieved using an N-EVAP apparatus (Organomation Associates Inc. MA, USA).

The analytical methods used for the analysis of flavonoid compounds (i.e. high performance-liquid chromatography- diode array detection (HPLC-DAD) and liquid chromatography- tandem mass spectrometry (LC-MS/MS), in scanning and multiple reaction monitoring (MRM) modes) were developed specifically for the compounds analyzed. Readers should consult the data chapters for details.

CHAPTER 3

Identification of a UDP-glucose:flavonoid 3-O-glucosyltransferase from the seed coat of black soybean (*Glycine max* (L.) Merr.)

Adapted from:

Kovinich N¹, Saleem A³, Arnason JT², and Miki B². 2010. Functional characterization of a UDP-glucose:flavonoid 3-O-glucosyltransferase from the seed coat of black soybean (*Glycine max* (L.) Merr.). *Phytochemistry* 71:1253-1263.

¹Designed and conducted research, analyzed data, and wrote the manuscript for publication

²Obtained funding for the research and revised the manuscript

³Developed the HPLC-DAD method

3.1. Introduction

Black soybean (*Glycine max* (L.) Merr.) accumulates relatively high amounts of cyanidin-, delphinidin-, petunidin-, and pelargonidin-based anthocyanins exclusively as 3-O-glucosides in the seed coat (Choung et al., 2001; Lee et al., 2009). Minor amounts of 3-O-galactosides of cyanidin and delphinidin, and 3-O-glucosides of peonidin and a cyanidin-catechin complex have also been reported from this tissue (Lee et al., 2009). By contrast, anthocyanins from *Arabidopsis* contain glucosyl moieties linked to the 3- and 5-positions of the cyanidin backbone that are decorated with *p*-coumaroyl, malonyl, and sinapoyl groups (Tohge et al., 2005). Several genes involved in the early steps of anthocyanin and general flavonoid biosynthesis in the seed coat of black soybean have been identified, but the gene catalyzing the final step in anthocyanin biosynthesis, namely UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT), has remained unreported (reviewed by Kovinich et al., 2011a).

UF3GT catalyzes the transfer of glucose, from uridine diphosphate (UDP)-glucose, to the 3-position of anthocyanidins to form the corresponding anthocyanins (Heller and Forkmann, 1988, 1993; Forkmann and Heller, 1999). UF3GTs belong to a large multigene family (Family 1) of inverting glycosyltransferases (UGTs) (CAZy, http://www.cazy.org/fam/acc_GT.html) defined by the presence of a conserved carboxy-terminal consensus sequence, the plant secondary product glycosyltransferase signature sequence (PSPG box) (Hughes and Hughes, 1994), which is involved in binding the UDP moiety of the sugar nucleotide (Offen et al., 2006) to the enzyme. UGT Family 1 consists of over 100 members in *Arabidopsis* (Ross et al., 2001) and approximately 150 members in the legume *Medicago truncatula* (Modolo et al., 2007). Presently, UGT substrate specificity or function cannot be predicted using amino acid sequence information alone (Modolo et al., 2007) and identity among UF3GTs is as low as 25% (Sarkar et al., 2007). Some UGTs that catalyze the transfer of glucose to the 3-position of anthocyanidins can also glucosylate flavonol (Ralston et al., 1988; Tanaka et al., 1996; Ford et al., 1998; Ogata et al., 2004; Almeida et al., 2007), dihydroflavonol, flavone, isoflavone (Owens and McIntosh, 2009), flavanone and coumestan substrates *in vitro* (Modolo et al., 2007). Identification and characterization of UGTs with high amino acid identities but different biochemical activities may provide insights into structure-function relationships and is the focus of ongoing studies (Osmani et al., 2009; Owens and McIntosh, 2009; Wang, 2009).

Anthocyanins are believed to be synthesized on the cytoplasmic face of the endoplasmic reticulum by metabolons (Saslowsky and Winkel-Shirley, 2001) and to be transported into the vacuole by systems that remain to be characterized (Grotewold and Davis, 2008). Glucosylation of the 3-position of anthocyanidins increases their chemical stability and glucosylation of secondary metabolites generally increases their solubility and enables access to active membrane transport systems that recognize glucosylated compounds but not their aglycones (Hostel, 1981). Glucosylation of the 3-position of anthocyanidins and the structurally-similar flavonols may be required for their accumulation in some plant species, as a null mutation in the Arabidopsis *UF3GT* gene *UGT78D2* (At5g17050) resulted in a drastic reduction of these compounds (79%) relative to the wildtype (Tohge et al., 2005). The molecular basis of the black phenotype in soybean seed coats is not well understood, but the accumulation of high amounts of anthocyanins may be involved (Kovinich et al., 2011a). Engineering reduced anthocyanin amounts in the seed coat of black soybean by suppression of the *UF3GT* gene could potentially be used to produce distinct seed colors to enable the visual identification and monitoring of transgenic grains (Kovinich et al., 2011a). However, before engineering reduced anthocyanin amounts by suppression of the *UF3GT* gene can be attempted, the gene(s) catalyzing the final step in anthocyanin biosynthesis must be identified from the black soybean seed coat.

The purpose of this study was two-fold: 1) to identify the gene that catalyzes the final step in anthocyanin biosynthesis from the seed coat of black soybean to

provide a possibility of engineering reduced pigment in transgenic grains, and 2) to characterize the catalytic properties of the recombinant enzyme to provide a basis for future glucosyltransferase structure-function analyses. We report here the isolation of a glucosyltransferase cDNA (*UGT78K1*) from the seed coat of black soybean. To determine the function of the corresponding recombinant enzyme, 28 flavonoid substrates previously identified in soybean were tested as substrates. To determine the *in vivo* function, the cDNA was transferred into the *Arabidopsis* mutant *ugt78d2* in an attempt to restore anthocyanin biosynthesis. To provide a basis for future structure-function analyses of UGT78K1, kinetics and specificity analyses were conducted with anthocyanidins and other flavonoid substrates.

3.2. Materials and Methods

3.2.1. Chemicals

Cyanidin was purchased from Indofine (Somerville, NJ, USA), delphinidin, myricetin, kaempferol, isorhamnetin, dihydroquercetin, apigeninidin, genistin, genistein and (-)-epicatechin were purchased from Sigma-Aldrich (Oakville, ON, CA), pelargonidin, malvidin, peonidin, kaempferol, quercetin, isorhamnetin and their 3-O-glucosides, petunidin, cyanidin 3-O-galactoside, (+)-catechin, apigenin from Extrasynthese (Lyon, FR) and 3-O-glucosides of delphinidin and petunidin from Polyphenols (Hanaveien, Norway).

3.2.2. Plant materials, growth conditions, and nucleic acid isolation

Black soybean (*Glycine max* (L.) Merr.) cv. Clark line (PI547438) was obtained from the U.S. Department of Agriculture Soybean Germplasm Collection (Agricultural Research Service, University of Illinois at Champaign-Urbana). Seeds were germinated in vermiculite and transferred to soil after one week. Plants were grown in a Conviron E15 cabinet under 16/8 h light/dark at 25 °C and 20 °C, respectively. Vegetative organs (obtained from 3 wko seedlings), seed coats, embryos and flower buds were dissected from plants, immediately frozen in liquid nitrogen, and stored at -80 °C. DNA was isolated from trifoliolate unexpanded soybean leaves or Arabidopsis seedlings using the DNeasy Plant Mini Kit (QIAGEN). RNA was isolated from all tissues using the RNeasy Plant Mini Kit (QIAGEN) or from black soybean seed coats as described previously (Wang and Vodkin, 1994).

Arabidopsis lines used in this study were of the Columbia ecotype. The T-DNA insertion line *ugt78d2* (CS25064) was obtained from the SALK collection (Alonso et al., 2003) at the ABRC (Columbus, OH, USA). Seeds were surface-sterilized in 2% Triton / 70% EtOH (v/v) for 5 min on a mixer wheel, rinsed thrice with EtOH, dried, and plated on culture media (1X Gamborg's salts, pH 5.8, 1X B-5 vitamins, sucrose (2%, w/v), agar (0.8%, w/v) and 10 mg ml⁻¹ benomyl). To release seed dormancy plates were incubated for 3 d at 4 °C, then transferred to light. Germination and seedling culture occurred in a Conviron I23L incubator (Conviron, Winnipeg, Canada) at 22 °C, under cool-white fluorescent lights (117

$\mu\text{E}\cdot\text{m}^2\cdot\text{s}$) with a photoperiod of 16 h light to favor vegetative growth. Seedlings were harvested for nucleic acid isolation or transferred to soil after 2 weeks.

3.2.3. Cloning of the *UF3GT* cDNA from black soybean

By searching the NCBI *Glycine max* EST collection using the megablast algorithm and the *Clitoria ternatea* Ct3GT-A mRNA (AB185904, N. Noda, Unpublished) as a query, forward and reverse gene-specific primers (B1F, B1R, B2F, B2R, E1F, E1R, M1F, M1R) (see Table 3.1) were designed to amplify partial *UGT78K1* sequences from seed coat cDNA using 5' and 3' RACE (CLONTECH), respectively. The resulting partial cDNA fragments were cloned into the TOPO-TA vector (Invitrogen) and sequenced. The full-length coding region was then amplified from seed coat cDNA by end-to-end PCR using primers UHF/UHR (Table 3.1) and the Pfx50 DNA polymerase (Invitrogen). The resulting 1517 bp amplicon was cloned into the *NdeI* and *BamHI* sites of the pET-14b vector (Novagen, ON, Canada) and sequenced to confirm its identity.

3.2.4. Cloning of the *UF3GT* gene from black soybean

The *UGT78K1* sequence was amplified from the soybean genome by PCR using primers UHF/UHR (Table 3.1) and the Pfx50 DNA polymerase (Invitrogen), cloned into the pENTR/D-TOPO vector (Invitrogen), and sequenced. PCR of the wild type *UGT78D2* allele, the T-DNA insertion *ugt78d2*, and the *UGT78K1* gene was performed with primer pairs ULP/URP, LB/URP, and UNSF/UNSR, respectively (Table 3.1).

Table 3.1: Primers.

Primer	Sequence
AtTubF	5'-GGGCTAAAGGACACTACACTGAAGG-'3
AtTubR	5'-CCTCCTGCACTTCCACTTCGTCTTC-'3
B1F	5'-TGTTCCACTTCCTTCCACGTTGTTGC-'3
B1R	5'-CACTTTCTTCCAATGCCTCTGCCACTG-'3
B2F	5'-GGCTTATGTTTGCTTTGGGACTGTGGTG-'3
B2R	5'-CACTATTTTCCCACGCTTCTTGGTCCTC-'3
[†] B _{UF}	5'-CACCG GATCC ACCA <i>AT</i> GGATCATCAAAACAAACACGTAGCA-'3
[†] B _{UR}	5'-ACTC GAGCTC ATCGCCGACTTATTTTAGGATCTTGTCG-'3
E1F	5'-CTTTGGCAGCCACCTTATGCCTCTCTTA-'3
E1R	5'-AGTTGGGTTTTTGCCTAGGACGTGACC-'3
GmTubF	5'-GTGACTTGAACCATCTGATCTAGC-3' -'3
GmTubR	5'-GTTGAAGCCATCCTCAAGCCAG-3' -'3
LB	5'-GGACCGCTTGCTGCAACT-'3
M1F	5'-AGACACAGATGCAACAGGGTGCTTGTC-'3
M1R	5'-GAGACCAAAGAAAGGGAACACCACTTGC-'3
[†] U _{HF}	5'-CACCC ATA<i>T</i> GGATCATCAAAACAAACACGTAGCA-'3
[†] U _{HR}	5'-ACTC GGATCC ATCGCCGACTTATTTTAGGATCTTGTCG-'3
ULP	5'-CTCTTCGTTATTTTCCTCCGG-'3
URP	5'-TCAAACCCATCTTTCGTGAAG-'3
UNSF	5'-TGGATCATCAAAACAAACACGTAGCAGT-'3
UNSR	5'-GTGCTGGCGAATCAAGTCAGTGTAGA-'3

[†]Restriction site in bold

[‡]Start codon italicized bold

3.2.5. Phylogenetic Analyses

A multiple alignment of the deduced amino acid sequences of UGT78K1 and other flavonoid glycosyltransferases was constructed using ClustalW (<http://www.align.genome.jp>). Node support was estimated by parsimony bootstrap analysis (1000 bootstrap replicates, 10 random addition sequences per bootstrap replicate with tree bisection-reconnection branch swapping, limited to a maximum of 10000 trees) using PAUP* 4.0b10 (Sinauer Associates).

3.2.6. RT-PCR

RNA samples (5 µg) were treated with DNase I (Amplification grade, Invitrogen) at 37 °C for 15 min prior to RT-PCR. First-strand cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. PCR of *UGT78D2*, *UGT78K1* (Glyma07g30190), Glyma08g07130, Glyma07g30190, Glyma07g30200, Arabidopsis β -tubulin, and *G. max* β -tubulin was performed with primers ULP/URP, UNSF/UNSR, 93F/93R, 86F/86R, 200F2/200R2, AtTubF/AtTubR, and GmTubF/GmTubR, respectively (Table 3.1).

3.2.7. Recombinant protein expression in *Escherichia coli*

The full-length *UGT78K1* coding region fused in-frame with the pET-14b vector (above) was transformed into the expression host *E. coli* BL21(DE3) pLysS (Novagen, ON, Canada) and a single colony was selected for production of recombinant proteins. Soluble recombinant proteins were isolated from *E. coli*

BL21(DE3) pLysS following growth and induction at 16 °C, and purified by ion metal-affinity chromatography as indicated (Ford et al., 1998). 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 a protein assay kit (Bio-Rad Laboratories Inc.; <http://www.bio-rad.com>).

3.2.8. *Recombinant Enzyme Assays*

Initial enzyme assays (total volume 100 μ l) consisted of 2 μ g of recombinant enzyme, 100 μ M of acceptor substrate and 5 mM UDPG in assay solution (50 mM HEPES pH 8, 14 mM β -mercaptoEtOH) and were incubated at 30 °C for 30 min. To study the relationship between activity and pH, reactions were incubated in the following buffers, each at 50 mM: MES, pH 5-6; Tris, pH 7-9; HEPES, pH 7-8; CAPS, pH 10. To determine the affects of temperature on enzyme activity reactions were carried out in a thermocycler at 10-55 °C. To measure for linearity of product formation, reactions were stopped at 20 s intervals up to 3 min, using 5 mM UDP-glucose with 10 μ M cyanidin or 100 μ M kaempferol.

The amount of each reaction product was determined by comparison of integrated peak areas of the glucoside (except for myricetin where the aglycone was used) to the corresponding standard curve analyzed on the same day. Standards were processed in the same manner as enzyme reactions with the exception that the recombinant enzyme was boiled prior to incubation. Standard curves were verified to be linear (COC > 98) over the range investigated using

Chem32 software (Agilent Technologies Inc., Montreal QC, Canada). All reaction solutions were brought to the specified assay temperature prior to the addition of the acceptor substrate and enzyme. Reactions containing anthocyanins or 3-deoxyanthocyanidins were stopped by vortexing in 500 μ L of 15% HCO₂H in MeOH for 20 s. Reactions were prepared for HPLC by centrifugation (21,000 g for 4 min at 4 °C), followed by removal of the supernatant and concentrated to 100 μ L under nitrogen gas. All other reactions were stopped by vortexing in 500 μ L of EtOAc for 20 s followed by removal of the organic phase, which was then evaporated under a stream of nitrogen gas. The residue was resuspended in 100 μ L of HPLC grade MeOH. All reactions were filtered through Teflon (0.2 μ m; Chromatographic Specialties) and 20 μ L injections were analyzed by HPLC-DAD.

3.2.9. Enzyme Kinetics

To measure the reaction velocity of the recombinant enzyme for acceptor substrates, the concentrations of cyanidin and kaempferol were varied from 3 to 100 μ M and from 5 to 500 μ M, respectively, and 5 mM UDP-glucose was used as the donor substrate. Enzyme assays were performed as described above but reactions were stopped at 3 min and 5 min to ensure linearity of data points. Lineweaver-Burk graphs were plotted for the calculation of K_m and V_{max} values.

3.2.10. Enzyme specificity

The recombinant enzyme was tested for activity against anthocyaninidins (cyanidin, delphinidin, pelargonidin, petunidin, peonidin, malvidin), anthocyanins

(3-O-glucosides of the anthocyanidins and cyanidin 3-O-galactoside), flavonols (kaempferol, quercetin, myricetin, isorhamnetin), flavonol-3-glucosides (3-O-glucosides of kaempferol, quercetin, and isorhamnetin), flavan-3-ols (+)-catechin and (-)-epicatechin, isoflavones (genistin, genistein and daidzein), a dihydroflavonol (dihydroquercetin), and a 3-deoxyanthocyanidin (apigeninidin), using 5 mM UDP-glucose as a donor substrate. UDP-galactose (5 mM) was also tested as a sugar donor using cyanidin and delphinidin acceptor substrates. Enzyme assays were performed as described above with either 10 or 100 μ M of acceptor substrate and stopped at 3 min.

3.2.11. Transgenic lines

To obtain *ugt78d2* lines transformed with *UGT78K1*, the full-length coding sequence of *UGT78K1* was amplified from the pET-14b-*UGT78K1* construct (described above) using primers BUF/BUR (Table 3.1) and cloned into the *Bam*HI and *Sac*I sites of a modified pCAMBIA2300 vector (<http://www.cambia.org>) harboring the *EntCUP4* constitutive promoter (Malik et al., 2002) and the NOS terminator between *Hind*III and *Eco*RI sites. Transformation of line *ugt78d2* with the pCAMBIA2300-tCUP4::*UGT78K1*-tNOS construct was achieved using the *Agrobacterium tumefaciens* strain GV3101 as described (Clough and Bent, 1998). T1 seeds were selected on culture media and 50 μ g ml⁻¹ kanamycin then transferred to soil to set T2 seeds. Independent lines were identified to carry a single transgene copy if T2 seedlings exhibited a viable to non-viable ratio of 3:1 on selection media. Seedlings were visually

screened for anthocyanin accumulation after 1 week of culture on anthocyanin gene induction media (pH 5.8) consisting of sucrose (3%, w/v), agar (0.8%, w/v) and 50 $\mu\text{g ml}^{-1}$ kanamycin. Three independent lines which carried a single copy of the transgene, and which accumulated visible levels of anthocyanins, were selected for further analysis.

3.2.12. HPLC-DAD and HPLC-ESI-MS analyses

The HPLC system (an Agilent 1100 series; Agilent Technologies Inc., Montreal QC, Canada) was 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 separations were achieved at 45 °C on a Luna C18(2), 4.6 x 150 mm, particle size 5 μm fitted with a corresponding guard-column (Phenomenex Inc, Santa Ana, CA). The mobile phase system consisted of 5% HCO_2H in HPLC grade water (solvent A) and HPLC grade MeOH (solvent B). The 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 at 100 % B, brought back to starting mobile phase composition in 0.1 min and equilibrated for 5 min before next injection. The HPLC separations were monitored at 254, 280, 350, 476, and 520 nm.

HPLC-ESI-MS analysis was done on an 3200QTRAP (ABI Sciex, Toronto, Canada). The characteristic fragments of C3G and K3G were detected in enhanced mass scan (EMS) mode in positive ionization. The optimal settings of the mass spectrometer were: curtain gas (N_2) 10 L min^{-1} , ion spray voltage 4500

V, source gas (N₂) 10 L min⁻¹, declustering potential +50 V, entrance potential 10 V, ionization energy 1.0 eV and detector 2200.

3.2.13. Metabolite Analysis

One-week-old *Arabidopsis* seedlings (100 mg) cultured on anthocyanin gene induction media (above) were pulverized in 200 µL of 80% MeOH in water using a FastPrep FP120 Homogenizer (Savant). The extracts were filtered through Teflon (0.2 µm; Chromatographic Specialties), 20 µl injections were analyzed by HPLC-DAD as described above, and 5 µl were measured by photospectroscopy using a NANODROP 2000 (Thermo Scientific) the formula $A_{530} - 0.25A_{657}$ to compensate for chlorophyll absorption at 530 nm (Mancinelli, 1990).

3.3. Results

3.3.1. Cloning and analysis of a glycosyltransferase gene from the seed coat of black soybean

As the Glyma1 soybean genome sequence was not available at the time this study was initiated, *Glycine max* ESTs from the GenBank collection were searched with the tBLASTn and BLASTn algorithms using every published *UF3GT* sequence as a query (September, 2007), including the only published legume *UF3GT* (UGT78G1, from *Medicago truncatula*) (Modolo et al., 2007) in order to identify *UF3GT* candidates. However, these searches failed to identify any sequences with exceptionally high similarity. Highest similarity to GenBank

G. max ESTs (up to 53%) was identified by tBLASTn using the PSPG box motifs of UGT78D2 from *Arabidopsis* (Tohge et al., 2005) and VvGT1 from *Vitis vinifera* (Ford et al., 1998). However, these searches yielded numerous unigenes with moderate similarity, and thus did not provide a sufficiently narrow pool of candidates for functional analysis. The GenBank database was searched using keywords such as anthocyanidin 3-O-glucosyltransferase which yielded the *Ct3GT-A* sequence (AB185904), representing a UF3GT from the legume *Clitoria ternatea* (N. Noda, personal communication). A megablast search using the *Ct3GT-A* mRNA as a query identified five ESTs with exceptionally high identity (68-81%). The ESTs were derived from various organs including seeds and flowers.

RNA was isolated from the seed coats of black soybean (*G. max* cv. Clark) and the three ESTs with highest identity to *Ct3GT-A* were extended by 5'- and 3'-RACE and sequenced. RACE fragments from the three ESTs were found to encode a single contiguous mRNA (1,661 nts in length). The 5'- and 3'-untranslated regions were 86 bp and 234 bp, respectively. Conceptual translation of the mRNA revealed a 447-amino acid protein, which we named UGT78K1. The presence of the plant secondary product glucosyltransferase (PSPG) consensus sequence in the C-terminal region of UGT78K1 identified it to be a type I glycosyltransferase (Vogt and Jones, 2000).

3.3.2. Genomic and phylogenetic analyses of soybean glycosyltransferases

To examine the potential for gene redundancy, a search for soybean genes with high identity to *UGT78K1* (Glyma07g30180) was performed. A BLASTn search of the soybean (Glyma1) genome sequence (www.phytozome.net/soybean) revealed four sequences (Glyma07g30200, Glyma06g39350, Glyma07g30190, and Glyma08g07130) with high identity to *UGT78K1* (83, 85, 86, and 93% respectively). Glyma08g07130, Glyma07g30190, and Glyma07g30200 had high amino acid identity (93, 79, and 72%, respectively) to *UGT78K1*, however conceptual translation of Glyma06g39350 revealed a highly truncated protein of 265 amino acids with two N-terminal deletions and the absence of the putative catalytic histidine residue equivalent to His20 of VvGT1 (Offen et al., 2006). This suggested that Glyma06g39350 may be non-functional, and thus it was excluded from further analysis.

Figure 3.1 demonstrates the genomic organizations of *UGT78K1* and the Arabidopsis gene *UGT78D2* with their respective paralogs. Interestingly, the *UGT78K1* genomic sequence is positioned in the same orientation and situated directly upstream from Glyma07g30190 and Glyma07g30200 (Figure 3.1A), with a fourth highly similar sequence (Glyma08g07130) located on a separate chromosome (not shown). A similar genomic organization has been reported for *UGT78D2* (At5g17050, *ANL1*) and three paralogs (At5g17030, At5g17040, and At1g30530) in the Arabidopsis genome (Figure 3.1B) (Kubo et al., 2007).

Figure 3.1. Genomic organisations of *UGT78K1* (Glyma07g30180), Glyma08g07130 and Glyma07g30190 from *Glycine max* (A) and *Arabidopsis thaliana* glycosyltransferases UGT78D2 (At5g17050), At5g17040, and At5g17030 (B).

However, unlike the Arabidopsis sequences, *UGT78K1* and its paralogous sequences lack the highly conserved glycosyltransferase (UGT) intron, equivalent to the Arabidopsis UGT intron 2 (Li et al., 2001). This data suggested that the gene triplication event that gave rise to *UGT78K1* and its paralogs on chromosome 7 (Glyma07g30190 and Glyma07g30200) most likely took place after divergence of *G. max* and Arabidopsis from a common ancestor, and thus the similar genomic organizations likely do not represent synteny. Furthermore, as Glyma08g07130 has greatest similarity to *UGT78K1*, this suggests that the gene duplication event that gave rise to this sequence occurred more recently than the triplication event that resulted in *UGT78K1*, Glyma07g30190, and Glyma07g30200 on chromosome 7.

Figure 3.2 demonstrates a phylogenetic analysis of the amino acid sequences of biochemically characterized flavonoid UGTs. The *in vitro* substrate specificities and *in vivo* functions of flavonoid UGTs cannot be accurately predicted using amino acid sequences alone. However, phylogenetic analysis resulted in five clusters that can be generally grouped based on common *in vitro* substrate specificities. Enzymes in Cluster V have anthocyanin 5-O- and/or flavonol 7-O- UGT activities, Cluster IV glycosylates flavonol and isoflavone substrates, and Cluster III includes isoflavone 7-O- and anthocyanidin 3,5-O-GT activities. Cluster II GTs have multiple substrate specificities generally for chalcones, flavones, and flavonols, but not anthocyanidins. Cluster I is exclusively UF3GTs. *UGT78K1* groups with Cluster I, as does its paralogs (Glyma08g07130, Glyma07g30190, and Glyma07g30200). *UGT78K1* groups more closely with its

paralogs located on chromosome 7 (Figure 3.1A) separately from UGT78D2 and its paralogs (Figure 3.2). This further suggests that the gene triplication event that resulted in similar genomic organizations for *UGT78K1* and *UGT78D2*, and their paralogs (Figure 3.1), likely occurred after speciation and is not a result of synteny.

3.3.3. Expression analysis of black soybean glycosyltransferases

To determine whether the expression of *UGT78K1* coincided spatially with anthocyanin and/or flavonol biosynthesis in black soybean, RT-PCR was performed on RNA isolated from individual organs (Figure 3.3). Anthocyanins accumulate to visible levels in the seed coat, mature stem, and purple flower petals of black soybean cv. Clark. By contrast, flavonol glycosides have been reported to accumulate in all aerial organs of soybean (Graham, 1991). RT-PCR identified *UGT78K1* transcripts in the seed coat, flower bud, stem, and all other organs examined (Figure 3.3). These results suggest that *UGT78K1* is expressed not only at the sites of anthocyanin biosynthesis, but also at sites of flavonol glycoside biosynthesis. A similar expression pattern was observed for *UGT78D2* (*ANL1*) in *Arabidopsis* (Kubo et al., 2007). RT-PCR demonstrated overlapping expression profiles of Glyma07g30200, Glyma07g30190, and Glyma08g07130 with *UGT78K1* in some organs, but only Glyma08g07130 was co-expressed with *UF3GT* in the seed coat (Figure 3.3).

Figure 3.3. Transcript detection of *UGT78K1* (Glyma07g30180), Glyma08g07130, Glyma07g30190, and Glyma07g30200 by RT-PCR with gene-specific primers.

3.3.4. *UGT78K1* encodes a *UF3GT* with strict specificity for anthocyanidins and flavonols *in vitro*

The open reading frame of *UGT78K1* was cloned into the pET-14b N-terminal hexahistidine fusion tag expression vector (Novagen, ON, Canada), transformed into BL21(DE3) pLysS *Escherichia coli* cells, and expressed at 16 °C for 24 h. Protein was extracted and his-tagged *UGT78K1* (r*UGT78K1*) was purified from the soluble fraction by ion-metal affinity chromatography (IMAC) using the Talon system (Clontech, Palo Alto, CA). Purified fractions obtained from a 250 ml *E. coli* culture contained 1.6-2.4 mg of the fusion protein. Separation of the IMAC fractions by SDS-PAGE followed by staining with Coomassie Blue revealed an apparently pure recombinant enzyme with an estimated molecular mass of ca 49.8 kDa (Figure 3.4A). This value matched well with the calculated molecular mass of *UGT78K1* (49.24 kDa) with the inclusion of its 2.18 kDa N-terminal tag. Preliminary assays using uridine diphosphate glucose (UDPG) as a sugar donor demonstrated that the purified r*UGT78K1* could transfer glucose to the 3-position of the anthocyanidin cyanidin (Figure 3.4B, D) and the flavonol kaempferol (Figure 3.4C, E), whereas the boiled enzyme and bacteria expressing the corresponding empty vector could not catalyze these reactions (not shown). Glycosylated products from the recombinant enzyme assays were identified and quantified by high performance liquid chromatography-diode array detection (HPLC-DAD) by comparison to authentic standards. The presence of cyanidin-3-O-glucoside (C3G) and kaempferol-3-O-glucoside (K3G) reaction products were verified by high performance liquid chromatography electrospray ionization mass

Figure 3.4. Purification of his-tagged UGT78K1 and identification of reaction products. (A) SDS-PAGE analysis of UGT78K1: total soluble protein from *E. coli* expressing UGT78K1 prior to induction with 100 μ M IPTG (lane 1), 24 h post-induction (lane 2); purified UGT78K1 (lane 3). (B, C) UGT78K1 enzyme reactions as revealed by HPLC chromatograms at 520 nm (D) and 350 nm (E), respectively. HPLC retention times: cyanidin (R_t : 10.0 min); cyanidin 3-O-glucoside (R_t : 7.0); kaempferol (R_t : 15.8); kaempferol 3-O-glucoside (R_t : 12.0).

spectrometry (HPLC-ESI-MS) in enhanced mass scan mode. The characteristic fragments detected in the C3G reaction mixture were 287.6 [cyanidin + H]⁺ and 450.1 [cyanidin-3-O-glucoside + H]⁺, and 287.1 [kaempferol + H]⁺ and 449.1 [kaempferol-3-O-glucoside + H]⁺ for the K3G reaction mixture. Further purification of the enzyme by the removal of its hexahistidine tag did not alter enzyme activity, as determined by measuring activity before and after cleavage. For this reason, assays were routinely performed without removal of the tag. The enzyme exhibited no significant loss in activity towards kaempferol after storage at 4 °C or –20 °C in a 50% glycerol solution for 24 h, however longer incubations resulted in a notable reduction in activity (not shown). To minimize the effect of storage on enzyme activity, assays were performed immediately following purification.

Optimal reaction conditions were determined using cyanidin and kaempferol as acceptor substrates with UDPG as the donor because cyanidin 3-O-glucoside and kaempferol glycosides are the most abundant anthocyanins and flavonols in the seed coats and purple petals of *G. max*, respectively (Choung et al., 2001; Iwashina et al., 2007; Lee et al., 2009). Investigation of a pH range from 5 to 10 and temperatures from 10 to 50 °C revealed the recombinant enzyme to be most active at pH 8 and 30 °C. rUGT78K1 exhibited a slight buffer preference for HEPES over Tris at pH 8 (not shown).

The kinetics of rUGT78K1 was determined for both cyanidin and kaempferol acceptor substrates (Figure 3.5). By using a concentration of 5 mM UDPG and varying the concentration of kaempferol between 5 and 400 µM, a hyperbolic saturation curve was obtained (Figure 3.5A), from which Lineweaver-Burk

transformation gave an apparent K_m for kaempferol of 174 μM and V_{max} of 24.8 $\text{pKat } \mu\text{g}^{-1}$. From these a k_{cat} of 1.25 s^{-1} was calculated. By contrast, when the concentration of UDPG was maintained at 5 mM and the concentration of cyanidin was varied from 5 to 100 μM , the reaction velocity increased with cyanidin concentrations up to circa 16 μM and progressively decreased with increasing cyanidin concentrations (Figure 3.5B), suggesting the enzyme was inhibited by this substrate.

The recombinant enzyme readily transferred glucose from UDPG to 3-position of all tested anthocyanidins and flavonol aglycones with variable specificities (Figure 3.6). When acceptor substrates were supplied below the inhibitory concentration of cyanidin the enzyme demonstrated highest activity towards the anthocyanidin delphinidin (Figure 3.6A). By contrast, when acceptor substrates were supplied above the inhibitory concentration of cyanidin, UGT78K1 demonstrated highest activity for flavonols (quercetin, myricetin, and kaempferol) (Figure 3.6B, white bars). No activity was observed for other flavonoid classes previously identified in soybean, including dihydroflavonol (dihydroquercetin), isoflavone (genistin, genistein and daidzein), (epi)-flavan-3-ol (catechin, epicatechin), 3-deoxyanthocyanidin (apigeninidin), flavonol conjugate (flavonol 3-O-glucosides) and anthocyanins (anthocyanidin 3-O-glucosides and cyanidin 3-O-galactoside) (data not shown). However, galactose could be transferred from UDP-galactose to the 3-position of cyanidin and delphinidin with low specificity relative to the analogous glucosyltransferase reactions (Figure 3.6, grey bars), as determined by HPLC-DAD retention times and absorbance spectra by

Figure 3.5. UGT78K1 kinetics for cyanidin and kaempferol acceptor substrates. Reaction kinetics for acceptor substrates kaempferol (A) and cyanidin (B) using 5 mM UDPG as a sugar donor. Points represent the mean of three assays $\pm$ the standard deviation.

Figure 3.6. Activities of UGT78K1 for anthocyanidins and flavonols. (A) Acceptor substrate concentration 10 μ M. (B) Acceptor substrate concentration 100 μ M. Anthocyanidins (black bars) and flavonols (white bars) assayed using 5 mM UDPG as a sugar donor. Cyanidin and delphinidin assayed using 5 mM UDP-galactose as a donor (grey bars). Each bar represents the mean of three assays + the standard deviation.

comparison to authentic standards. These results strongly suggest that rUGT78K1 acts exclusively on anthocyanidins and flavonols *in vitro* preferentially as a glucosyltransferase with strict regiospecificity for the 3-position.

3.3.5. *UGT78K1* functions as a 3-O-glucosyltransferase in anthocyanin and flavonol biosynthesis *in vivo*

To confirm that *UGT78K1* has flavonoid 3-O-glucosyltransferase activity *in vivo*, the cDNA was transferred into the Arabidopsis mutant *ugt78d2* with highly reduced anthocyanidin and flavonol 3-O-glucosyltransferase activity (Tohge et al., 2005). T1 seedlings were selected on media containing kanamycin, transferred to soil and grown to maturity. Thirty percent of mature T1 plants (12 independent lines) selected on kanamycin media exhibited pigmentation at the rosette base similar to the wild type, whereas the *ugt78d2* mutant transformed with the empty vector lacked visible pigments (not shown). Three lines (U4-8, U4-25, and U4-28) were identified that carry a single copy of the *UGT78K1* cDNA by selection of T2 seedlings on kanamycin media ($\chi^2_{0.05, 1}$ (3:1, viable:non-viable) = 1.7, 0.1, and 0.005, respectively) and were chosen for further analysis. Presence of the *UGT78K1* transgene and T-DNA insertions in the *UGT78D2* gene were verified by PCR using primers UHF/UHR and LB/URP, respectively, whereas the *UGT78D2* was identified to lack the T-DNA insertion using primers ULP/URP (Figure 3.7A). RT-PCR was used to verify that each transgenic line expressed *UGT78K1* and lacked the expression of *UGT78D2* (Figure 3.7B).

To examine anthocyanin pigmentation phenotype more clearly, T2 seedlings were cultured on anthocyanin induction media (see Materials and methods) and were visualized using light microscopy. The majority of T2 seedlings displayed highly pigmented cotyledons and hypocotyls similar to the wild type, whereas the *ugt78d2* mutant transformed with the empty vector had minimal amounts of visible pigments (Figure 3.7C). To examine the amounts of total anthocyanins in transgenic lines relative to wild type and *ugt78d2* controls, 100 mg of seedlings from each line were pooled, extracted with 80% MeOH, and total anthocyanins were quantified spectrophotmetrically using a formula to compensate for chlorophyll absorption. On average, extracts from transgenic lines contained five (line U4-8) to six times (line U4-28) the anthocyanin absorbance relative to the *ugt78d2* mutant (Figure 3.7D) and 65% (U4-8) to 75% (U4-28) absorbance relative to the wild type. This suggested restoration of anthocyanin biosynthesis in transformed seedlings, as 25% of the segregating T2s on non-selective anthocyanin induction media would not carry the *UGT78K1* gene.

Figure 3.7. The *UGT78K1* gene complements anthocyanin biosynthesis in the *Arabidopsis ugt78d2* mutant. (A) PCR analysis for the *UGT78K1* and *UGT78D2* genes and the T-DNA-inserted allele *ugt78d2* from genomic DNA of wild type (Columbia), mutant *ugt78d2* (transformed with empty pCambia1300tCUP plasmid), and the *ugt78d2* mutant transformed with the *UGT78K1* gene (three

independent T2 lines; U4-8, U4-25, U4-28) . (B) RT-PCR analysis of total RNA from 100 mg of pooled seedlings from each line. (C) T2 lines cultured on anthocyanin induction media for 1 week (see materials and methods). (D) Amount of anthocyanins in 100 mg of pooled T2 seedlings cultured on anthocyanin induction media.

To determine the relative amounts of individual anthocyanins and flavonols in transgenic lines relative to the wildtype and *ugt78d2* controls, 100 mg of seedlings (T2s) from each line were pooled, extracted with 80% MeOH, and analyzed by HPLC-DAD. The *ugt78d2* mutant transformed with the empty vector had reduced peak area for all peaks measured at 520 nm (anthocyanins) and some peaks measured at 350 nm (flavonoids) relative to the wildtype (Figure 3.8). Highly similar profiles were reported previously for Col-0 and the *ugt78d2* mutant, and the peaks reduced at 520 nm and 350 nm in the *ugt78d2* mutant relative to the wildtype were identified to be anthocyanins and flavonols, respectively, that contained a glucose at the 3-position (Tohge et al., 2005). The pooled T2 seedlings expressing the *UGT78K1* cDNA in the *ugt78d2* background had restored peaks, albeit to lower levels than the wild type. These results strongly suggest that *UGT78K1* has similar activity to UGT78D2, a flavonoid 3-O-glucosyltransferase involved in anthocyanin and flavonol biosynthesis *in vivo*.

Figure 3.8. Functional analysis of the *UGT78K1* gene by complementation of the Arabidopsis *ugt78d2* null mutation. (A-J) HPLC chromatograms of 80% aq. MeOH extracts of seedlings cultured on anthocyanin induction media for 1 week. (A, F) Col-0, (B, G) *ugt78d2*, (C, H) U4-8, (D, I) U4-25, (E, J) U4-28. (A-E) Absorbance at 520 nm for analysis of anthocyanins. (F-J) Absorbance at 350 nm for analysis of flavonoids. HPLC retention times: peak 1 (Rt: 7.8); peak 2 (Rt: 9.0); peak 3 (Rt: 9.6); peak 4 (Rt: 11.6); peak 5 (Rt: 11.9); peak 6 (Rt: 7.7); peak 7 (Rt: 8.5).

3.4. Discussion

Several genes involved in common flavonoid biosynthesis steps have been characterized from black soybean, however the gene catalyzing the final step in anthocyanin biosynthesis, namely UDP-glucose:flavonoid 3-O-glucosyltransferase (*UF3GT*), remained unreported (reviewed by Kovinich et al., 2011a). The purpose of this study was two-fold: 1) to identify the gene catalyzing the final step in anthocyanin biosynthesis from the seed coat of black soybean to provide a possibility of engineering reduced pigment in transgenic grains, and 2) to characterize the catalytic properties of the recombinant enzyme to provide a basis for future glucosyltransferase structure-function analyses.

The first UGT gene isolated from plants was the UDP-glucose:flavonol glucosyltransferase from *Zea mays*, encoded by the bronze locus (Fedoroff et al., 1984). Since then, UF3GT enzymes have been partially purified from *Brassica oleracea* (Saleh et al., 1976), *Haplopappus gracilis* (Saleh et al., 1976), *Senecio x hybridus* (Ogata et al., 1998), and cDNAs have been cloned into expression vectors for characterization of recombinant UF3GT activities from *Gentiana triflora* (Tanaka et al., 1996), *Vitis vinifera* (Ford et al., 1998), *Dianthus caryophyllus* (Ogata et al., 2004), *Iris hollandica* (Yoshihara et al., 2005), *Arabidopsis* (Tohge et al., 2005), *Fragaria x ananassa* (Almeida et al., 2007), *Medicago truncatula* (Modolo et al., 2007; Peel et al., 2009) and *Citrus paradisi* (Owens and McIntosh, 2009). Homology-based searches of the GenBank database using each reported UF3GT as a query identified numerous soybean

unigenes with equally low similarity. This suggested that soybean UF3GT(s) and other putative glycosyltransferases are highly divergent from published UF3GTs, including the only previously reported legume UF3GT UGT78G1 from *Medicago truncatula* (Peel et al., 2009).

All recombinant UF3GTs characterized to date have unique substrate specificities *in vitro*. Generally, some prefer anthocyanidins (Tanaka et al., 1996; Ford et al., 1998; Ogata et al., 2004; Yoshihara et al., 2005; Almeida et al., 2007), while others prefer flavonols or (iso)flavonoids (Modolo et al., 2007; Owens and McIntosh, 2009). UGT78K1 glucosylated only anthocyanidins and flavonols, however its relative substrate preference was dependent on the concentration of acceptor substrate (Figure 3.6). Kinetic analyses suggested that the enzyme is inhibited by cyanidin at higher concentrations (above circa 16 μ M) (Figure 3.5B). The fact that high amounts of cyanidin remained after enzyme reactions (not shown) supports the notion of substrate inhibition rather than degradation of cyanidin as the cause of the observed kinetics. Future studies will include full kinetics analyses for all 10 acceptor substrates to determine whether they are similarly inhibiting UGT78K1 activity, and kinetics assays with an acceptor substrate (e.g. kaempferol) and an inhibitor (cyanidin) to gain more insight into the underlying mechanism of UGT78K1 inhibition.

UF3GTs from red cabbage (*B. oleracea*) and *H. gracilis* (Saleh et al., 1976; Saleh et al., 1976) have also been shown to be inhibited by cyanidin. However, it remains unclear whether inhibition of UF3GT activity by cyanidin could have a

role in the regulation of flavonoid biosynthesis *in vivo*. The possibility exists that inhibition of UGT78K1 by cyanidin could prevent the cytotoxic effects of high cytosolic cyanidin 3-O-glucoside concentrations as observed in glutathione S-transferase mutants defective for vacuolar sequestration of anthocyanins (Marrs et al., 1995; Alfenito et al., 1998). Alternatively, inhibition of UGT78K1 by cyanidin 3-O-glucoside could function to make available more anthocyanidin substrates for proanthocyanidin biosynthesis, as anthocyanidins are precursors of both pathways (Lepiniec et al., 2006), and anthocyanins and PAs co-accumulate in palisade cells of black soybean during seed development (Kovinich, unpublished data). However, another possibility is that localized cyanidin concentrations are not high enough in soybean to cause the substrate inhibition of UGT78K1 that is observed *in vitro*. Flavonoid enzymes are thought to physically associate in metabolons (Saslowsky and Winkel-Shirley, 2001; Winkel, 2009). Unfortunately, it is presently not possible to measure the localized concentrations of flavonoid intermediates within metabolons to determine whether these concentrations are comparable to those which are observed to cause inhibition *in vitro*.

Transfer of UGT78K1 into the Arabidopsis mutant *ugt78d2* restored both anthocyanin and flavonol glycoside biosyntheses (Figure 3.8), confirming the *in vivo* function of UGT78K1 as a UF3GT. Anthocyanin levels were not restored to a lesser degree than flavonols in Arabidopsis despite the ability of cyanidin to inhibit UGT78K1 *in vitro* (Figure 3.5B). This suggested that substrate inhibition by cyanidin was not operating *in vivo* and may have been a result of excessive

amounts of the UGT78K1 enzyme relative to the amount of cyanidin substrate. Similar to UGT78K1, the *M. truncatula* UF3GT UGT78G1 exhibited relatively low activity towards cyanidin compared to other flavonoid substrates *in vitro* (Modolo et al., 2007) , but was demonstrated to act in anthocyanin biosynthesis *in vivo* (Peel et al., 2009). These results raise the possibility that *in vitro* specificities may better reflect UF3GT capability rather than major *in vivo* function(s).

The V_{max} value of UGT78K1 for kaempferol ($24.8 \text{ nKat } \mu\text{g}^{-1}$) was slightly higher than those reported previously for UF3GTs and flavonol substrates (11.6 to $20.5 \text{ nKat } \mu\text{g}^{-1}$) (Ford et al., 1998; Owens and McIntosh, 2009). The K_m values for flavonoid substrates from characterized UF3GTs vary greatly among species with values ranging from $1.5 \text{ } \mu\text{M}$ to $400 \text{ } \mu\text{M}$ (Saleh et al., 1976; Saleh et al., 1976; Ford et al., 1998; Modolo et al., 2007; Owens and McIntosh, 2009). The K_m of UGT78K1 for kaempferol ($174 \text{ } \mu\text{M}$) was within this range. Interestingly, UF3GTs that are inhibited by cyanidin ($174\text{-}400 \text{ } \mu\text{M}$) (Saleh et al., 1976a; Saleh et al., 1976b; the present study) have greater K_m values for flavonoid substrates than UF3GTs that do not exhibit substrate inhibition ($12\text{-}90 \text{ } \mu\text{M}$) (Ford et al., 1998; Modolo et al., 2007; Owens and McIntosh, 2009). The identification of more UF3GTs and characterization of their kinetics for cyanidin may help determine whether there is a relationship between K_m and inhibition.

Anthocyanins accumulate to visible levels in the seed coat, mature stem, and purple flower petals of black soybean cv. Clark. By contrast, flavonol glycosides have been reported to accumulate in all aerial organs of soybean (Graham, 1991). *UGT78K1* was expressed transcriptionally in all organs, not just the sites

of visible anthocyanin accumulations (Figure 3.3). This expression pattern is similar to the UGT78D2 gene (At5g17050) in Arabidopsis (Tohge et al., 2005; Kubo et al., 2007) and may suggest that UGT78K1 also has a role in anthocyanin and flavonol glycoside biosyntheses *in vivo*.

UGT78K1 was demonstrated to catalyze the transfer of galactose to the 3-position of cyanidin or delphinidin at low rates relative to the corresponding glucosyltransferase reactions (Figure 3.6). These findings are consistent with previous reports of mainly 3-O-glucosylated and minor amounts of 3-O-galactosylated cyanidins and delphinidins in the seed coat of black soybean (Lee et al., 2009). However, knock-out or suppression of *UGT78K1* would be needed to determine whether this minor activity has a role in the accumulation of anthocyanin galactosides in the seed coat of black soybean. We are currently attempting to obtain black soybean lines expressing an RNAi vector for silencing the UGT78K1 gene to test this hypothesis and to potentially change the color of the soybean seed coat.

3.5. Concluding Remarks

In conclusion, we have isolated a cDNA from the seed coat of black soybean and have constructed a recombinant enzyme expression plasmid and transgenic Arabidopsis lines to determine the function of the encoded enzyme. Our results suggest that UGT78K1 is a UF3GT involved in anthocyanin and flavonol glycoside biosynthesis. Engineering reduced anthocyanin levels in the seed coat of black soybean by suppressing the expression of UGT78K1 could potentially

produce distinct seed colors to enable the visual identification and monitoring of transgenic grains (Kovinich et al., 2011a). However, RT-PCR identified the co-expression of Glyma08g07130 (Figure 3.3), which was demonstrated to be closely related to UGT78K1 by phylogenetic analyses (Figure 3.2) and may suggest functional redundancy in this tissue. Future work will determine the acceptor substrate specificities of recombinant Glyma08g07130 for anthocyanidins to determine whether overlapping substrate specificities exist with UGT78K1. This will determine whether both genes must be silenced to reduce anthocyanin accumulations in the seed coat of black soybean.

CHAPTER 4

A combined analysis of transcriptome and metabolite data to identify pigment genes and to understand the molecular control of black soybean grain color

Adapted from:

Kovinich N¹, Saleem A³, Arnason JT², and Miki B². 2011. 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. *BMC genomics* 12:381. doi:10.1186/1471-2164-12-381.

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²Obtained funding for the research and revised the manuscript

³Developed the HPLC-DAD method

4.1 Introduction

Flavonoids represent a class of plant secondary metabolites that have evolved a variety of physiological functions including pigmentation, pathogen defense, and UV protection (Winkel-Shirley, 2001). 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 (Dixon, 2005; Butelli et al., 2008; Kovinich et al., 2011a).

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 (Todd and Vodkin, 1993). A goal for biosafety is to engineer a novel red seed coat color as a marker for transgenic soybean grains to facilitate their identification (Kovinich et al., 2011a), 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*) (Bernard and Weiss, 1973) 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^h*, *I^k*, *i*); *I* gives completely non-pigmented seed coat, *I^h* restricts pigment to the hilum and *I^k* to a saddle-shaped region, whereas the *i* allele results in a fully pigmented seed coat (Bernard and Weiss, 1973). The recessive *i* allele results from spontaneous deletion of *CHS4* or *CHS1* 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 (Todd and Vodkin, 1996; Tuteja et al., 2004).

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 (Woodworth, 1921; Buzzell et al., 1987; Todd and Vodkin, 1993). Genetic polymorphisms that affect the expression or

function of the flavonoid 3'-hydroxylase gene (*F3'H1*) have been shown to co-segregate with recessive *t* alleles (Toda et al., 2002; Zabala and Vodkin, 2003).

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 (Bernard and Weiss, 1973). The *W1* allele for purple flower color was shown to code flavonoid 3',5'-hydroxylase (*F3'5'H*), as a 65-bp insertion in the gene (*F3'5'H*) co-segregated with white flower color (*w1*) (Zabala and Vodkin, 2007).

The *Wp* locus was suggested to code the flavonone 3-hydroxylase gene (*F3H1*) by microarray analysis as high levels of *F3H1* transcripts co-segregated with purple flower color (*Wp*), and low levels with pink (*wp*) flowers (Zabala and Vodkin, 2005). In the seed coat, recessive *wp* resulted in a change from black (*iRTWp*) to a lighter grayish (*iRTwp*) color (Zabala and Vodkin, 2005).

The *O* locus affects the color of brown (*irTO*) seed coats, with the recessive *o* allele giving a red-brown (*irTo*) phenotype (Bernard and Weiss, 1973). 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 (Yang et al., 2010). 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 (Nagai, 1921). 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 (Ku, 2009). 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* (Kovinich et al., 2010). 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 (Schmutz et al., 2010). 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 (Hirai et al., 2004; Hirai et al., 2005; Tohge et al., 2005). 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 (Hirai et al., 2004; Tohge et al., 2005; Pienkny et al., 2009).

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.

4.2 Materials and Methods

4.2.1 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).

4.2.2 Plant Materials and Growth Conditions

Black (*iRT*) soybean (*G. max* (L.) Merr.) variety Clark (PI547438) and the nearly isogenic brown (*irT*) 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% triton/70% EtOH–H₂O (7:3, v/v) for 5 min on a mixer wheel, rinsed three times with EtOH, dried, and germinated in autoclaved vermiculite in a Conviron E15 cabinet with a photoperiod of 16 h light (590 $\mu\text{E m}^2 \text{ s}$) 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.

4.2.3 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 (Thermo Scientific, ON, Canada) and incubated at 4 °C for 2 h. The slurry was centrifuged at 20,000g for 10 min, and 5 μL of supernatant from

each sample was measured by photospectroscopy using a NANODROP 2000 (Thermo Scientific, ON, Canada) according to the formula $A_{530} - 0.25A_{657}$ 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 μ l of supernatant was vortexed with HCO₂H-MeOH (1 mL, 15:85, v/v) for 20 sec, centrifuged at 20,000g for 10 min, and the supernatant was evaporated under a stream of nitrogen gas (to 50 μ L), filtered through PVDF (0.45 μ m; Millipore), and 20 μ l and 5 μ l 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 (Downey et al., 2003; Kennedy and Jones, 2003) 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 (Li et al., 1996) using procyanidin B2 and cyanidin as standards for the respective assays.

4.2.4 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 Bioanalyser 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 (Gentleman et al., 2004). The raw data was adjusted for background signal, and normalized across all GeneChips using the Robust Multi-array Average (RMA) method (Irizarry et al., 2003). To identify differentially expressed genes, the SAM algorithm, which minimizes variation across arrays and incorporates an estimate of false discovery rate (FDR) (Benjamini and Hochberg, 1995), 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 (Brazma et al., 2001). 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 µl 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 MgCl₂, 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 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 iQ SYBR Green Supermix (BioRad, ON, Canada). 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, QC, Canada). 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, ON, Canada). 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 Appendix A.

4.2.5 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 (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/UAR and HO5F/HO5R, respectively (Appendix A) and a high-fidelity DNA polymerase (Invitrogen). The resulting UGT78K2 and OMT5 amplicons (1466 bp and 836 bp, respectively) were cloned into *Nde*I and *Bam*HI sites, and *Nde*I and *Xho*I sites of the pET-14b vector (Novagen, ON, Canada), respectively, and sequenced to confirm their identities.

4.2.6 Expression of Recombinant Proteins in E. coli

The full-length CDSs of UGT78K2 and OMT5 were independently fused in-frame to the N-terminal hexahistidine tag encoded by the pET-14b vector (above). Plasmids were transformed into the expression host *E. coli* BL21(DE3) pLysS (Novagen, ON, Canada) 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, ON, Canada). 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 (BioRad, ON, Canada).

4.2.7 Recombinant Enzyme Assays

The UGT78K2 assay (total volume 100 µl) was performed as described previously (Kovinich et al., 2010) 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 a 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.

4.2.8 HPLC-DAD and HPLC-MS/MS Analyses

HPLC-DAD analysis of anthocyanins was performed as described previously (Kovinich et al., 2010). Briefly, separations were achieved at 45 °C on a Luna C18(2), 4.6 x 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% HCO₂H 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.

4.3 Results

4.3.1 *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 (Ku, 2009). 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* (Kovinich et al., 2010) 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 (Sugimoto et al., 1992; Tuteja et al., 2004).

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 4.1). Stereomicroscopy and photospectroscopic measurements confirmed these developmental stages to coincide with the stages of anthocyanin biosynthesis in the black (*irT*) seed coat (Figure 4.2). In contrast to *UGT78K1*, *DFR1* was not differentially expressed at the stages of anthocyanin biosynthesis (Figure 4.1). *ANS2/ANS3* mRNAs were confirmed to be overexpressed in the black (*irT*) seed coat at most stages (Figure 4.1) as shown previously (Ku, 2009).

The seed coat palisade cells of black (*irT*) soybean are unusual compared to those of other model plants used for the study of anthocyanin biosynthesis (e.g. *Arabidopsis*, Maize, and *Petunia*) because black (*irT*) soybean palisade cells synthesize both anthocyanins and proanthocyanidins (PAs). ANR and LAR

Figure 4.1. Transcript profiles from black (iRT) and brown (irT) seed coats during development measured by qRT-PCR. Black (iRT) soybean (black bars) and brown (irT) soybean (white bars). Student's t test significant at $P < 0.01$ (**), student's t test significant at $P < 0.05$ (*).

enzymes have been identified to catalyze the conversion of cyanidin to epicatechin and leucocyanidin to catechin, respectively, which are the putative initial biochemical steps of PA biosynthesis (Tanner et al., 2003; Xie et al., 2003). To investigate how a seed tissue may coordinate the biosynthesis of these parallel pigment pathways, the expressions of putative PA genes *ANR1* [GenBank:JF433915] and *LAR1* [GenBank:JF433916] were examined in relation to anthocyanin gene expressions. qRT-PCR demonstrated *ANR1* expressions to decrease throughout seed coat development in both genotypes and to be almost undetectable by the stages of *UGT78K1* overexpressions and anthocyanin biosynthesis (Figure 4.1). By contrast, *LAR1* expressions were downregulated at the stages of anthocyanin biosynthesis in the black (*iRT*) seed coat (Figure 4.1). 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.

4.3.2 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

Figure 4.2. Anthocyanin accumulations in black (*iRT*) and brown (*irT*) soybean seed coats during seed development. The fresh weight stages of seed development are indicated. (A) Seed color phenotypes during development. (B) Total amounts of anthocyanins in the seed coat of black (*iRT*) soybean during seed development. No anthocyanin absorbance was measured from brown (*irT*) seed coat extracts.

The structures of anthocyanins from only a few (Korean) black soybean varieties have been described (Choung et al., 2001; Lee et al., 2009) and information regarding other phenolic compositions may be limited to proanthocyanidins (PAs), where acid-catalyzed hydrolysis of black (*iRT*) and brown (*irT*) seed coats showed the PAs to be 3',4'-hydroxylated (Todd and Vodkin, 1993). 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 with decreasing expression during mid- to late-development (Ranathunge et al., 2010), and the metabolism gene *DFR1* were ensured to be equivalent between black (*iRT*) and brown (*irT*) tissues (Appendix B).

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 cyanic 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 4.1). 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 4.3. 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 (Downey et al., 2003; Kennedy and Jones, 2003). 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 (Appendix C, Panel A – Panel C). Reaction with DMACA reagent demonstrated similar amounts of soluble PAs from both black (*iRT*) and brown (*irT*) seed coats ($P=0.08$) (Appendix C, Panel F). Further extraction of seed coat residues at 50°C in the presence of acid and phloroglucinol yielded only low levels of epicatechin-

phloroglucinol adduct, epicatechin terminal units, and several unidentified compounds (Appendix C, Panel B), and acid catalyzed cleavage of seed coat

Table 4.1. HPLC-DAD analysis of anthocyanins from the seed coat of black (iRT) soybean variety Clark.

Peak	Rt (min)	Area (%) (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	Cyanidin
10	9.7	2.0	Diosmetinidin

*For chemical structures see Figure 4.3.

Figure 4.3. Anthocyanins from the seed coat of black (*iRT*) soybean variety Clark. Numbers correspond to chromatographic peaks identified by HPLC-DAD (see Table 4.1).

residues at 95°C identified relatively high, but equivalent amounts of 3',4'-hydroxylated solvent-insoluble PAs (Appendix C, Panel E).

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 4.2). Twenty-eight compounds were detected from the black (*iRT*) seed coat (Table 4.2); 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 4.2); 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 4.4.

The 14 compounds exclusive to the black (*iRT*) seed coat were anthocyanins (8 compounds), procyanidins (4 compounds), and unknown compounds (2 compounds) (Table 4.2). HPLC-MS/MS (Table 4.2) confirmed the identity of

Table 4.2. 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)	Genotype ^a
1	Unknown 1	413	183, 115	1.4	B
2	Unknown 2	381	184, 125	1.6	<i>irT</i>
3	Unknown 3	183	-	1.7	<i>iRT</i>
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	<i>irT</i>
11	Unknown 10	331	248	4.2	<i>irT</i>
12	Procyanidin trimer 1	867	579, 309, 299	4.7	<i>iRT</i>
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	<i>iRT</i>
17	Procyanidin trimer 3	867	579	6.6	<i>iRT</i>
18	Epicatechin*	291	-	6.9	B
19	Cyanidin-3-O-galactoside* Daidzein O-hexoside	449	287	7.1	<i>iRT</i>
20	malonylated	503	417	7.4	<i>irT</i>
21	Unknown 12	481	319	7.4	<i>irT</i>
22	Cyanidin-3-O-glucoside*	449	287	7.5	<i>iRT</i>
23	Petunidin-3-O-glucoside*	479	319, 303	8.0	<i>iRT</i>
24	Unknown 13	341	278, 111	8.1	<i>irT</i>
25	Pelargonidin-3-O-glucoside*	433	-	8.3	<i>iRT</i>
26	Peonidin-3-O-glucoside*	463	301, 286	8.7	<i>iRT</i>
27	Procyanidin dimer 2	579	409, 291	9.2	B
28	Unknown flavonol 1	331	-	9.0	<i>irT</i>
29	Procyanidin trimer 4	867	579, 699	9.2	<i>iRT</i>
30	A-type procyanidin dimer	593	-	9.7	<i>iRT</i>
31	Unknown anthocyanin 1	317	-	9.6	<i>iRT</i>
32	Unknown flavonol 2	615	-	9.6	<i>irT</i>
33	Cyanidin*	287	-	10.0	<i>iRT</i>
34	Unknown 14	487	-	10.8	B
35	Diosmetinidin*	285	-	10.3	<i>iRT</i>
36	Unknown benzoic acid 1	633	331, 315	11.0	<i>irT</i>
37	Quercetin-3-O-glucoside*	465	303	11.0	B ^b

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

^aBlack soybean (*iRT*), brown soybean (*irT*), both (B).

^bTrace amounts in *iRT*.

Figure 4.4. Distribution of compound classes from black (*iRT*) and brown (*irT*) soybean seed coats identified by LC-MS/MS. *iRT*, black bars; *irT*, white bars; common to both genotypes, grey bars.

anthocyanins found by HPLC-DAD (Table 4.1). 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 trimer 1, 2, and 4) and the A-type procyanidin dimer were exclusive to the black (*iRT*) seed coat (Table 4.2).

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 4.2).

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.

4.3.3. 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 (Schmutz et al., 2010), 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 (Ku, 2009). 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 (Appendix B).

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 (Appendix D).

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%) (Appendix E). 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 4.3).

A BLASTP search of the soybean genome sequence Glyma1 (www.phytozome.net/soybean) using the conserved plant secondary product glycosyltransferase signature sequence (PSPG box) (Hughes and Hughes, 1994)

Table4.3. Putative phenylpropanoid/flavonoid gene sets upregulated more than 2-fold in the seed coats of black (IRT) soybean compared to the brown (IRT) isolate^{a,b}

Gene Family	Probe Sets	Name	Target Description	Black/ brown fold change	SAM	
					P-value	d- score
Phenylpropanoid/ Flavonoid pathway	GmaAfx 80720 1 S1_at	DFR2*	Dihydroflavonol -4-reductase	62 45	0 000002	24 70
	GmaAfx 42116 1 S1_at	CHS4*/ CHS5*	Chalcone synthase (LOC732575)	10 99	0 000020	8 45
	GmaAfx 42116 1 S1_x_ at			8 06	2 62E-05	7 12
	Gma 1163 1 S1_at	ANS2*/ ANS3*	Anthocyanidin synthase	6 77	0 000018	8 77
	Gma 17141 1 S1_at	F3H-like	Moderately similar to A thaliana putative flavonone 3- β - hydroxylase clone U20744 (74%)	6 43	0 004849	2 03
	Gma 7423 2 S1_a_at	4CL-like	4-coumarate- CoA ligase-like protein	2 80	0 005815	1 96
	Gma 5621 1 S1_at	G4DT*	Pterocarpan 4- dimethylallyltra nsferase	2 36	0 009232	1 80
Glycosyltransferase	Gma 8472 1 S1_at	4CL2*	4-Coumarate- CoA ligase 2	2 27	0 000108	4 60
	GmaAfx 71999 1 S1_at	UGT78K 2	Glycosyltransfe rase	3 09	0 000124	4 50
	Gma 1002 2 S1_at	UGT78K 1*	UDP- glucose flavono id 3-O- glucosyltransfer ase	2 81	0 000229	4 03
O-methyl-transferase	Gma 9647 1 S1_at	OMT-like	O- methyltransfera se, putative	2 58	0 000098	4 74
	GmaAfx 57777 1 S1_at	OMT5	Caffeoyl-CoA O- methyltransfera se 5	2 48	0 000157	4 33
Glutathione S-transferase	GmaAfx 71212 1 A1_at	GST26	Glutathione S- transferase	234 11	0 000003	21 22
	Gma 5139 1 S1_at	GST21	Glutathione S- transferase	2 05	0 000656	3 09

^aExpression values were obtained using RMA (Irizarry et al , 2003)

^bSee Appendix D for the complete list of upregulated genes

*Previously characterized flavonoid biosynthesis genes

identified 214 annotated Family 1 glycosyltransferases (UGTs)(Appendix F). 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 *UGT78K1* (Kovinich et al., 2010) was upregulated more than 2.8-fold and a second uncharacterized glycosyltransferase, which we named *UGT78K2* in accordance with UGT nomenclature (Mackenzie et al., 1997), was upregulated more than 3-fold (Table 4.3).

A search of the soybean genome sequence with the conserved OMT-domain sequence (PLN02589; NCBI) and the ontology ‘O-methyltransferase’, identified 84 annotated OMTs (Appendix G). 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 4.3).

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 4.4; Appendix H). 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%) (Appendix I). 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

Table 4 4 Putative phenylpropanoid/flavonoid gene probe sets downregulated more than 2-fold in the black (iRT) compared to the brown (iRT) soybean seed coat^{a,b}

Gene Family	Probe Sets	Name	Target Description	Black/brown fold change	SAM	
					P-value	d-score
Phenylpropanoid/Flavonoid pathway	Gma 17605 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
	Gma 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
	Gma 4340 2 S1_a_at	Unknown	Moderately similar to P trichocarpa flavonoid O-methyltransferase-related (73 9%)	0 462	0 004180	-2 08
	Gma 10949 1 S1_s_at	Unknown	Moderately similar to A thaliana 3-chloroallyl aldehyde dehydrogenase/aldehyde dehydrogenase (NAD)/coniferyl-aldehyde dehydrogenase (ALDH2C4) (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

case of the *CHS1* (Table 4.4). 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 4.4).

Of the differentially regulated probe sets, 12 represented transcription factor genes, 8 were upregulated and 4 downregulated (Appendix D and Appendix I, 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) (Song et al., 2004). Nineteen differentially-regulated probe sets were associated with genes located on chromosome 9 of the soybean genome sequence Glyma1 (Appendix J). 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 4.5).

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

Figure 4.5. Genomic organization of differentially regulated genes from chromosome Gm09 of the Glyma1 sequence. (A) All differentially expressed genes were located in a 5.16 Mb region of the distal end of chromosome Gm09. (B) Enlargement of the 5.16 Mb region showing the locations of the upregulated genes (blue) and downregulated genes (red) relative to sequence-based markers BARC-025669-04989 and Sat_293 (black) that are situated upstream and downstream, respectively, nearest the R locus in the Glyma1 sequence. Chromosome positions of differentially regulated genes are listed in Table SX. Chromosome positions of BARC-025669-04989 (Gm09:41,620,379..41,620,751) and Sat_293 (Gm09:42,848,788..42,849,068).

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* (Appendix K). 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 (Appendix K).

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.

4.3.3 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 (Tohge et al., 2005). 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 4.6). In addition to 7 previously characterized soybean flavonoid genes, several uncharacterized genes

Figure 4.6. Summary of flavonoid/phenylpropanoid gene and metabolite accumulations from black and brown soybean seed coats*. Transcripts upregulated and metabolites exclusive to the black (*iRT*) seed coat are shown in blue. Transcripts downregulated and metabolites absent in the black (*iRT*) seed coat but present in the nearly-isogenic brown (*irT*) seed coat are shown in red. 4CL, 4-coumarate-CoA ligase; OOMT, orcinol O-methyltransferase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; UF3GT, UDP-glucose:flavonoid 3-O-glycosyltransferase; AOMT, anthocyanin O-methyltransferase; GST, glutathione S-transferase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; MATE, multidrug and toxic extrusion protein; IFR, isoflavone reductase; G4DT, pterocarpin 4-dimethylallyltransferase. *For the complete lists of differentially regulated transcripts see Appendix D and Appendix H, and for a complete list of differentially accumulated metabolites see Table 4.2.

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 4.6).

Anthocyanins in the seed coat of black (*iRT*) soybean are glycosylated at the 3-position of the C-ring (Figure 4.4). The glycosyltransferase UGT78K2 is assigned to code a UDP-glucose:flavonoid 3-O-glycosyltransferase (UF3GT) protein (Figure 4.6) and may have functional redundancies in anthocyanin biosynthesis with the previously characterized UGT78K1 (Kovinich et al., 2010), which was also upregulated in the black (*iRT*) seed coat (Table 4.3). Two anthocyanins (petunidin-3-O-glucoside and peonidin-3-O-glucoside) and one deoxyanthocyanidin (diosmetinidin) from the black (*iRT*) seed coat are methylated at the 3-position of the B-ring (the 3'-position) (Figure 4.3). The two upregulated O-methyltransferases were assigned to code anthocyanin 3'-O-methyltransferase (AOMT) proteins (Figure 4.6) and may have redundant function in anthocyanin biosynthesis this tissue.

4.3.4 *UGT78K2 and OMT5 Code UDP-Glycose: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 4.3) and the upregulation paralleled the accumulation of anthocyanins with 3-O-glycosylated structures (Figure 4.2), suggesting the function of these genes as UDP-glycose:flavonoid 3-O-glycosyltransferases (UF3GTs). We have characterized *UGT78K1* previously and shown it to possess UF3GT activity towards anthocyanidins *in vitro* and *in vivo* (Kovinich et al., 2010). 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 4.7A). In assays containing UDP-glucose as the sugar donor and cyanidin as the acceptor, the

Figure 4.7. Purification of his-tagged UGT78K2 and determination of the reaction product and kinetics for cyanidin. (A) SDS-PAGE analysis of UGT78K2: total soluble protein from *E. coli* expressing UGT78K2 prior to induction with 100 mM

IPTG (lane 1), 24 h post-induction (lane 2); IMAC-purified UGT78K2 (lane 3). (B) The UGT78K2 enzyme reaction as revealed by HPLC-DAD chromatograms at 520 nm (C). HPLC retention times: cyanidin (Rt: 10.1 min); cyanidin 3-O-glucoside (Rt: 7.1 min). (D) UGT78K2 kinetics for the cyanidin acceptor substrate using 5 mM UDPG as a sugar donor. Points represent the mean of three assays $\pm$ the standard deviation.

recombinant enzyme transferred glucose to the 3-position of cyanidin to form cyanidin-3-O-glucoside (Figure 4.7B, 4.7C), 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]⁺¹ and the MS/MS fragment [287.3 cyanidin]⁺¹ corresponding to the loss of glucose (-162 amu).

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 4.7D) despite the enzymes having 93% amino acid similarity (Appendix L).

Methylation of the B-ring of anthocyanins has a reddening effect on flower color (reviewed by Tanaka et al., 2009) 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 4.3) and the upregulation paralleled the accumulation of 3'-O-methylated anthocyanins in this tissue (Figure 4.2), 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 4.8A). 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 4.8B, 4.8C), 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

Figure 4.8. Purification of his-tagged OMT5 and determination of the reaction product for cyanidin 3-O-glucoside. (A) SDS-PAGE analysis of OMT5: total soluble protein from *E. coli* expressing OMT5 prior to induction with 100 IM IPTG (lane 1), 24 h post-induction (lane 2); IMAC-purified OMT5 (lane 3). (B) The OMT5 enzyme reaction as revealed by HPLC-DAD chromatograms at 520 nm (C). HPLC retention times: cyanidin 3-O-glucoside (Rt: 7.3 min); peonidin 3-O-glucoside (Rt: 8.6 min).

loss of glucose (-162 amu).

To further investigate the function of 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 4.9) and expressions were compared to anthocyanin accumulation profiles throughout seed development (Figure 4.2). 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 4.9), the stages at which anthocyanins accumulate (Figure 4.2), and were low or almost undetectable at early stages of seed development in the black (*iRT*) seed coat and at all stages in the brown (*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.

Figure 4.9. Seed coat transcript profiles of *UGT78K2* and *OMT5* during seed development measured by qRT-PCR. Black (*iRT*) seed coat (black bars), brown (*irT*) seed coat (white bars). Student's *t* test significant at $P < 0.01$ (**), student's *t* test significant at $P < 0.05$ (*).

4.4 Discussion

The seed coat compositions of black (*iRT*) and brown (*irT*) soybean (*Glycine max*) were previously 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 phenylpropanoid content (Table 4.2). 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 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 4.4). 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 4.2).

The anthocyanins identified from variety Clark were mainly similar to those reported previously from various Korean varieties (Choung et al., 2001; Lee et al., 2009) however Clark was the first shown to contain a 3-deoxyanthocyanidin (diosmetinidin) in the seed coat (Table 4.2). 3-deoxyanthocyanidins are relatively rare intensely coloured pigments found in *Sorghum bicolor* in response to fungal attack (Snyder and Nicholson, 1990), and from the cotyledons of yellow soybean in response to UV-C treatment (Boveris et al., 2001). The biosynthesis of 3-deoxyanthocyanidins from flavonones has been shown to be a minor activity of a

DFR isogene in Sorghum (Liu et al., 2010). 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 4.3). This is almost double the number of probe sets upregulated by overexpression of the MYB transcription factor PAP1 in Arabidopsis (38 probe sets) (Tohge et al., 2005), 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) (Peel et al., 2009). 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 (Schmutz et al., 2010). 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*, *UGT78K2* and *UGT78K1*, *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 (Todd and Vodkin, 1996; Toda et al., 2002; Zabala and Vodkin, 2003; Zabala and Vodkin, 2005; Zabala and Vodkin,

2007; Kavinich et al., 2010). 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 4.6).

Transcriptome analysis has previously demonstrated a critical role of *CHS7* and *CHS8* genes for isoflavonoid biosynthesis in developing embryos of soybean (Dhaubhadel et al., 2007). 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 4.3). Interestingly, it was *CHS4* that was upregulated and *CHS1* and *CHS9* that were downregulated in the black (*iRT*) seed coat (Table 4.3 and Table 4.4, 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 (Todd and Vodkin, 1996). 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 4.6). 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 leucocyanidin to (+)-catechin *in vitro* (Tanner et al., 2003; Xie et al., 2003; Pang et al., 2007; Paolocci et al., 2007). 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 (Pang et al., 2007). Similarly, our metabolite data showed that soybean PAs lack catechin subunits (Appendix C) and that *LAR1* expressions (Figure 4.1) 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 4.2) 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 (Appendix C). 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 4.7). A UF3GT cDNA (*UGT78K1*) has recently been isolated from the black seed coat and functionally characterized (Kovinich et al 2010). *UGT78K2* was 93% similar to *UGT78K1* but possessed increased catalytic activity towards cyanidin (Figure 4.7D). The

identification of 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 4.3). *OMT5* was cloned and found to code an AOMT enzyme *in vitro* (Figure 4.8). 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 (Tohge et al., 2005) 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 Tanaka et al., 2009). 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 (Yang et al., 2010). 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 4.5). 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/phoein gene was commonly upregulated in the black (*iRT*) soybean seed coat and Arabidopsis seedlings overexpressing PAP1 (Tohge et al., 2005). 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 4.5). 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.

4.5 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-glucose:flavonoid-3-O-glucosyltransferase and anthocyanin 3'-O-methyltransferase proteins *in vitro*, respectively. Duplicate gene copies for several enzymes were overexpressed in

the black (*iRT*) seed coat suggesting that more than one isogene may have to be silenced to engineer seed coat color using RNA interference.

CHAPTER 5

Biochemical analyses to understand red-brown soybean seed coat color and the identification of proanthocyanidin genes

Adapted from:

Kovinich N¹, Saleem A³, Arnason JT², and Miki B². Submitted. Identification of two anthocyanidin reductase genes and three red-brown soybean accessions with reduced *ANTHOCYANIDIN REDUCTASE1* mRNA, activity, and proanthocyanidin amounts. *Plant Physiology and Biochemistry*.

¹Designed and conducted research, analyzed data, and wrote the manuscript for publication

²Obtained funding for the research and revised the manuscript

³Developed the HPLC-DAD method

5.1. Introduction

Flavonoids are plant secondary metabolites that have a variety of ecophysiological functions including inter-organism signalling, pathogen defense, and protection from abiotic stresses (Winkel-Shirley, 2001; Gould, 2004; Grotewold, 2006). Furthermore, metabolic engineering of flavonoids has become an important target for plant biotechnology as they provide agronomic traits to crops, health benefits to foods, and may be used to color commercial genetically modified materials to facilitate their identification and monitoring (Dixon, 2005; Butelli et al., 2008; Kovinich et al., 2011a).

In soybean (*Glycine max* (L.) Merr.), flavonoids including proanthocyanidin (PA, a.k.a. condensed tannin) and anthocyanin pigments can be synthesized in the seed coat (Figure 5.1). Six seed coat colors have been identified to associate

Figure 5.1. Flavonoid biosynthesis in the soybean seed coat.

with spontaneous mutations in genetic loci by classical genetics (*I*, *R*, *T*, *Wp*, *W1*, and *O*) (Bernard and Weiss, 1973). The presence or absence of flavonoid-based color in the seed coat has been associated with four alleles of the *I* locus (*I*, *I*^{*j*}, *I*^{*k*}, *i*); the *I* allele results in the absence of pigmentation in the seed coat, giving a yellow seed due to the color of the underlying embryo, the *I*^{*j*} and *I*^{*k*} alleles restrict pigmentation to the hilum and to a saddle-shaped region, respectively, and the *i* allele results in a fully pigmented seed coat (Bernard and Weiss, 1973).

Mutations *I*→*i* (or *I*^{*j*}→*i*) have been associated with deletions of *CHS4* or *CHS1* promoter sequences and result in the abolishment of an RNA interference mechanism that silences chalcone synthase genes (*CHS*) specifically in the seed coat, allowing the accumulation of *CHS7* and *CHS8* transcripts (Todd and Vodkin, 1996; Tuteja et al., 2004).

Black (*iRT*) or brown (*irT*) soybean lines that differ in alleles of the *R* locus differentially accumulate specific anthocyanins, PAs, isoflavones, and phenylpropanoids in the seed coat (Kovinich et al., 2011b). Alleles of the *R* locus are associated with the differential expressions of 20 flavonoid/phenylpropanoid isogenes (Kovinich et al., 2011b), suggesting that *R* encodes a regulatory gene.

Black (*iRT*) and imperfect black (*iRt*) seed coat color is associated with different alleles of the *T* locus. The *T* locus is pleiotropic controlling the type and abundance of flavonoid pigments in the seed coat and trichomes in addition to seed coat structural integrity (Woodworth, 1921; Buzzell et al., 1987; Todd and Vodkin, 1993). The recessive *t* allele for tawny pubescence is associated with

genetic polymorphisms that affect the function or expressions of the flavonoid 3'-hydroxylase (*F3'H1*) gene (Toda et al., 2002; Zabala and Vodkin, 2003).

Imperfect black (*W1*) and buff (*w1*) seed coat colors in an *iRt* background are associated with alleles of the *W1* locus, which also controls flower color (Bernard and Weiss, 1973). A 65-bp insertion in the flavonoid 3',5'-hydroxylase (*F3'5'H*) gene gives white (*w1*) flower color, while an uninterrupted *F3'5'H* sequence is associated with purple (*W1*) flower color (Zabala and Vodkin, 2007).

Pigmented soybeans carrying the *Wp* allele are black (*iRTWp*), whereas *wp* gives a lighter grayish (*iRTwp*) grain color (Zabala and Vodkin, 2005). Microarray analysis demonstrated that high levels of transcripts of the flavonone 3-hydroxylase gene (*F3H1*) were associated with purple (*Wp*) flower color, and low levels with pink (*wp*) color (Zabala and Vodkin, 2005).

Brown (*O*) and red-brown (*o*) grain color can be controlled by the *O* locus in an *irT* background (Bernard and Weiss, 1973). Anthocyanidin reductase (*ANR*) catalyzes the first committed biochemical step to PA biosynthesis in *Arabidopsis thaliana* and *Medicago truncatula* (Xie et al., 2003). Recently, a partial soybean sequence with 86% sequence identity to the *M. Truncatula ANR* gene mapped to the genomic region coding the *O* locus (Yang et al., 2010). However, the function of the putative *ANR* sequence was not investigated, nor was it determined whether there existed biochemical differences relating to PA biosynthesis in the brown (*O*) and red-brown (*o*) seed coats. In *Arabidopsis*, knock-out mutations in the *ANR* (a.k.a. *BANYULS*) gene result in the absence of PAs in the seed coat,

the temporary accumulation of cyanic pigments during early seed development, and a transparent testa (*tt*) of the dried grain (Albert et al., 1997).

Coloring soybeans with endogenous pigments such as anthocyanins has been proposed as a strategy to enable effective detection and monitoring of adventitious presence of genetically modified (GM) soybeans in non-GM grain shipments (Kovinich et al., 2011a). Recent studies aimed at elucidating the molecular mechanisms that control soybean seed coat color have identified late-stage pigmentation genes and biochemical differences associated with grain color. A UDP-glucose:flavonoid-3-O-glucosyltransferase (UF3GT; EC 2.4.1.115) gene (*UGT78K1*) was isolated from black soybean seed coat cDNA using homology-based methods, and its function was confirmed *in vitro* and by complementation of the Arabidopsis mutation *ugt78k2* (Kovinich et al., 2010). *UGT78K1*, a second UF3GT gene (*UGT78K2*), and anthocyanidin 3'-O-methyltransferase (AOMT) gene (*OMT5*) were found to be upregulated with anthocyanin biosynthesis in the seed coat of black soybean during seed development and to have low expression levels in brown soybean (Kovinich et al., 2011b). However, genetic and biochemical differences underlying brown versus red-brown grain color remained unexamined.

Here we employed non-targeted and targeted approaches to identify differences in metabolite compositions between brown and red-brown soybean seed coats. Liquid chromatography tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode revealed quantitative differences in flavan-3-ol, procyanidin, and other flavonoids. We also identified two ANR genes

(*ANR1* and *ANR2*) from soybean seed coat tissue and compared their expressions and activity in relation to seed coat color. We show that the three publicly available red-brown soybean varieties exhibit alterations in seed coat PA content, *ANR1* transcript profiles and/or recombinant enzyme activity. Based on our results, we suggest that the manipulation of *ANR1* gene expressions or activity may be a rationale approach for engineering red-brown soybean color, which would ultimately enable the visual identification of GM grains.

5.2. Materials and Methods

5.2.1. Chemicals

Cyanidin was purchased from Indofine (Somerville, NJ, USA), NADPH from Fisher Scientific (Ottawa, ON, CAN), (-)-epicatechin, procyanidin B2, cyanidin 3-O-galactoside, and the 3-O-glucosides of cyanidin, quercetin and isorhamnetin were purchased from Extrasynthese (Lyon, FR). All chemicals were of the highest available purity.

5.2.2. Plant materials, growth conditions, and nucleic acid isolation

Glycine max (L.) Merr.) varieties M100 and M11 were obtained from the Agriculture and Agri-Food Canada Soybean EMS-mutagenesis collection 2005 (M. Morrison, Eastern Ontario Oilseed Research Centre, Ottawa), varieties Clark (ir; PI547438), Soysota (r17; PI 548417), Kuro Daizu (KD; PI 81041 -1) and Agate (Ag; PI 548296) were obtained from the U.S. Department of Agriculture

Soybean Germplasm Collection (Agricultural Research Service, University of Illinois at Champaign-Urbana). Seeds were germinated in vermiculite and transferred to soil after one week. Plants were grown in a Conviron E15 cabinet under 16/8 h light/dark at 25 °C/20 °C for 1 month, following 12/12 h light/dark at 25 °C/20 °C to promote reproductive development. Pods were harvested into crushed ice. Seed coats were dissected from pod and embryo material, immediately frozen in liquid nitrogen, lyophilized, and stored at -80 °C. DNA was isolated from trifoliolate unexpanded leaves using a kit (QIAGEN). RNA was isolated from seed coats as described (Wang and Vodkin, 1994), then washed using the RNeasy Plant Mini Kit (QIAGEN) to remove impurities.

5.2.3. Metabolite analysis and LC-MS/MS

For measurement of total soluble and insoluble PAs, lyophilized seed coats (400 – 500 mg FSW) were ground to a fine powder in liquid nitrogen, extracted (10 mg mL⁻¹) with 70% acetone (v/v), 0.1% ascorbic acid (w/v) in HPLC grade water, and analyzed as described previously (Li et al., 1996). For compound identification, 10 – 12 mg lyophilized seed coats were extracted (20 µL mg⁻¹) with MeOH:H₂O:HCO₂H (80:15:5, v/v) on ice with shaking (250 rpm) for 16 h. The supernatant was filtered through Teflon (0.2 µm; Chromatographic Specialties) and 5 µL aliquots were analyzed by LC-MS/MS in scanning mode as described previously (Kovinich et al., 2011b). For compound quantification, 2 µL aliquots were analyzed by LC-MS/MS in multiple reaction monitoring (MRM) mode in positive polarity at unit resolution. The system consisted of a 1200 series

Agilent liquid chromatograph with high performance autosampler (model G1376B), binary pump (model G1312A), column thermostat (model G1316A), and Peek 0.12 mm ID lines from column inlet onwards till tri-way splitter of a 3200 QTRAP with Peek 0.17 mm ID x 60cm lines on splitter outlet.

For MRM, two Q1 (parent) and Q3 (product) transitions were developed per authentic standard. The most sensitive transition was used for quantification and the second for authentication of identity. For quantitative optimization, authentic standards ($10\text{-}20\text{ }\mu\text{g mL}^{-1}$ dissolved in methanol) were infused into the turbo ion source using an electrospray probe at a flow rate of $10\text{ }\mu\text{g mL}^{-1}$. Flow Injection Analysis (FIA) was applied to optimize source conditions. LC conditions consisted of a linear gradient of 32-70% MeOH in 5% HCO₂H over 22 min at a flow rate of 1 mL min^{-1} . The column (Luna C18 (2), 150x 4.6 mm, 5 μm particle size, (Phenomenex Inc., CA, USA) was washed by ramping up the MeOH % from 70-100% for 8 min. The initial conditions were brought back in 0.1 min and the column was equilibrated for 6 min before next injection.

5.2.4. Cloning ANR1 and ANR2 cDNAs

By searching the GenBank *Glycine max* EST collection using the BLASTn algorithm and the *Lotus corniculatus ANR1-1* sequence as a query, forward and reverse gene-specific primers (AEV1F, AEV1R, AB4F, AEB4R) were designed to amplify partial ANR sequences from seed coat cDNA using 5' and 3' RACE (CLONTECH, CA, USA), respectively. The resulting partial cDNA fragments were cloned into the TOPO-TA vector (Invitrogen, ON, Canada) and sequenced. The

full-length *ANR1* coding region was then amplified from seed coat cDNA by end-to-end PCR using primers AHF/AHR and the Pfx50 DNA polymerase (Invitrogen, ON, Canada). The *ANR2* cDNA was similarly cloned using primers designed based on the Glyma08g06640 sequence. The resulting 1108 bp (*ANR1*) and 1017 bp (*ANR2*) amplicons were cloned into the *NdeI* and *BamHI* sites of the pET-14b vector (Novagen, ON, Canada) and sequenced to confirm their identities. Primers for this study are listed in Appendix M.

5.2.5. Cloning *ANR1* genes

ANR1 sequences were amplified from genomic DNA by PCR using primers OGF/OGR and the Pfx50 DNA polymerase (Invitrogen), cloned into the *BamHI* and *EcoRI* sites of the pUC19 vector, and 5 clones per variety were sequenced.

5.2.6. Phylogenetic analyses

A multiple alignment of the deduced amino acid sequences was performed using MAFFT (Kato et al., 2009). Node support was estimated by parsimony bootstrap analysis (1000 bootstrap replicates, 10 random addition sequences per bootstrap replicate with tree bisection-reconnection branch swapping, limited to a maximum of 10000 trees) using PAUP 4.0b10 (Sinauer Associates, MA, USA).

5.2.7. RT-PCR and qRT-PCR

RT-PCR and qRT-PCR were performed essentially as described previously (Kovinich et al., 2011b). Briefly, for RT-PCR, RNA samples (3 µg) were treated

with DNase I (Amplification grade, Invitrogen) and first-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. For quantitative RT-PCR (qRT-PCR), cDNA template was prepared as described above and 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 iQ SYBR Green Supermix (BioRad, ON, Canada). qRT-PCR of each target gene for each seed coat sample was performed in triplicate on cDNA samples or untreated RNA samples. qRT-PCR data and PCR efficiencies were analyzed using the Opticon Monitor 3 software (BioRad, ON, Canada). 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. RT-PCR and qRT-PCR experiments were performed in triplicate. Primers used in this study can be found in Appendix M.

5.2.8. Recombinant protein expression in Escherichia coli

The full-length *ANR1* and *ANR2* coding regions were fused in-frame with the pET-14b vector (above), transformed into the expression host *E. coli* BL21(DE3) pLysS (Novagen), and single colonies were selected for production of recombinant proteins. Growth, expression, and purification of soluble recombinant proteins by ion metal-affinity chromatography were performed essentially as described (Kovinich et al., 2010) with the exception that 1 L of *E. Coli* were routinely cultured to have enough recombinant protein for kinetics analysis. 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 a protein assay kit (Bio-Rad Laboratories Inc.; <http://www.bio-rad.com>).

5.2.9. Recombinant enzyme assays and kinetics

To measure recombinant enzyme activity for cyanidin, the concentrations of cyanidin were varied from 3 to 400 μ M, and 2 mM NADPH was used as a hydride donor. Enzyme assays (total volume 500 μ l) consisted of 75 μ g of recombinant enzyme, cyanidin and 2 mM NADPH in buffer (50 mM MES pH 5) and were incubated at 45 °C for 60 min. Gentle agitation (100 rpm) and occasional vortexing (15 – 20 min intervals) were provided to minimize precipitation. The amount of each reaction product was determined by comparison of integrated peak areas of epicatechin to the corresponding standard curve analyzed on the same day. Standards were processed in the same manner as enzyme reactions with the exception that the recombinant enzyme was boiled for 10 min prior to incubation. Standard curves were verified to be linear ($R^2 > 0.99$) over the range investigated using Chem32 software (Agilent Technologies Inc., Montreal QC, Canada). All reaction solutions were brought to 45 °C prior to the addition of cyanidin. Cyanidin stocks were 10 mM in MeOH and stored at -80 °C. Reactions were stopped by vortexing in EtOAc (1 mL) for 60 s, followed by removal of the organic phase, which was then evaporated under a stream of nitrogen gas. The residue was resuspended in 100 μ l of LC-MS grade MeOH. All reactions were

filtered through Teflon (0.2 µm; Chromatographic Specialties) and 20 µl injections were analyzed by HPLC-DAD as described previously (Kovinich et al., 2010).

5.3. Results

5.3.1. *The seed coats of three red-brown soybean varieties have reduced amounts of soluble proanthocyanidins (PAs) compared to brown soybean*

In *Arabidopsis*, knock-out mutations of the anthocyanidin reductase (*ANR*, a.k.a. *BANYULS*) gene result in the loss of proanthocyanidins (PAs) in the seed coat and the temporary accumulation of cyanic pigments (Devic et al., 1999). As the red-brown soybean also exhibits a cyanic grain color phenotype, we wanted to determine whether this may be associated with reduced PA amounts compared to brown soybean. The total amounts of soluble and solvent-insoluble PAs were measured from the seed coats of 3 brown, 2 red-brown, and 1 red-brown saddle variety (listed in Table 5.1) using previously described methods (Li et al., 1996). The 3 red-brown varieties that were available from public collections had similar seed coat luster (shiny), seed size (large), and relative timing of seed maturation (early) as the brown variety M100 (Table 5.1). However, brown soybean seed color phenotypes range from dark- to light-brown. To better represent the range of brown seed coat color intensities, the light-brown variety Clark (ir) and mid-brown variety Soysota (r17) were selected to serve as comparators to the red-brown varieties, in addition to M100 which has a dark-brown seed coat. Varieties Kuro Daizu (KD) and M11 (a line produced from EMS mutagenesis of Linia N145) had red-brown color distributed across the entire seed coat surface,

Table 5.1. Soybean lines, alleles, and phenotypic properties of their seeds.

Variety	Abbreviated Name	Alleles ¹	Seed Coat Color	Seed Coat Luster	Seed Size	Relative Timing of Seed Maturation	Source/Origin
Clark	ir	<i>irTO</i>	Light brown	Dull	Medium	Late	i-mutation in L11, found in 1965 at Urbana, USA Collected in 1910 in Italy EMS-mutagenized Linia N145 (PI 522192 A), collected in 2005 at Ottawa, Canada Collected at Hokkaido, Japan, donated in 1951 Collected in 1929 at Hokkaido, Japan EMS-mutagenized Linia N145 (PI 522192 A), collected in 2005 at Ottawa, Canada
Soysota	r17	<i>irTO</i>	Mid-brown	Mod-erate	Small	Mid	
Linia N145	M100	Unknown	Dark brown	Shiny	Large	Early	
Agate	Ag	<i>*rTo</i>	Red-brown saddle	Shiny	Large	Early	
Kuro Daizu	KD	<i>irTo</i>	Red-brown	Shiny	Large	Early	
Linia N145	M11	Unknown	Red-brown	Shiny	Large	Early	

¹All lines used in this study were homozygous for the *I*, *R*, *T* and *O* alleles listed.

whereas variety Agate (Ag) with red-brown saddle (*krTo*) had pigmentation confined to a saddle-shaped region that encompassed 60-71% of the seed coat surface area (not shown).

Reaction of seed coat extracts with DMACA reagent showed that red-brown varieties KD and M11 had 46-71% of the amount of soluble PAs compared to the brown varieties (Figure 5.2A). The red-brown saddle variety Ag had 32-45% soluble PAs compared to brown varieties (significantly less than what may be attributed to the incomplete (saddle-shaped) distribution of pigments across 60-71% of the seed coat surface area) (Figure 5.2A). By contrast, the amounts of solvent-insoluble PAs measured following acid-catalyzed hydrolysis of seed coat residues were not found to differ significantly between red-brown and brown varieties (Figure 5.2B).

PA and anthocyanin branchpoint enzymes use the same flavonoid intermediate, cyanidin, as a substrate, suggesting the potential for competition between these pathways. To investigate whether the decrease in soluble PA amounts may be associated with an increase in the levels of cyanic pigments (i.e. anthocyanidins or anthocyanins), seed coats were extracted with aqueous methanol and cyanic pigments were quantified by photospectroscopy in comparison to the commercial standard cyanidin (Figure 5.2C).

Red-brown seed coats had an increase in cyanic pigment amounts, however the increase ($0.5\text{--}1.4\text{ mg g}^{-1}$ lyophilized seed coat (LSC)) was much less than the decrease in soluble PA amounts ($15.3\text{--}50.4\text{ mg g}^{-1}$ LSC) (compare Figure 5.2C to Figure 5.2A).

Figure 5.2. Flavonoid amounts from the seed coats of brown (O) and red-brown (o) soybean varieties expressed in units mg flavonoid per gram lyophilized seed coat (LSC) (A) Total soluble PAs by DMACA assay, (B) total insoluble PAs by acid-catalyzed hydrolysis, (C) cyanic pigments by photospectroscopy (Abs 520 nm), (D) individual flavonoids by LC-MS/MS in multiple reaction monitoring (MRM) mode. White bars represent brown (O) soybean varieties (ir, r17, and M100) and grey bars represent red-brown (o) varieties (Aq, KD, and M11). Values are mean and SD from three biological replicates.

To determine the identities of the differentially accumulated metabolites and to measure their amounts, we performed high performance liquid

chromatography-tandem mass spectroscopy (HPLC-MS/MS) in scanning and multiple reaction monitoring (MRM) modes, respectively, in comparison to authentic standards. Seven flavonoids had accumulated to different amounts in red-brown seed coats compared to the brown lines (Figure 5.2D). Red-brown seed coats had reduced amounts of (-)-epicatechin, procyanidin B2 (line M11), and an unknown procyanidin dimer (Figure 5.2D top panels), elevated amounts of the anthocyanins cyanidin-3-O-glucoside (C3G) and cyanidin-3-O-galactoside (C3Ga) (Figure 5.2D middle panels) and the flavonol quercetin-3-O-glucoside (Figure 5.2D bottom left). The red-brown lines Ag and KD, but not the EMS mutagenized line M11 had elevated amounts of an unknown compound that had the same m/z (479.0) but different chromatographic retention time as isorhamnetin-3-O-glucoside (Figure 5.2D bottom right panel).

Overall, these results demonstrated that red-brown soybean varieties had major reductions in the amount of soluble PAs in their seed coat compared to the brown varieties, accompanied by minor increases in specific anthocyanin, flavonol, and unknown metabolites.

5.3.2. Identification of *ANR1* and *ANR2* genes from the *G. max* seed coat

Yang et al (2010) recently mapped a putative anthocyanidin reductase (*ANR*) gene sequence to the genomic region coding the *O* locus (Weiss, 1970) and speculated that *O* may correspond to *ANR* (Yang et al., 2010). We have shown that red-brown soybean seed coats have reduced amounts of soluble PAs (Figure 5.2A); further suggesting the color phenotype may be associated with a

defect in the PA pathway. These results prompted us to identify the functional *ANR* gene sequence(s) from soybean to determine whether red-brown seed coat color may correspond to defects in the *ANR* gene(s).

As the Glyma1 soybean genome sequence (Schmutz et al., 2010); for sequence see www.phytozome.net/soybean) was not available at the time our study was initiated, *G. max* ESTs from the GenBank collection were searched with the tBLASTn algorithm using the sequence of every reported catalytically characterized ANR protein as a query in order to identify *G. max* ANR candidates (September, 2007). Two *G. max* ESTs (GenBank accessions EV264997 and BM092813) were identified by tBLASTn that had high identity (>80%) to ANR1-1 from *Lotus corniculatus* (LcANR1-1; ABC71332). 5'- and 3'-RACE using seed coat cDNA from brown soybean variety Clark (ir) as a template was performed to extend the EST sequence information, and revealed that both ESTs coded the same cDNA, which we named *ANR1* (JF433915). The full-length *ANR1* cDNA was 1324 bp and the deduced amino acid sequence had high identity (83%), and similar length to LcANR1-1 (337 residues vs. 338 for LcANR1-1).

Primers AHF and AHR designed to amplify from the 5'-coding region to the 3'-UTR of the *ANR1* cDNA (Appendix N) were used to amplify and clone the corresponding genomic regions from variety ir to gain insight into the structural organization of the *ANR1* gene in the *G. max* genome. The genomic *ANR1* sequence coded six exons and five introns (not shown), similar to the banyuls (*BAN*) gene from Arabidopsis (NC_003070). A BLASTn search of the Glyma1 soybean genome sequence using genomic *ANR1* as a query found it to be 100%

identical to the predicted gene Glyma08g06630 (www.phytozome.net/soybean), and 70% identical to a second predicted gene Glyma08g06640 (76% identical at the amino acid level), which we named *ANR2* (JN098512) due to its sequence similarity.

To determine the relationship of putative *G. max* ANR proteins with other catalytically characterized reductase epimerase dehydratase (RED)-superfamily proteins, we performed a phylogenetic analysis. The phylogenetic tree demonstrated that *ANR1* and *ANR2* were most closely related to ANR proteins than to other reductase-epimerase-dehydrogenase (RED) superfamily proteins (Figure 5.3). Gene expression analysis by RT-PCR demonstrated highly divergent expression patterns; with *ANR2* expressed at low levels in all organs, and *ANR1* expressed preferentially and highly in the seed coat (Figure 5.4) consistent with the identification of (-)-epicatechin and (-)-epicatechin-based procyanidins in the seed coat of brown soybean (Kovinich et al., 2011b).

To confirm the putative catalytic function of *ANR1* and *ANR2* proteins for the reduction of cyanidin to (-)-epicatechin, their open reading frames were separately cloned from brown soybean (ir) seed coat cDNA into the pET-14b N-terminal hexahistidine fusion tag expression vector. The resultant plasmids were transformed into BL21(DE3) pLysS *Escherichia coli*, expressed at 16 °C and the recombinant his-tagged proteins were purified from the soluble fraction by ion-metal-affinity chromatography (IMAC). Separation of the IMAC fractions by SDS-PAGE followed by staining with Coomassie Blue showed apparently pure

Figure 5.3. Phylogenetic tree of ANR and other reductase-epimerase-dehydrogenase (RED) superfamily proteins. Amino acid sequences of characterized enzymes from *Arabidopsis*, AtBAN (NP_176365), AtDFRL (CAP08805); *Camellia sinensis*, CsANR (AAT68773); *Cicer arietinum*, CalFR (Q00016); *G. max*, ANR1, ANR2, GmDFR1 (AAD54273), GmDFR2 (ABM64803), GmIFR1 (AAF17577), GmIFR2 (AAF17578); *L. corniculatus*, ANR1-1 (ABC71332), LAR1-1 (ABC71324), LAR2-1 (ABC71328); *L. japonicus*, PTR (BAF34844); *Malus x domestica*, MdANR (AAX12184); *M. truncatula*, ANR (AAN77735), DFR1 (AAR27014), LAR (CAI56327); *Phaseolus coccineus*, LAR (CAI56322); and *Vitis vinifera* ANR (BAD89742) were aligned using MAFFT (Katoh et al., 2009) and the phylogenetic tree was produced using Dendroscope (Huson et al., 2007). The scale bar represents 1.0 substitutions per site.

Figure 5.4. Transcript detection of ANR1 and ANR2 from Clark (ir) cDNA by RT-PCR with gene-specific primers.

recombinant enzymes with estimated molecular masses of ca. 38.7 kDa and 38.9 kDa for ANR1 and ANR2, respectively (Figure 5.5A). These values matched well with their respective calculated molecular masses (38.72 kDa for ANR1 and 39.30 kDa for ANR2) with the inclusion of the 2.18 kDa N-terminal tag.

Recombinant enzyme assays were conducted using NADPH as a hydride donor and cyanidin as an acceptor. Incubation of ANR1 or ANR2 recombinant enzymes with cyanidin and NADPH resulted in the production of (-)-epicatechin, as identified by the identical LC retention time (Rt: 6.9 min), parent ion ([M+] 291.4 m/z) and major ion fragment(s) (139.1 m/z and/or 123.1 m/z) compared to the authentic standard (Figure 5.5B). The boiled enzyme and bacteria expressing the corresponding empty vector could not catalyze this reaction (not shown).

In summary, two functional ANR genes were identified from the Glyma1 soybean genome sequence that exhibit different expression patterns, with *ANR2* being expressed at low levels in all organs, and *ANR1* preferentially and highly expressed in the seed coat. These results suggest that *ANR1* has a major role in PA biosynthesis in the soybean seed coat.

5.3.3. Red-brown soybean varieties M11 and KD have non-synonymous substitutions in the ANR1 gene that confer minor reductions in enzyme activity

Flavan-3-ol and PA contents from the seed coat of brown soybean were shown to be (-)-epicatechin and (-)-epicatechin-based, respectively (Kovinich et al., 2011b). Figure 5.2A demonstrated that the three publically available red-brown soybean varieties have reduced amounts of soluble PAs in the seed coat

Figure 5.5. Purification of his-tagged ANR1 and ANR2 and identification of reaction products. (A) SDS-PAGE analysis of ANR1 and ANR2: total soluble protein from *E. coli* expressing ANR1 prior to induction with 100 μ M IPTG (lane 1), 24 h post-induction (lane 2); purified ANR1 (lane 3); total soluble protein from

E. coli expressing ANR2 prior to induction with 100 μ M IPTG (lane 4), 24 h post-induction (lane 5), and purified ANR2 (lane 6). (B) ANR1 and ANR2 enzyme reactions as revealed by LC-MS/MS. LC retention times: cyanidin (Rt: 10.0 min); (-)-epicatechin (Rt: 6.9). The m/z 575.3 (lower right panel) corresponded to a cyanidin (Cy) dimer formed non-enzymatically, as indicated by its presence in control (boiled enzyme) and enzyme reactions at higher Cy concentrations.

compared to brown varieties. To determine whether reduced soluble PA amounts in the red-brown seed coat may be associated with sequence-based defects in the *ANR1* gene, the 3-kb genomic region coding *ANR1* from variety M11 was cloned using primers OGF/OGR (Appendix N) and compared to *ANR1* sequences from the 5 other varieties (Table 5.1). To ensure accurate sequence information, five individual clones per variety were sequenced with at least three times coverage.

Genomic *ANR1* sequences from all brown varieties were identical, and were the same as the predicted gene Glyma08g06630 from the reference soybean genome sequence Glyma1 (www.phytozome.net/soybean). By contrast, each red-brown variety had at least one sequence polymorphism compared to the *ANR1* sequence from brown genotypes (Table 5.2). Red-brown saddle variety Ag had a single base pair insertion in intron 1 at position 550. Red-brown variety KD had two non-synonymous substitutions; T to C in exon 3 at position 1269, and G to C in exon 4 at position 1957. These mutations corresponded to the predicted amino acid changes of V122A and G214A (Table 5.2) in conserved and non-conserved residues, respectively (Figure 5.6). Red-brown EMS-mutagenized variety M11 had the same G to C substitution in exon 4 at position 1957, and had

Table 5.2. Comparison of ANR1 genomic and amino acid sequences among brown (O) and red-brown (o) soybean varieties*.

Variety	Percent identity to ir (genomic DNA)	Mutation	Position	Amino acid substitution
Ir	100	None	N/A	N/A
r17	100	None	N/A	N/A
M100	100	None	N/A	N/A
Ag	99	A insertion	550 (intron 1)	N/A
KD	99	T → C	1269 (exon 3)	V122A
		G → C	1957 (exon 4)	G214A
M11	99	A → T	1870 (exon 4)	N185I
		G → C	1957 (exon 4)	G214A

*See Table 5.1 for a description of varieties.

NADPH

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ANR1-1r  MATIKPTGKKACVIGGSGFMASLLIKOLLEKGYAVNTTVRDPONTKKIPHLLALQSLGEL 60
ANR1-r17 MATIKPTGKKACVIGGSGFMASLLIKOLLEKGYAVNTTVRDPONTKKIPHLLALQSLGEL 60
ANR1-M100 MATIKPTGKKACVIGGSGFMASLLIKOLLEKGYAVNTTVRDPONTKKIPHLLALQSLGEL 60
ANR1-Ag  MATIKPTGKKACVIGGSGFMASLLIKOLLEKGYAVNTTVRDPONTKKIPHLLALQSLGEL 60
ANR1-KD  MATIKPTGKKACVIGGSGFMASLLIKOLLEKGYAVNTTVRDPONTKKIPHLLALQSLGEL 60
ANR1-M11 MATIKPTGKKACVIGGSGFMASLLIKOLLEKGYAVNTTVRDPONTKKIPHLLALQSLGEL 60
*****

ANR1-1r  NIFGADLTGEKDFDAPLAGCELVFQLATPVNFASEDPENDMIKPAITGVNLVLKACVRAK 120
ANR1-r17 NIFGADLTGEKDFDAPLAGCELVFQLATPVNFASEDPENDMIKPAITGVNLVLKACVRAK 120
ANR1-M100 NIFGADLTGEKDFDAPLAGCELVFQLATPVNFASEDPENDMIKPAITGVNLVLKACVRAK 120
ANR1-Ag  NIFGADLTGEKDFDAPLAGCELVFQLATPVNFASEDPENDMIKPAITGVNLVLKACVRAK 120
ANR1-KD  NIFGADLTGEKDFDAPLAGCELVFQLATPVNFASEDPENDMIKPAITGVNLVLKACVRAK 120
ANR1-M11 NIFGADLTGEKDFDAPLAGCELVFQLATPVNFASEDPENDMIKPAITGVNLVLKACVRAK 120
*****

ANR1-1r  GVKRVILTSSAAAVTINQLKGTDLVMDSESNWTDVEYLSLAKPPTWGYFASKALAEKAANK 180
ANR1-r17 GVKRVILTSSAAAVTINQLKGTDLVMDSESNWTDVEYLSLAKPPTWGYFASKALAEKAANK 180
ANR1-M100 GVKRVILTSSAAAVTINQLKGTDLVMDSESNWTDVEYLSLAKPPTWGYFASKALAEKAANK 180
ANR1-Ag  GVKRVILTSSAAAVTINQLKGTDLVMDSESNWTDVEYLSLAKPPTWGYFASKALAEKAANK 180
ANR1-KD  GVKRVILTSSAAAVTINQLKGTDLVMDSESNWTDVEYLSLAKPPTWGYFASKALAEKAANK 180
ANR1-M11 GVKRVILTSSAAAVTINQLKGTDLVMDSESNWTDVEYLSLAKPPTWGYFASKALAEKAANK 180
*****

ANR1-1r  FAENHIDLITVIPTLTGFSVTDDIPSSVGMAGSLITGNDFLINALKGMQLLSGSISIT 240
ANR1-r17 FAENHIDLITVIPTLTGFSVTDDIPSSVGMAGSLITGNDFLINALKGMQLLSGSISIT 240
ANR1-M100 FAENHIDLITVIPTLTGFSVTDDIPSSVGMAGSLITGNDFLINALKGMQLLSGSISIT 240
ANR1-Ag  FAENHIDLITVIPTLTGFSVTDDIPSSVGMAGSLITGNDFLINALKGMQLLSGSISIT 240
ANR1-KD  FAENHIDLITVIPTLTGFSVTDDIPSSVGMAGSLITGNDFLINALKGMQLLSGSISIT 240
ANR1-M11 FAENHIDLITVIPTLTGFSVTDDIPSSVGMAGSLITGNDFLINALKGMQLLSGSISIT 240
*****

ANR1-1r  HVEDICRAQIFVAEKESASGRYICCAHNTSVPELAKFLSKRYPQYKIPTEFDDCPSKAKL 300
ANR1-r17 HVEDICRAQIFVAEKESASGRYICCAHNTSVPELAKFLSKRYPQYKIPTEFDDCPSKAKL 300
ANR1-M100 HVEDICRAQIFVAEKESASGRYICCAHNTSVPELAKFLSKRYPQYKIPTEFDDCPSKAKL 300
ANR1-Ag  HVEDICRAQIFVAEKESASGRYICCAHNTSVPELAKFLSKRYPQYKIPTEFDDCPSKAKL 300
ANR1-KD  HVEDICRAQIFVAEKESASGRYICCAHNTSVPELAKFLSKRYPQYKIPTEFDDCPSKAKL 300
ANR1-M11 HVEDICRAQIFVAEKESASGRYICCAHNTSVPELAKFLSKRYPQYKIPTEFDDCPSKAKL 300
*****

ANR1-1r  IISSEKLVKEGFSFKYGIEEIIDOTLEYLKSKEGALNN 337
ANR1-r17 IISSEKLVKEGFSFKYGIEEIIDOTLEYLKSKEGALNN 337
ANR1-M100 IISSEKLVKEGFSFKYGIEEIIDOTLEYLKSKEGALNN 337
ANR1-Ag  IISSEKLVKEGFSFKYGIEEIIDOTLEYLKSKEGALNN 337
ANR1-KD  IISSEKLVKEGFSFKYGIEEIIDOTLEYLKSKEGALNN 337
ANR1-M11 IISSEKLVKEGFSFKYGIEEIIDOTLEYLKSKEGALNN 337
*****

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Figure 5.6. Alignment of *G. max* ANR1 proteins from brown (O) seed coat varieties (ir, r17, M100) and red-brown (o) seed coat varieties (Ag, KD, and M11) using the ClustalW program with default parameters. Mutations are shown with black background. Grey background represents amino acid residues that are conserved among all catalytically validated ANR proteins: (AtBAN (NP_176365), CsANR (AAT68773), *G. max* ANR1-ir, LcANR1-1 (ABC71332MdanR (AAX12184); *M. truncatula*, ANR (AAN77735); and *Vitis vinifera* ANR

(BAD89742). The putative NADPH binding site and the catalytic triad (Gomez et al., 2009) are represented by a thick black line above the sequences and boxed amino acids, respectively.

a unique substitution in exon 4 (A to T at position 1870) that conferred a predicted amino acid change of N185I (Table 5.2) in a conserved region (Figure 5.6).

To determine whether these amino acid substitutions altered enzyme activity, the respective *ANR1* ORFs from varieties KD and M11 were individually cloned into an N-terminal hexahistidine fusion tag vector for comparison of enzyme kinetics with ANR1 from brown soybean variety *ir* *in vitro*. Recombinant enzyme assays using NADPH as a hydride donor and cyanidin as an acceptor revealed minor reductions ($1.8 - 26.2 \text{ fmol epicatechin s}^{-1} \text{ ug}^{-1}$) in the activity of the ANR1 proteins from varieties KD and M11 compared to ANR1 from variety *ir* (Figure 5.7). Calculated K_m and V_{max} values are not given as the enzymes were intensely inhibited by cyanidin concentrations above $\sim 100 \text{ }\mu\text{M}$ (not shown).

These results suggest that reduced ANR1 activity could (at least in part) account for some of the reduction of soluble PAs in the red-brown seed coats of varieties KD and M11 (Figure 5.2A), however additional defects in PA biosynthesis would likely be required to account for the major reductions in soluble PA amounts.

Figure 5.7. *In vitro* kinetics of recombinant ANR1 enzymes from brown soybean variety ir (◇) and red-brown soybean varieties M11 (■) and KD (Δ). Activity was measured as amount of epicatechin produced per second per microgram enzyme.

5.3.4. Red-brown soybean seed coats of varieties Ag and KD have reduced *ANR1* transcript levels

To determine whether reduced soluble PA amounts may be associated with transcript levels of the ANR genes, we compared *ANR1* and *ANR2* transcript profiles between red-brown and brown lines by quantitative RT-PCR (qRT-PCR) using seed coat cDNA as a template. The 25-50 mg FSW stage of seed development was chosen as the developmental stage for qRT-PCR analysis because *ANR1* transcripts were previously shown to be at maximum levels at this stage in brown (*irTO*) variety Clark (*ir*) and its black (*irTO*) isoline (Kovinich et al., 2011b). Phosphoenol pyruvate carboxylase (*PEPC*) was used as an endogenous reference for normalization of the qRT-PCR measurements of target genes across seed coat samples, as *PEPC* has been used previously as a reference for gene expressions from pigmented and non-pigmented soybean seed coats, and is expressed at similar levels in a wide range of soybean tissues (Sugimoto et al., 1992; Tuteja et al., 2004; Kovinich et al., 2011b). The expressions of other genes situated at the branchpoint between PA and anthocyanin biosynthesis, (namely *UGT78K2* (HM591298) coding UF3GT (Kovinich et al., 2011b), *LAR1* (JF433916) putatively coding leucoanthocyanidin reductase (Kovinich et al., 2011b), and *ANS2/ANS3* (AY382829/AY382830) coding anthocyanidin synthases (Ku, 2009) were also measured to investigate for a relationship with seed coat color.

qRT-PCR analysis demonstrated that only *ANR1* and *ANS2/ANS3* were expressed at consistently high levels among all brown varieties, with the other

genes showing highly variant expression levels with no consistent correlation with seed color (Figure 5.8). The EMS-mutagenized variety M11 had similar *ANR1* expression levels compared to the brown varieties, however out of all the genes investigated, only *ANR1* expressions were significantly reduced in spontaneous red-brown mutants KD and Ag with the recessive *o* locus (Figure 5.8). These results suggest that reduced *ANR1* gene expressions have a role in the reduction of soluble PA content in the seed coats of naturally-occurring (spontaneous) red-brown varieties KD and Ag with the recessive *o* locus.

Figure 5.8. Seed coat transcript profiles of PA and anthocyanin branchpoint genes measured by qRT-PCR. PA genes *ANR1*, *ANR2* and *LAR1*, anthocyanin genes *UGT78K2* and *ANS2/ANS3*. Student's *t* test significant at $P < 0.05$ (*).

5.4. Discussion

The seed coats of yellow (*l*) soybean have reduced amounts of flavonoids compared to those of pigmented (*i*) genotypes due to differences in the expressions of chalcone synthase genes mediated by a post-transcriptional gene silencing mechanism (Todd and Vodkin, 1996; Tuteja et al., 2004). The seed coats of brown (*r*) soybean have reduced amounts of specific anthocyanins, PAs, isoflavones, and phenylpropanoids compared to black (*R*) soybean, due to differences in the expressions of a putative regulatory gene (Kovnich et al., 2011b). Here we have shown that three red-brown soybean accessions have major reductions in the amounts of soluble PAs compared to brown soybean lines (Figure 5.2A). A previous study demonstrated that red faba beans (*Vicia faba*) have reduced PA content compared to the brown variety with normal flower color (Martin et al., 1995). Similarly, red to orange-brown and dark-red varieties of cowpea (*Vigna unguiculata*) had reduced PA content compared to their beige comparators (Price et al., 1980). These results have significant implications for the metabolic engineering of seed color for the purposes of marking commercial transgenic legume grains; they suggest that cyanic (e.g. red-brown) color may be engineered to mark genetically modified (GM) grains by the suppression of PA biosynthesis in the seed coat. Further, the reduction of PA amounts would be favourable for post-harvest processing of the GM grain, as PAs bind proteins and other metabolites, interfering with their extraction.

Our LC-MS/MS analysis of red-brown seed coat metabolites demonstrated that the major reduction in soluble PA content was accompanied by minor

increases in the amounts of anthocyanin pigments (C3G and C3Ga) (Figure 5.2). However, red color in beans may not always be associated with increased anthocyanin content, as commercial dark red kidney beans (*Phaseolus vulgaris* L.) were identified to consist mainly of flavonol-glycosides and PAs with no anthocyanins identified by HPLC-DAD (Beninger and Hosfield, 1999). Other pigments identified from soybean seed coats that could provide a cyanic color include epiflavan-3-ol-anthocyanin conjugates, such as catechin-cyanidin-3-O-glucoside identified from the seed coat of black soybean cultivar Cheongja 3 (Lee et al., 2009), or 3-deoxyanthocyanidins, such as diosmetinidin identified from the seed coat of black soybean variety Clark (Kovinich et al., 2011b). However, these compounds were not identified as major pigments from the red-brown soybean seed coat by HPLC-DAD measuring absorbance at 520 nm (not shown) or as metabolites that have accumulated to different amounts compared to brown soybean by LC-MS/MS. Our LC-MS/MS results demonstrated that 3-O-glycosylated anthocyanins (C3G and C3Ga) were the main cyanic pigments present at significantly higher levels in the seed coat of red-brown soybean compared to the brown lines (Figure 5.2D).

Anthocyanin and PA biosynthesis are linked by the requirement for the common flavonoid intermediates, anthocyanidins; the PA enzyme anthocyanidin reductase (ANR) and the anthocyanin enzyme UDP-glucose:flavonoid-3-O-glycosyltransferase (UF3GT) both utilize cyanidin as a substrate (Figure 5.1). This suggests the possibility that the inhibition of PA biosynthesis due to a defect in ANR would render more cyanidin substrate available to UF3GT for

anthocyanin biosynthesis, and could result in a red-brown seed coat phenotype. In *Arabidopsis*, mutations causing loss-of-function of the *ANR* gene (a.k.a. *BANYULS*) resulted in the loss of PAs in the seed coat and the temporary accumulation of red pigments (not formally identified as anthocyanins) during seed development (Devic et al., 1999). In soybean, our results demonstrated that defects in the *ANR1* gene were accompanied by major reductions in PA content and minor elevations in the amounts of anthocyanins in the red-brown seed coat (Figure 5.2D). Although gene expression analysis demonstrated that *ANR1* was highly expressed in the seed coat compared to *ANR2* (Figure 5.4), it remains unclear whether the differences observed in gene expression or enzyme activity are sufficient to explain the phenotypic variation between brown and red-brown seed coats. Recently, a partial soybean sequence with 86% sequence identity to the *M. truncatula* *ANR* gene mapped to the genomic region coding the *O* locus (Yang et al., 2010). The genes coding *ANR1* (Glyma08g06630) and *ANR2* (Glyma08g06640) were located in the same region of the Glyma1 soybean genome sequence, further supporting that either may encode the *O* gene. However, further analysis, such as gene complementation or gene suppression will be required to substantiate that defects in the *ANR1* gene are responsible for red-brown seed coat color and that *ANR1* may be the *O* gene.

5.5. Conclusion

A better understanding of the biochemistry of red-brown seed coat color and the role of the soybean *ANR1* gene in PA biosynthesis has been achieved in this

work. We confirmed the *in vitro* catalytic function of two ANR genes isolated from the seed coat of brown soybean. Of the two genes, ANR1 was preferentially and highly expressed in the seed coat, suggesting a principal role in PA biosynthesis in this tissue. ANR1 exhibited reduced gene expression profiles in the seed coat of red-brown soybean relative to brown soybean and/or minor reductions in recombinant enzyme activity. Further analysis will be required to determine whether the differences observed in *ANR1* gene expression or enzyme activity are sufficient to explain the phenotypic variation between red-brown and brown seed coats. Based on these results, we are presently attempting to suppress the *ANR1* gene in brown soybean using RNA interference (RNAi) to determine whether such a mechanism will be sufficient to engineer a red-brown soybean grain color. We predict that the suppression of *ANR1* transcript profiles will result in the redirection of metabolic flux into the anthocyanin (and possibly other metabolite) pathways to produce a red-brown soybean grain color. If successful, the RNA interference construct could be linked to commercial transgenic traits to enable the visual detection of GM soybeans in the marketplace and abroad.

CHAPTER 6

Coloring genetically modified soybean grains with anthocyanins by the suppression of proanthocyanidin genes

Adapted from:

Kovinich N¹, Saleem A², Rintoul TL³, Brown DCW⁵, Arnason JT^{4,5}, and Miki B^{4,5}. Submitted. Aposomatic GM soybeans: Coloring genetically modified soybean grains with anthocyanins by suppression of the proanthocyanidin genes *ANR1* and *ANR2*. *Transgenic Research*.

¹Invented the approach of suppressing ANR genes to redirect metabolic flux into anthocyanin biosynthesis for coloring plant grains, designed and conducted research, analyzed data, and wrote the manuscript for publication

²Developed the multiple reaction monitoring (MRM) method for LC-MS/MS

³Transformed soybeans

⁴Invented the concept of 'coloring soybeans with anthocyanins' with V. De Luca (Brock University)

⁵Obtained funding for the research and revised the manuscript

6.1. Introduction

The increase in cultivation (Lemaux, 2008) and the diversification of commercial genetically modified (GM) crops for pharmaceutical (Karg and Kallio, 2009) and industrial (Basaran and Rodriguez-Cerezo, 2008) uses has brought added pressure for effective detection and monitoring of the adventitious presence of GM grains in non-GM materials (Demeke et al., 2006). Cases of adventitious presence are the basis for the opposition to GM/non-GM coexistence (Ramessar et al., 2010) and typically result in severe economic penalties for exporters (Demeke et al., 2006; Karg and Kallio, 2009), international trade barriers (Smyth and McHughen, 2008), non-science-based regulations (Krueger and Le Buanec, 2008; Ramessar et al., 2010) and elevated public

concern over the capability of regulatory bodies to effectively detect and monitor GM grains. Presently, the detection and quantification of the levels of adventitious presence are based on sophisticated tests (e.g. PCR, qPCR, and ELISA) (Demeke et al., 2006) that require a knowledge of molecular biology for proper interpretation of results and are not always reproducible (Morisset et al., 2009). Coloring transgenic plants, plant organs, and other transgenic materials intended for commercial markets to enable their visual identification could simplify the detection process and facilitate more effective monitoring and quantitation (Kovinich et al., 2011a).

Anthocyanins are flavonoid pigments found in plants that provide red-to-blue color to flowers, leaves, and grains. Proanthocyanidins (PAs) represent another class of flavonoid pigments that if present in plant grains provide a brown color when oxidized during desiccation. The biosynthesis of anthocyanins and PAs has been well-characterized over the past several decades, with numerous structural, regulatory, and transport genes identified (reviewed by Mol et al., 1998; Winkel-Shirley, 2001; Lepiniec et al., 2006; Zhao and Dixon, 2010). Although often studied separately, the biosynthesis of anthocyanin and PA pigments are intimately linked by their requirement for supply of flavonoid intermediate, cyanidin (Figure 6.1). The anthocyanin branchpoint enzyme UDP-glyucose:flavonoid-3-O-glycosyltransferase (UGT; EC 2.4.1.115) and the PA branchpoint enzyme anthocyanidin reductase (ANR; EC 1.3.1.77) both utilize the unstable flavonoid precursor, cyanidin, as a substrate (Figure 6.1).

Figure 6.1. Simplified diagram of flavonoid biosynthesis in the seed coat of soybean variety Clark. See Kovich et al. (2011b) for a more extensive diagram.

Numerous studies have reported the successful manipulation of flower color as a result of engineering anthocyanin biosynthesis (reviewed by Holton, 1995; Tanaka et al., 1998; Nishihara and Nakatsuka, 2011). Further, flower color has been manipulated by redirecting metabolic flux into the anthocyanin pathway by suppression of the branchpoint gene for flavonol biosynthesis (*FLS*) (Holton et al., 1993; Tsuda et al., 2004; Nakatsuka et al., 2007). However, successful reports of engineering novel grain color are limited to studies that have overexpressed anthocyanin transcription factors in Maize (Shen and Petolino, 2006), Arabidopsis, and Tobacco (Geekiyana, 2009). Engineering grain color by manipulating PA biosynthesis has not been reported, and PA gene knock-outs produce a transparent seed coat (testa) in the model plant Arabidopsis (Lepiniec et al., 2006) which suggests little potential for engineering grain color by manipulating PA genes.

We previously identified the anthocyanin branchpoint gene *UGT78K1* encoding UDP-glycosyltransferase (UGT) from the black soybean seed coat and demonstrated its encoded activity *in vitro*, and by complementation of the Arabidopsis *ugt78d2* knock-out mutation (Kovach et al., 2010). Subsequently, a second UGT gene *UGT78K2* was identified from the black seed coat by comparing metabolite and transcriptome data between isogenic black and brown soybean seed coats differing in alleles of the *R* locus (Kovach et al., 2011b). The PA branchpoint genes *ANTHOCYANIN REDUCTASE1* (*ANR1*) and *ANR2* were identified from the brown soybean seed coat using homology-based methods, and the activity of the encoded proteins for

(-)-epicatechin synthesis were confirmed *in vitro* (Kovinich et al., 2011c). The same study demonstrated that reduced ANR1 gene expressions or activity to coincide with reduced amounts of soluble PAs, and elevated anthocyanin levels, in the red-brown seed coats of 2 naturally occurring and one EMS-mutagenesis mutant compared to 3 brown varieties (Kovinich et al., 2011c). Elevated anthocyanin levels in the red-brown seed coats did not coincide with elevated anthocyanin branchpoint gene expression, suggesting that a red-brown soybean grain color could be engineered simply by the suppression of the *ANR1* gene.

In the present study, we found that a distinct red-brown soybean grain color could be engineered by the simultaneous suppression of the PA branchpoint genes *ANR1* and *ANR2*. Gene suppression resulted in the increased accumulation of metabolites with expected and unexpected identities. Further genetic analysis identified the upregulation of flavonoid genes, suggesting positive feedback and feedforward mechanisms to be responsible for the increased accumulation of metabolites with unexpected identities in the soybean seed coat.

6.2. Materials and Methods

6.2.1. Chemicals

Cyanidin was purchased from Indofine (Somerville, NJ, USA), NADPH from Fisher Scientific (Ottawa, ON, CAN), (-)-epicatechin, procyanidin B2, cyanidin 3-O-galactoside, and the 3-O-glucosides of cyanidin, quercetin and isorhamnetin were purchased from Extrasynthese (Lyon, FR). All chemicals were of the

highest available purity. Solvents for metabolite extraction and LC-MS/MS were LC-MS grade (Fisher Scientific, Ottawa, ON, CAN).

6.2.2. Plant materials and growth conditions

Glycine max (L.) Merr. Variety Clark (PI547438) was obtained from the U.S. Department of Agriculture Soybean Germplasm Collection (Agricultural Research Service, University of Illinois at Champaign-Urbana). Seeds were surface-sterilized as described previously (Kovinich et al., 2011b), dried, and germinated in vermiculite in a Conviron E15 cabinet with a photoperiod of 16 h light (590 $\mu\text{E m}^2 \text{ s}$) at 25 °C, and 8 h dark at 20 °C. After 12 days, seedlings were transplanted to soil and 30 days later the photoperiod was changed to 12 h light/12 h dark to encourage reproductive development. Pods were routinely harvested on ice and seed coats were dissected from pod and embryo material, immediately frozen in liquid nitrogen, lyophilized, and stored at -80 °C for future analysis.

6.2.3. Nucleic acid isolation, vector construction, soybean transformation and PCR genotyping

RNA was isolated from soybean seed coats using a previously described method (Wang and Vodkin, 1994), and further purified using the RNeasy Plant Mini Kit (QIAGEN, ON, Canada). The quantity and purity of RNA samples were determined by spectrophotometry using a NANODROP 2000 (Thermo Scientific, ON, Canada). RNA samples (3 μg) were treated with DNase I (Amplification grade, Invitrogen, ON, Canada) at 37 °C for 15 min prior to cDNA

synthesis using SuperScript III Reverse Transcriptase (Invitrogen, ON, Canada) according to the manufacturer's instructions.

For assembly of the *ANR*-RNAi construct, 254 bp of the 3'-CDS of *ANR1* was amplified from seed coat cDNA isolated from the 25 – 50 mg fresh seed weight (FSW) stage of seed development using primers ASF/ASR (Appendix O). The amplicon was cloned into the pENTR/D-TOPO entry vector (Invitrogen), recombined into the pANDA35HK vector (Miki and Shimamoto, 2004) as described by the suppliers using the LR clonase enzyme mix (Invitrogen, ON, Canada), and the resultant *ANR*-RNAi construct was sequenced to confirm the integrity of the *ANR1* hairpin. Soybean variety Clark was transformed with the *ANR*-RNAi construct by bombardment of embryonic suspension culture tissues as described by Finer and McMullen (1991). For genotyping, DNA was isolated and amplified using the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, Oakville, ON, CA) and the primers GUSF/GUSR (Appendix O).

6.2.4. *qRT-PCR*

qRT-PCR was performed on seed coat cDNA isolated from the 25 – 50 mg FSW stage using 250 nM of forward and reverse primers and the iQ SYBR Green Supermix (BioRad, ON, Canada) as described previously (Kovinich et al., 2011b). Parallel reactions were performed in the absence of Superscript III to test for contamination of genomic DNA. Gene expressions from each sample were normalized to the endogenous reference *PEPC16*. PCR cycling and qRT-PCR analysis of gene expressions and PCR efficiencies were analyzed using the

Opticon Monitor 3 software (BioRad) as described previously (Kovinich et al., 2011b). To verify the specificity of the qRT-PCR reactions, melting curves were performed subsequent to each reaction in addition to fractionation of RT-PCR products on agarose gels. Primer sequences used in this study are listed in Appendix O.

6.2.5. Metabolite Analysis

Lyophilized seed coats (400 – 500 mg FSW) were ground to a fine powder in liquid nitrogen using an electric drill. Samples were aliquotted into 10 – 12 mg, and extracted with MeOH:H₂O:HCO₂H (80:15:5, v/v; 20 μ L mg⁻¹) for 16 h on ice with shaking (250 rpm). Subsequent to centrifugation the supernatant was filtered through Teflon (0.2 μ m; Chromatographic Specialties). For compound identification, 5 μ L aliquots were analyzed by LC-MS/MS in scanning mode as described previously (Kovinich et al., 2011b). For compound quantification, 2 μ L aliquots were analyzed by LC-MS/MS in multiple reaction monitoring (MRM) mode in positive polarity at unit resolution. The system consisted of a 1200 series Agilent liquid chromatograph with high performance autosampler (model G1376B), binary pump (model G1312A), column thermostat (model G1316A), and PEEK 0.12 mm ID lines from column inlet onwards till tri-way splitter of a 3200 QTRAP with PEEK 0.17 mm ID x 60cm lines on splitter outlet.

For MRM, two Q1 (parent) and Q3 (product) transitions were developed per authentic standard. The most sensitive transition was used for quantification and the second for authentication of identity. For quantitative optimization, authentic

standards (10-20 $\mu\text{g mL}^{-1}$ dissolved in methanol) were infused into the turbo ion source using an electrospray probe at a flow rate of 10 $\mu\text{g mL}^{-1}$. Flow Injection Analysis (FIA) was applied to optimize source conditions using the same LC conditions as for sample analysis; a linear gradient of 32-70% MeOH in 5% HCO_2H over 22 min at a flow rate of 1 mL min^{-1} . The column (Luna C18 (2), 150x4.6 mm, 5 μm particle size, (Phenomenex Inc., CA, USA) was washed by ramping up the MeOH % from 70-100% for 8 min. The initial conditions were brought back in 0.1 min and the column was equilibrated for 6 min before next injection. Samples were injected at a draw/inject speed of 200 $\mu\text{L min}^{-1}$. The injection needle was washed in a flush port for 3 sec after every injection to minimize carry over. The column thermostat was 20 °C.

Detailed QTRAP settings for MRM analysis of the 6 investigated compounds are listed in Appendix P. Optimal signal-to-noise ratio was achieved with vertical and horizontal probe settings of 7mm and 5mm, respectively. Quantitation of the 6 target compounds was performed using Analyst software 1.5.1. The integration of peaks was performed after applying a bunching factor/smoothing of 5. The calibration curves were generated by diluting authentic standard mixes serially in methanol. Calibration curves were linear fit ($R^2 \geq 0.998$).

6.3. Results

6.3.1. Engineering red-brown soybean seed coats using the RNAi construct ANR-RNAi

An RNA interference (RNAi) construct (*ANR*-RNAi) designed to silence *ANTHOCYANIDIN REDUCTASE 1* (*ANR1*) was transferred into brown (*irTO*) soybean variety Clark using a biolistics protocol (Finer and McMullen, 1991). The *ANR*-RNAi construct consisted of 254-bp inverted repeats of the 3'-coding region of the *ANR1* gene (Kovinich et al., 2011c) driven by the 35S constitutive promoter and separated by a GUS linker sequence derived from the pANDA-35HK vector (Miki and Shimamoto, 2004) (Figure 6.2A). Regenerated T0 plants were screened by PCR of the GUS linker sequence, and by the visual inspection of immature seeds dissected from pod material at various stages of development.

The three regenerated T0 lines (A1A1, A1A2, and A1A3) tested positive for the presence of the GUS linker sequence by PCR (GUS+), however only line A1A1 survived to reproductive maturity. Line A1A1 exhibited a novel red pigmentation in the hilum of immature seeds in the <25 – 200 mg fresh seed weight (FSW) stage of development (Figure 6.2B). This phenotype was not observed in the control (untransformed Clark) (Figure 6.2B), or in spontaneous or EMS-mutagenized red-brown varieties previously shown to have reduced *ANR1* gene expression or enzyme activity (Kovinich et al., 2011c). The red pigment became visible over the entire seed coat surface by 200 – 300 FSW stage of seed development (Figure 6.2C).

Selfing line A1A1 produced T1 progeny with 3 combinations of traits: 1) GUS+ progeny with red-brown grains, 2) GUS+ with brown grains identical to the

Figure 6.2. Structure of the *ANR*-RNAi DNA construct and phenotypes associated with its genomic integration in soybean. (A) Structure of the *ANR*-RNAi DNA construct: 35S promoter (35S); 3'-CDS of the *G. max* *ANTHOCYANIDIN REDUCTASE1* (*ANR1*); GUS linker (*GUS Linker*); transcription terminator (T); herbicide resistance gene *hygromycin phosphotransferase* (*HPT*). (B, C) Seed coat phenotypes at the 100 – 200 mg fresh seed weight stage (FSW) and the 200 – 300 mg FSW stage, respectively. Untransformed variety Clark (Control); T0 line A1A1 (A1A1). (D) PCR detection of GUS linker sequence from genomic DNA and dried grain phenotype of selected T1 lines.

control, and 3) GUS- (tested negative for the presence of GUS by PCR) progeny with brown grains. The red-brown and brown grain color phenotypes observed in the T1 generation segregated stably with GUS linker sequences in the T2 and T3 generations, suggesting that multiple transgene insertions had been present in the parental T0 line. T2 lines representing all combinations of traits and containing single or multiple transgene insertions (as determined by inheritance of GUS and seed coat color in the T3 generation) were selected for in-depth analysis. Line A1A1-3-4 had a single transgene insertion and red-brown grain color, line A1A1-1-2 had multiple transgene insertions and a red-brown phenotype, line A1A1-2-2 was GUS- with a brown grain and was selected to represent the null segregating control, and line A1A1-4-2 had a multiple transgene insertions and a brown grain color. The grain phenotype and the detection of GUS linker sequence from genomic DNA by PCR are shown for each selected line in Figure 6.2D. GUS PCR products were sequenced to confirm their identity.

6.3.2. High-level suppression of ANR1 and ANR2 genes by the ANR-RNAi construct confers a red-brown grain phenotype while low-level suppression fails to modify seed coat color

To measure the level of gene suppression in transgenic lines carrying the ANR-RNAi construct, quantitative RT-PCR (qRT-PCR) of ANR genes was performed using seed coat cDNA isolated at the 25-50 mg FSW stage of seed development. The 25-50 mg FSW stage was selected as the developmental time

point for this analysis because *ANR1* transcripts have been shown to be at maximum levels at this stage in the seed coat of brown (*irT*) soybean variety Clark (*ir*) (Kovinich et al., 2011b). To ensure the seeds of all lines were at the same stage of development, the days post anthesis, pod length, embryo morphology, and transcript levels of the developmental marker gene Gm-r1083-1191 were ensured to be equivalent among all materials prior to testing (Appendix Q). Comparisons of the *ANR*-RNAi sequence with other reductase-epimerase-dehydrogenase (RED) superfamily genes previously shown to be expressed in the seed coats of pigmented soybeans (Kovinich et al., 2011b, c) found *ANR*-RNAi to have high identity only with *ANR1* (100%) and *ANR2* (86%), and moderate identity (55-47%) to other RED superfamily genes (Appendix R). For this reason we decided to include the RED superfamily genes with highest identity to *ANR*-RNAi (i.e. *LAR1* and *DFR1*) in our qRT-PCR analysis to determine the specificity of gene silencing. Phosphoenol pyruvate carboxylase 16 (*PEPC16*) was used as the endogenous reference for normalization of qRT-PCR measurements of target genes across seed coat samples as described previously (Sugimoto et al., 1992; Tuteja et al., 2004; Kovinich et al., 2011, 2011b).

Quantitative RT-PCR (qRT-PCR) revealed a 35.2 – 38.8 fold (97.1 – 97.4%) reduction in the levels of *ANR1* transcripts, and a 3.8 – 7.0 fold (73.5 – 85.7%) reduction in *ANR2* transcripts in transgenic lines with a red-brown seed coat (A1A1-1-2 and A1A1-3-4), compared to the untransformed control or the segregating line (A1A1-2-2) that lacked the *ANR*-RNAi construct (Figure 6.3). By

Figure 6.3. qRT-PCR measurement of transcript profiles from the seed coat of transgenic soybean carrying the *ANR*-RNAi construct. *ANTHOCYANIDIN REDUCTASE1* (*ANR1*), *ANR2*, the *ANR*-RNAi construct *GUS* linker sequence (*GUS*), *LEUCOANTHOCYANIDIN REDUCTASE1* (*LAR1*), and

DIHYDROFLAVONOL 4-REDUCTASE1 (DFR1) profiles relative to the *PEPC16* gene. Transgenic lines with red-brown seed coat color (grey bars), lines with control seed color (white bars). Values are mean and SD from three biological replicates. Student's *t* test compared to the control significant at $P < 0.01$ (**), $P < 0.05$ (*).

contrast, only a 2.1 and 1.7 fold reduction in the levels of *ANR1* and *ANR2* transcripts, respectively, was observed in line A1A1-4-2 (Figure 6.3), that tested GUS+ but contained the control phenotype (brown seed coat color) (Figure 6.2D). The magnitude of GUS linker sequence expression was inversely proportional to the level of *ANR* gene expression (Figure 6.3), supporting that *ANR*-RNAi expression was the causal agent of reduced *ANR1* and *ANR2* transcript levels in transgenic lines.

qRT-PCR demonstrated no significant decrease in the levels of *LAR1* or *DFR1* transcripts in transgenic lines relative to the control or the segregating GUS- line (Figure 6.3), suggesting that *ANR*-RNAi-mediated gene suppression was highly specific to *ANR1* and *ANR2* genes. Interestingly, *DFR1* expressions were upregulated 1.2 – 1.6 fold in transgenic lines with a red-brown seed coat, suggesting that an endogenous positive feedback mechanism may have been stimulated in response to engineered *ANR* gene suppressions.

In summary, our results show high-level suppression of *ANR1* and *ANR2* genes in transgenic lines carrying the *ANR*-RNAi construct that have a red-brown seed coat color, and low levels of suppression in the GUS+ transgenic line that has the control (brown) seed coat phenotype.

6.3.3. Red-brown transgenic grains undergoing high-level suppression of ANR1 and ANR2 genes have major reductions in the amount of PAs along with elevated levels of cyanic pigments in the seed coat

We previously demonstrated that proanthocyanidins (PAs) from brown or black seed coats of variety Clark are exclusively (-)-epicatechin based (Kovinich et al., 2011b). As ANTHOCYANIDIN REDUCTASE1 (ANR1) and ANR2 enzymes from *G. max* have been shown to convert cyanidin to (-)-epicatechin (Kovinich et al., 2011c), high-level suppression of *ANR1* and *ANR2* expressions by the *ANR*-RNAi construct would be expected to cause significant reductions in the amounts of epicatechin-based PAs in the soybean seed coat.

Reaction of seed coat extracts with DMACA reagent revealed a 71.5-71.7% reduction (37.3-37.4 mg g⁻¹ LSC) in amounts of soluble PAs in the transgenic lines that had red-brown grain color and high-level *ANR* gene suppressions (A1A1-1-2 and A1A1-3-4), in comparison to the untransformed or segregating null controls (Figure 6.4A). By contrast, no significant change in soluble PA amounts relative to the controls was observed in the transgenic brown-colored

Figure 6.4. Total PA and cyanic pigment amounts from the seed coat of transgenic soybean carrying the *ANR*-RNAi construct expressed in units mg per gram lyophilized seed coat (LSC). (A) Soluble PAs measured by reaction of seed

coat extracts with DMACA reagent, using the PA dimer procyanidin B2 (B2) as the quantitative standard. (B) Solvent insoluble PAs measured by acid catalyzed hydrolysis of previously extracted seed coat residues, quantitatively compared to the authentic standard cyanidin chloride. (C) Cyanic pigments (anthocyanins or anthocyanidins) measured by photospectrometry in comparison to cyanidin chloride. Transgenic lines with red-brown seed coat color (grey bars), lines with control seed color (white bars). Values are mean and SD from three biological replicates. Student's *t* test compared to the control significant at $P < 0.01$ (**).

line (A1A1-4-3) (Figure 6.4A) that had only low levels of *ANR* gene suppression (Figure 6.2).

Measurement of solvent insoluble PAs by subjecting pre-extracted seed coat residues to acid-catalyzed hydrolysis revealed a 73.9-82.9% reduction ($1.3\text{-}1.4\text{ mg g}^{-1}$ LSC) in the amounts of solvent insoluble PAs in the red-brown transgenic lines (A1A1-1-2 and A1A1-3-4), and no significant change in amounts in the transgenic line that had the brown grain phenotype (A1A1-4-3), compared to the untransformed or segregating null controls (Figure 6.4B).

The red component of the red-brown grain color suggested elevated levels of cyanic pigment molecules (either anthocyanidins or their glycosides, the anthocyanins) in the red-brown transgenic seed coat. Measurement of the total amount of cyanic pigments from seed coat extracts was performed by photospectroscopy in comparison to the authentic standard cyanidin. Red-brown transgenic lines (A1A1-1-2 and A1A1-3-4) had a 7.9-8.3 fold increase ($3.2\text{-}3.4\text{ mg g}^{-1}$ LSC) in the total amounts of cyanic pigments compared to the control (Figure 6.4C). By contrast, the transgenic line with brown grain color (A1A1-4-3)

that was phenotypically identical to the control exhibited no significant change in the amount of cyanic pigments compared to the control.

Overall, these results demonstrated that transgenic lines with engineered red-brown grain color by high-level suppression of *ANR1* and *ANR2* genes had major reductions in the amounts of soluble and solvent insoluble PAs, along with significant increases in the amounts of cyanic pigments (either anthocyanins or anthocyanidins).

6.3.4. High-level suppression of soybean ANR1 and ANR2 genes redirects metabolic flux from PA biosynthesis into the anthocyanin and flavonol-3-O-glucoside pathways

The enzymes anthocyanidin reductase (ANR) and UDP-glycose:flavonoid-3-O-glycosyltransferase (UF3GT) utilize cyanidin as a substrate for proanthocyanidin (PA) and anthocyanin biosynthesis, respectively (Figure 6.1). As the ANR gene *ANR1* and the UF3GT gene *UGT78K2* were shown to be expressed at high and low levels in the brown soybean seed coat, respectively (Kovinich et al., 2011b), we hypothesized that suppression of ANR genes would redirect a moderate amount of metabolic flux from the PA pathway into the anthocyanin pathway, and would result in a phenotypic change from brown to a cyanic-colored soybean grain.

To identify metabolites that had accumulated differentially between transgenic and control seed coats, we analyzed seed coat extracts by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in scanning mode. Six

compounds were identified by LC-MS/MS to be present in different amounts in the transgenic red-brown seed coats compared to the untransformed control: (-)-epicatechin, procyanidin B2, cyanidin-3-O-glucoside (C3G), cyanidin-3-O-galactoside (C3Ga), quercetin-3-O-glucoside (Q3G), and isorhamnetin-3-O-glucoside (I3G) (Figure 6.5A).

To compare the amounts of each of these compounds between transgenic and control lines, LC-MS/MS was performed in multiple reaction monitoring (MRM) mode optimized for the detection of each of the six compounds. MRM revealed significant reductions in the amounts of (-)-epicatechin (38.8-41.5%; 9.0-9.6 mg g⁻¹ LSC) and procyanidin B2 (52.1-62.2%; 1.9-2.3 mg g⁻¹ LSC), along with increases in the amounts of the 3-O-glycosylated anthocyanins C3Ga (13.0-14.3 fold; 0.76-0.84 mg g⁻¹ LSC) and C3G (2.5-2.7 fold; 0.27-0.32 mg g⁻¹ LSC), and the flavonol-3-O-glucosides Q3G (166.0-178.6 fold; 25.9-27.9 mg g⁻¹ LSC) and I3G (8.0-9.6 fold; 1.9-2.3 mg g⁻¹ LSC) from red-brown transgenic seed coat extracts (Figure 6.4B).

These results demonstrate that high-level suppression of soybean *ANR1* and *ANR2* genes redirects metabolic flux from PA biosynthesis into the anthocyanin pathway to produce a red-brown soybean seed coat. The major increase in flavonol-3-O-glucoside amounts in the transgenic seed coat was an unexpected result of engineering the suppression of ANR genes, as the branch into the

Figure 6.5. Identification and quantification of seed coat metabolites that accumulate to different amounts in transgenic lines with red-brown grain color compared to the untransformed control. (A) Mass spectra of (-)-epicatechin (Rt: 6.9 min), cyanidin-3-O-galactoside (Rt: min), quercetin-3-O-glucoside (Rt: 10.6 min), procyanidin B2 (Rt: min), cyanidin-3-O-glucoside (Rt: min), isorhamnetin-3-O-glucoside (Rt: 12.4 min). (B) Multiple reaction monitoring (MRM) of the

compounds listed in (A) expressed in units mg per gram lyophilized seed coat (LSC). Transgenic lines with red-brown seed coat color (grey bars), lines with control seed color (white bars). Values are mean and SD from three biological replicates. Student's *t* test compared to the control significant at $P < 0.01$ (**).

flavonol-3-O-glucoside pathway precedes the biochemical step catalyzed by ANR (Figure 6.1). These results suggest that metabolite differences resulting from the suppression of ANR genes may not be attributed solely to the increased accumulation of the ANR substrate, cyanidin.

6.3.5. High-level suppression of soybean ANR1 and ANR2 genes causes the upregulation of anthocyanin and flavonol-3-O-glucoside isogene transcripts

Metabolomes have a tendency to rebalance themselves in response to metabolic engineering events by mechanisms that are poorly understood. The overaccumulation of flavonol-3-O-glucosides in red-brown transgenic seed coats undergoing ANR gene suppression (Figure 6.5B) represented one such example. However, the unexpected upregulation of *DFR1* transcripts (Figure 6.3) suggested transcriptional responses are involved in the mechanism responsible for re-routing metabolic flux. To gain further insight into this mechanism, we compared the expressions of several anthocyanin and flavonol-3-O-glucoside isogenes between transgenic red-brown seed coats and the control. As the suppression of ANR may be predicted to result in the accumulation of cyanidin, (the substrate of ANR and the product of the anthocyanidin synthase (ANS) enzyme) (Figure 6.1), we investigated whether ANR suppression may cause

alterations in the expressions of ANS genes (*ANS2/ANS3*). Furthermore, cyanidin is also the substrate for UDP-glucose:flavonoid-3-O-glucosyltransferase (UF3GT), which produces anthocyanins from anthocyanidins (Figure 6.1). For this reason, we also investigated whether ANR suppression may cause alterations in the expressions of UF3GT genes (*UGT78K1* and *UGT78K2*).

qRT-PCR demonstrated 2.0-2.1 and 1.7-1.8 fold increases in the expressions of *UGT78K2* and *GST26*, respectively, in red-brown transgenic seed coats undergoing ANR gene suppression compared to the control (Figure 6.6). By contrast, no significant differences in the expressions were observed for the UF3GT isogene *UGT78K1*, or the ANS genes *ANS2/ANS3* (Figure 6.6).

Overall, the results from Figure 6.3 and Figure 6.6 demonstrate the upregulation of 3 flavonoid isogenes (*DFR1*, *UGT78K2*, and *GST26*) from transgenic red-brown soybean seed coats undergoing ANR gene suppressions. These results identify the upregulation of a network of gene transcripts as a component of positive feedback and feedforward mechanisms responsible for the redistribution of metabolic flux in response to ANR gene suppressions in the soybean seed coat.

6.4. Discussion

Previously we proposed that soybean grain color could be engineered by the suppression of anthocyanin and/or proanthocyanidin (PA) genes in the seed coat

Figure 6.6. qRT-PCR measurement of transcript profiles identified positive feedforward/feedback induction of flavonoid gene expressions in the seed coat of transgenic soybean carrying the *ANR*-RNAi construct. *ANTHOCYANIDIN SYNTHASE1/ANTHOCYANIDIN SYNTHASE2* (*ANS1/ANS2*), *UDP-GLYCOSE:FLAVONOID-3-O-GLYCSYLTRANSFERASE* isogene 1 (*UGT78K1*), *UDP-GLYCOSE:FLAVONOID-3-O-GLYCSYLTRANSFERASE* isogene 2 (*UGT78K2*), and putative anthocyanin *GLUTATHIONE S-TRANSFERASE26* (*GST26*) profiles relative to the *PEPC16* gene. Transgenic lines with red-brown seed coat color (grey bars), lines with control seed color (white bars). Values are

mean and SD from three biological replicates. Student's *t* test compared to the control significant at $P < 0.01$ (**), $P < 0.05$ (*).

to enable the visual detection of commercial transgenic grains (Kovinich et al., 2011a). Here we have reported the successful modification of soybean grain color (Figure 6.2D). This was achieved by the simultaneous and high-level suppression of the proanthocyanidin (PA) genes *ANTHOCYANIDIN REDUCTASE1* (*ANR1*) and *ANR2* (Figure 6.3). *ANR1* and *ANR2* are branchpoint genes into PA biosynthesis (Figure 6.1), and their high-level suppressions resulted in the reduction of total PAs (Figure 6.4), and the overaccumulation of specific anthocyanins (and unexpectedly flavonol-3-O-glucosides) in the soybean seed coat (Figure 6.5).

Since the first report of engineering novel flower color by the expression of the maize *DFR* gene in petunia (Meyer et al., 1987), there have been numerous publications describing successful flower color manipulations in various plant species (reviewed by Holton, 1995; Tanaka et al., 1998; Nishihara and Nakatsuka, 2011). By contrast, successful reports of engineering novel grain color remain scarce, and may have been limited to the overexpression of anthocyanin transcription factors in Maize (Shen and Petolino, 2006), *Arabidopsis*, and Tobacco (Geekiyana, 2009). The suppression of PA branchpoint genes in soybean to redirect metabolic flux into anthocyanin biosynthesis represented a novel approach for engineering pigmentation in plant grains.

The re-channeling of flux between branches of the flavonoid metabolism has been observed previously. The *dfr* mutant had increased levels of flavonols in the Arabidopsis seed (Routaboul et al., 2006). The flavonol triple regulatory mutant *pfg1 pfg2 pfg3* and the *fls1-2* knock-out mutant each had increased accumulation of anthocyanins in the seedling (Stracke et al., 2007; Stracke et al., 2009). And the *M. truncatula* anthocyanin transporter mutant *mate2* had elevated levels of epicatechin-3'-O-glucoside in the flower (Zhao et al., 2011). Further, previous engineering experiments in soybean, Arabidopsis, and petunia have redirected metabolic flux from anthocyanin biosynthesis into the isoflavone pathway, from lignin biosynthesis into the flavonoid pathway, and from flavonol biosynthesis into the anthocyanin pathway, by suppressing anthocyanin, lignin, and flavonol branchpoint genes, respectively (Yu et al., 2003; Besseau et al., 2007; Nakatsuka et al., 2007). The overexpression of the ANR gene from *Medicago truncatula* in tobacco resulted in reduced anthocyanin pigmentation in the flower and elevated PA levels (Xie et al., 2003). These results demonstrated the potential for ANR to compete with the anthocyanin biosynthesis enzyme UDP-glucose:flavonoid-3-O-glycosyltransferase (UGT) for the substrate anthocyanidin. Our results demonstrated the converse scenario; that suppression of ANR genes results in increased anthocyanin accumulations (Figure 6.4, Figure 6.5).

The Arabidopsis *ANR* (a.k.a. *BAN*) knockout mutant displayed precocious accumulation of cyanic pigments in the seed coat during early seed development (Albert et al., 1997). The accumulations were only temporary, and resulted in a

transparent testa (tt) phenotype with black pigmentation confined to the raphe of the dried grain (Albert et al., 1997). This contrasts the phenotype in soybean, where high-level suppression of ANR genes gives a red-brown grain (Figure 6.2D). There may exist underlying mechanistic and metabolite differences that could explain the differences in grain phenotypes between these species. In *Arabidopsis*, the UF3GT gene (*UGT78D2*) and the *ANR* gene are regulated reciprocally, with *UGT78D2* expressed with anthocyanins in the seedling, and *ANR* expressed with PAs in the seed coat (Lee et al., 2005). By contrast, soybean UF3GT genes (*UGT78K1* and *UGT78K2*) and ANR genes (*ANR1* and *ANR2*) are both expressed in the seed coat (Figure 6.3 and Figure 6.6). Thus, it is possible that the difference in phenotype between the soybean grain undergoing high-level ANR gene suppressions and the *Arabidopsis* *ANR* knock-out grain, may be attributed to the presence and absence of UF3GT expressions, respectively, that stabilize anthocyanins in soybean allowing their accumulations to provide the red-brown grain phenotype. Consistent with this hypothesis, high-level suppression of ANR genes in the soybean seed coat resulted in increased amounts of the enzymatic products of UF3GT (3-O-glycosylated anthocyanins) (Figure 6.5). It should be noted that the cyanic pigments that accumulated temporarily in the seed coat of the *Arabidopsis* *ANR* knockout mutant were identified by photospectrometric methods that could not distinguish between anthocyanins and the unstable anthocyanidins (Albert et al., 1997; Devic et al., 1999), and thus may not be 3-O-glycosylated anthocyanins. This hypothesis suggests that the expression of the UF3GT gene(s) may be an important

consideration when designing strategies for the manipulation of grain pigmentation by the suppression of ANR genes in other species.

Feedback and feedforward mechanisms that operate during flavonoid biosynthesis remain poorly understood, and their elucidation could allow for more precise control over the outcomes of flavonoid engineering. Mechanisms of feedback and feedforward regulation of flavonoid and phenylpropanoid metabolism operate at the enzymatic level or the level of gene expression. L-phenylalanine ammonia lyase was shown to be inhibited by its own product, *trans*-cinnamic acid (Bolwell et al., 1986; Blount et al., 2000). The regulation of the bean CHS promoter by the first two intermediates of the phenylpropanoid pathway, *trans*-cinnamic acid and *trans-p*-coumaric acid, was observed in fungal elicitor treated alfalfa protoplasts (Loake et al., 1991). The Arabidopsis flavonol synthase (FLS) knockout mutant *fls1-2* demonstrated the induction of *DFR* and *LDOX* (a.k.a. *ANS*) genes (Stracke et al., 2009). Our results demonstrated the upregulation of *DFR1*, *UGT78K2*, and the putative anthocyanin glutathione S-transferase gene *GST26* in response to ANR gene suppressions (Figure 6.3, Figure 6.6). These results suggest the upregulation of anthocyanin and flavonol gene transcripts to, at least in part, be responsible for the elevated levels of anthocyanins and flavonol-3-O-glucosides (Figure 6.5).

Interestingly, the transgenic line (A1A1-4-2) undergoing moderate levels of ANR gene suppression (Figure 6.3) did not exhibit a change in seed coat pigmentation relative to the untransformed or null segregating controls (Figure 6.2). This was consistent with the previous finding that a threshold of flavonoid 3'-

hydroxylase gene down-regulation by virus-induced gene silencing (VIGS) had to be exceeded to cause a change in pubescence pigmentation in soybean (Nagamatsu et al., 2009). *DFR1*, *UGT78K2* and *GST26* were not upregulated in line A1A1-4-2 which suggested that the accumulation of upstream metabolites (possibly cyanidin), rather than suppression of ANR genes themselves, was responsible for the feedback/feedforward gene induction. The induction of *DFR1*, *UGT78K2* and *GST26* suggests the involvement of a regulatory factor in the feedback/feedforward induction mechanism, which is consistent with flavonoid genes being coordinately regulated (Hahlbrock et al., 1976; Pelletier et al., 1997; Hartmann et al., 2005). Further work is needed to determine how the overaccumulation of a flavonoid intermediate is perceived by the plant cell and the subsequent mechanisms that trigger the induction of flavonoid structural genes.

6.5. Concluding Remarks

The adventitious presence of genetically modified (GM) grains in non-GM shipments is the basis for opposition to GM/non-GM coexistence (Ramessar et al., 2010) and has resulted in elevated public concern over the capability of government bodies to effectively detect and monitor GM technologies. The use of fluorescent proteins (FPs) as transgene markers has been proposed to enable the optical detection of commercial genetically modified plant materials (Stewart, 2005, 2006), however FPs do not have a history of safe use for human consumption and their detection requires specialized equipment which may not

allow for convenient detection by consumers, producers, processors, and importers. An alternative approach would be to color transgenic soybeans by altering their endogenous pigment metabolism to produce distinct colors of natural products that are currently already part of the food supply and that are generally recognized as safe (Kovinich et al., 2011a). Recently discovered branchpoint genes for soybean proanthocyanidin (PA) biosynthesis (namely, *ANTHOCYANIDIN REDUCTASE1* (*ANR1*) and *ANR2*) and anthocyanin biosynthesis (*UGT78K1* and *UGT78K2*) have been identified in addition to comparative biochemical analyses of soybean lines differing in their expressions and seed coat color (Kovinich et al., 2010, 2011b, 2011c). This has enabled the design of a rationale approaches for engineering soybean grain color. Here we have demonstrated one such approach; the production of a red-brown grain by the suppression of PA branchpoint genes *ANR1* and *ANR2* to redirect metabolism into anthocyanin pigment biosynthesis. Coloring transgenic soybean grains intended for commercial markets to enable their visual detection may provide increased safety features to consumers and increased freedom to producers (Kovinich et al., 2011a). The use of recombinant DNA technology, rather than traditional breeding or EMS-mutagenesis, offers the advantage of physically linking the DNA element for “grain-coloring” to the transgene(s) that encode the desired commercial trait. Reducing the amount of PAs in transgenic soybean grains by the suppression of PA biosynthesis represents an advantageous approach to engineering a visible marker for industrial, pharmaceutical, or other commercial grains designed to produce proteins, as PA

pigments are known to bind proteins and complicate their extraction during processing. As the prevalence and diversity of transgenic materials increases, the use of different color markers to distinguish materials engineered for pharmaceutical, industrial, and other non-food/feed commercial uses may become increasingly warranted.

CHAPTER 7

General discussion

7.1. How does this study fit in existing societal, methodological and theoretical debates?

7.1.1. The societal debate

Numerous incidents have occurred where GM materials have contaminated non-GM shipments. In October 2009, the European Union halted the importation of all North American soybeans due to contamination by tiny amounts of unapproved GM maize types (Michael Hogan, Reuters – HAMBURG, Nov 2, 2009). The European Union stopped importation of flax (*Linum usitatissimum* L.) seed from Canada due to illegal contamination by GMOs, threatening a lucrative \$320-million annual market (Martin Mittelstaedt, Globe and mail, October 2009). The biotechnology giant ProdiGene Inc was forced to purchase 500,000 bushels of potentially contaminated soybeans at a cost of \$2.8 million and pay a \$250,000 fine due to their failure to contain corn producing a pig vaccine (Karg and Kallio 2009). These and other unfortunate incidents have resulted in elevated public concern over the capability of government bodies to effectively detect GM grains and could potentially have been prevented.

Presently, the detection and quantification of the levels of adventitious presence are based on sophisticated tests (e.g. PCR, qPCR, and ELISA) (Demeke et al., 2006) that require a knowledge of molecular biology for proper interpretation of results and are not always reproducible (Morisset et al., 2009).

The use of fluorescent proteins (FPs) as transgene markers has been proposed to enable the optical detection of commercial genetically modified plant materials (Stewart, 2005, 2006). Although green fluorescent protein (GFP) has been shown to be safe for consumption in tests using laboratory rats (Richards et al., 2003), FPs do not have a history of safe use for human consumption nor are they present in commercial GM crops presently on the market. Further, the detection of FPs requires specialized equipment that may not allow for convenient detection by importers, processors, producers, and consumers.

The main objective of our study, to engineer colored soybeans by suppression of anthocyanin and/or PA genes, was intended to provide a safe, simple, and effective alternative technology to enable the detection of GM grains. Several lines of GM carnation (*Dianthus caryophyllus*) with modified flower color are commercially available (www.cera-gmc.org). This exemplifies consumer acceptance of GM plant materials colored by genetic engineering. However, it remains to be determined whether consumers would similarly accept a safe edible plant material colored by genetic engineering.

As the soybean market becomes more fragmented through the introduction of new attribute-enhanced (a.k.a. identity preserved) varieties, there will be a need to expand the current storage and handling infrastructure in Ontario to accommodate more soybean varieties than are currently handled (Soy 20/20, 2010). The use of grain coloring technologies to enable the efficient detection of adventitious presence may be economically advantageous for Canada. Such technologies would enable more effective identification and monitoring of GM

grains intended for specific uses, and would reduce the incidence of adventitious presence. This in turn, would likely increase consumer confidence and could ease the GM/non-GM coexistence debate.

7.1.2. The methodological debate

Numerous studies have reported the successful manipulation of flower color as a result of engineering anthocyanin biosynthesis (reviewed by Holton, 1995; Tanaka et al., 1998; Nishihara and Nakatsuka, 2011). However, successful engineering of target colors is generally uncommon, as exemplified by more than one decade of attempts to engineer a *true* blue rose (Holton and Tanaka, 1994; Katsumoto et al., 2007). Reports of successfully engineering novel grain color have remained scarce (Shen and Petolino, 2006; Geekiyanage, 2009). For these reasons, our objective to engineer red-brown or red soybean grain colors by the suppression of anthocyanin or PA genes, respectively, was continuously met with skepticism from experts of metabolic engineering. This positioned our work in the center of a methodological debate as to whether grain color could be engineered simply by gene suppressions.

Prior to initiating our work we had two hypotheses: 1) The suppression of the UF3GT gene(s) in the seed coat of black soybean would result in reduced anthocyanin accumulations and produce a red-brown grain color, and 2) the suppression of the ANR gene(s) in the seed coat of brown soybean would redirect metabolic flux into the anthocyanin pathway and produce a red grain color. We were unable to test the first hypothesis because no viable

transformants were obtained from transformation of soybean with a constitutively-expressed construct (i.e. pANDA35HK encoding the 35S promoter) designed to silence the UF3GT gene *UGT78K1*. This may be attributed to the low efficiency of transformation of soybean variety Clark, or it may suggest that UF3GT may be essential for soybean viability. However, we were able to test hypothesis 2) and were successful in demonstrating that GM soybean grain color could be modified by the suppression of ANR genes. As PAs are believed to be responsible for the brown color of seeds (Lepiniec et al., 2006), we hypothesized that suppression of PA genes in a brown seed coat would remove the brown pigmentation result in an anthocyanin-based red grain color. However, the result of suppression of ANR genes was actually a red-brown grain color, suggesting that flavonols may also oxidize to brown in the dried grain.

Suppression of PA genes represents a novel approach for engineering pigmentation in plant tissues that had not been reported previously. The possibility exists that suppression of PA genes could modify flower color in some species. The possibility also exists that this technology could be adapted for coloring the GM grains of other important crop species. For example, the pigmented husks of wheat (*Triticum* spp.) and canola (*Brassica napus* L.) accumulate PAs (McCallum and Walker, 1990; Naczek et al., 1994) and ESTs representing *ANR* (a.k.a. *BANYULS*) gene(s) have been recently identified from the seed coat of flax (*Linum usitatissimum*) (Venglat et al., 2011).

PA deposition in the seed coat has a role in defense against predators and pathogens (Winkel-Shirley 1998). However, commercial yellow soybeans lack

PAs, so red-brown seed coats with reduced PA amounts may not be expected to have reduced levels of pathogen defense compared to the yellow varieties. Also, no differences in plant or seed development were observed to result from the constitutive expression of the *ANR*-RNAi construct by the 35S promoter. This may be due to the fact that PAs accumulate in the seed coat of soybean and are not essential for development, as exemplified by their absence in yellow soybean genotypes.

Soybean is a self-pollinating plant species. However, in the rare event that red-brown GM soybean plants were to outcross to commercial yellow varieties, the red-brown trait would not be visible due to the natural RNA interference (RNAi) of the *CHS* gene by the *I* locus. For this reason, transformation of elite commercial yellow soybean varieties with a *CHS* gene that evades RNA interference by the *I* locus in addition to the *ANR*-RNAi element would likely be a superior approach for coloring commercial GM soybean grains.

Presently, there are no commercial GM crop plants that utilize RNAi as the transgenic trait. This is likely due to ongoing patent issues rather than safety reasons. Rice contains a very large number of short double-stranded RNA (dsRNA), many of which have identical sequences to segments of various human genes (Ivashuta et al., 2009). This means that humans have a long history of safe exposure to dsRNA's that can mediate RNAi in foods, such as plant-derived RNAs that are identical to segments of our own genes.

The suppression of *ANR1* and *ANR2* in the GM soybean seed coat caused a reduction of PA amounts to levels that are within the range found in brown and

yellow soybean seed coats. However, the suppression of PA genes was accompanied by the unexpected overaccumulation of high amounts (25.9-27.9 mg g⁻¹ LSC) of quercetin-3-O-glucoside (Q3G). Onion (*Allium cepa*) represents a food that has relatively high amounts of Q3G (upto 1 mg g⁻¹ fresh weight) (Hertog et al., 1992; Crozier et al. 1997), however our engineered seed coats had roughly 26 times this amount. This data suggests that Q3G levels in the engineered seed coats may be out of the range that is found in foods, and should undergo additional safety testing before the GM grain can be considered safe for consumption. As quercetin has been shown to have a range of activities (e.g. anti-carcinogenic, anti-hypertensive, anti-diabetic, and antioxidant) in vivo, this data also suggests that our engineered red-brown seed coats could be a valuable commercial source for the quercetin-3-O-glucoside.

7.1.3. The theoretical debate

Feedback and feedforward mechanisms have remained largely ignored in models describing the control of flavonoid biosynthesis. The unexpected feedback and feedforward mechanisms observed in our work support the view that such mechanisms must be elucidated to enable more predictive outcomes of metabolic engineering. These unexpected results illustrate the importance of basic research prior to product development.

7.2. Original contributions

Our comparative analyses of metabolites from black, brown, and red-brown soybean seed coats by LC-MS/MS provided a better understanding of the relationship between seed coat composition and grain color.

Previous to our work, genes for only 4 flavonoid enzymes (CHS, F3H, F3'H, and F3'5'H) were identified to be involved in pigment biosynthesis in the soybean seed coat (Todd and Vodkin, 1996; Toda et al., 2002; Zabala and Vodkin, 2003; Zabala and Vodkin, 2005; Zabala and Vodkin, 2007; Kavinich et al., 2010). We identified more than 20 isogenes for 14 proteins to have putative roles in flavonoid biosynthesis in black (*iRT*) or brown (*irT*) seed coats using a combined analysis of microarray and metabolite data. Further, the functions of 5 genes (*UGT78K1*, *UGT78K2*, *OMT5*, *ANR1* and *ANR2*) were characterized *in vitro*, by assay of recombinant enzyme activities, and *in vivo* by complementation (*UGT78K1*) and gene suppression (*ANR1* and *ANR2*). Our combined analyses of metabolite and gene expression data have provided a better understanding of soybean seed coat metabolism and grain color.

Our work demonstrated the concept that soybean seed coat color could be modified by metabolic engineering to enable the visible identification of the genetically modified (GM) grain.

The suppression of proanthocyanidin (PA) branchpoint genes in soybean to redirect metabolic flux into anthocyanin biosynthesis represented a novel approach for engineering pigmentation in plants.

We identified positive feedback and feedforward mechanisms in the flavonoid biosynthetic pathway in the soybean seed coat. We identified that the induction

of flavonoid genes in response to ANR gene suppressions was, at least in part, responsible for the redirection of metabolic flux into anthocyanin biosynthesis.

7.3. Future research

The successful engineering of a red-brown soybean grain color raises the question of whether these results may be extended to produce additional seed coat colors for GM soybeans that have alternative uses. For example, could a blue grain color be produced by introducing into the ANR-*RNAi* construct an element that would silence the flavonoid 3'-hydroxylase (*F3'H*) gene? This may be expected to redirect metabolic flux into the delphinidin (blue) branch of the anthocyanin pathway. Alternatively, could a red grain be produced by expressing a synthetic or orthologous chalcone synthase (*CHS*) gene with the ANR-*RNAi* construct in a yellow grain? The synthetic or orthologous *CHS* gene could be designed to evade the post-transcriptional silencing response of the *I* locus, and its expression may be expected to drive metabolic flux into the flavonoid pigmentation pathways.

Feedback and feedforward mechanisms that operate during flavonoid biosynthesis remain poorly understood, and their elucidation could allow for more precise control over the outcomes of flavonoid engineering. Our results suggested that the overaccumulation of a flavonoid intermediate (possibly cyanidin), rather than suppression of ANR genes themselves, was responsible for triggering the feedback/feedforward gene induction. The identification of the exact metabolite (or metabolites) that trigger the induction of flavonoid structural

genes, and the elucidation of the mechanisms that lead to gene induction, would represent significant advancements in our understanding of flavonoid metabolism, and should be considered as important objectives for future research.

7.4. Implications and concluding remarks

Our work demonstrated that the seed coat color could be engineered to enable the visible identification of the GM soybean grain. We identified numerous pigmentation genes that could be manipulated to provide different colors to GM soy grains that are intended for different uses. This type of technology would simplify GM grain detection and may lead to fewer incidences of adventitious presence, which would undoubtedly have favourable economic, regulatory, and social implications.

Our work demonstrated that the suppression of PA biosynthesis caused the overaccumulation of anthocyanins and unexpectedly flavonol glycosides. We identified positive feedback and feedforward mechanisms as a component of the underlying mechanism. Harnessing feedback and feedforward controls in addition to other levels of metabolism regulation will likely expand the boundaries of what can be achieved by metabolic engineering.

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APPENDIX A

Primers				
Experiment	Corresponding probe set	Target	Primer	Sequence (5' to 3') ^{†‡}
Semi-qRT-PCR	GmaAfx 39483 1 A1_at	MYB159	159F	TGACTCAGCCTCACCCTTG
			159R	CAAAGGCATTGACCCAAATAA
	Gma 15972 1 A1_at	4CL2	4C2F	ACAAACATGGTGGTGGACAA
			4C2R	GGTAGAAACCGCTGCCACT
	Gma 7423 2 S1_a_at	4CL	4CF	GCAAGACCAATCTTGATCC
			4CR	TTTTGCCCAGGCATATATGTAA
	Gma 2605 1 S1_at	9012b	901F	CACTTTGACGTCGATCCAAAC
			901R	GAATCAGATGTCAAACCGTTCA
	Gma 2605 2 S1_at	9012a	902F	GCACGAGGGAAAATGTGTTT
			902R	GTTGGAACGGATTGGTGT
		ANR1	qAF	CCCTCAATATAAAATCCAACTGAA
			qAR	CAGAGCCCTTTGCTCTTTA
	GmaAfx 42116 1 S1_at	CHS4	CHS4F	CCTTCCAAGCCACTTTGCA
			CHS4R	CTGGAGCAAAGGATGAAAGTGA
	GmaAfx 42116 1 S1_at	CHS5	CHS5F	CACTTTGCCACATTCATCC
			CHS5R	TGTGAATGAACTAATGAAGCTATAGC
		CHS7/ CHS8	CHS78F	TAGGCAAGACATGGTGGTGG
			CHS78R	CTTTGACTTTGGCTGGCCC
	GmaAfx 80720 1 S1_at	DFR2	DFR2F	CCCCTTTCTTATGCCAACAA
			DFR2R	ATGTACCTCCCTTCGGCTTT
	Gma 15972 1 A1_at	EF-Hand	EHF	ACGCCATCTCCATTCAAATC
			EHR	CAGCAGGGGAAGATGAGAAG
	Gma 5139 1 S1_at	GST 21	G21F	ACTTGATGCAGACCCCTTGT
			G21R	TTCAAGAACACCCAACACCA
	GmaAfx 71212 1 A1_at	GST1	GST1F	CCAGGGCTTGAACATCTCAT
			GST1R	TCCACGCCATGTATTTGTTG
	GmaAfx 90491 1 A1_s_at	Protein kinase	KIF	GAAAGGTTTGAACGCACCAT
			KIR	ATTTTGGCGGAGTTTCACAC
	GmaAfx 73273 2 S1_s_at	Lipase	LIPF	CCGCATGGTTCTATCTTGGT
			LIPR	TGCATTTGACAAACCCTTCA
	GmaAfx 45454 1 S1_at	LRR	LRF	TTACATCAATGGCGGGATCT
			LRR	TTGGTGGGAACCTCTTTTGG
	GmaAfx 81605 1 S1_at	MYB50	M50F	TTTCAATCTGGAATTGGTTCG
			M50R	TTTTACTCATTCAACCAATAACAACAC
	GmaAfx 57777 1 S1_at	OMT5	OMT2F	CCTCACTGCACTTACCATTCC
			OMT2R	TCTTTATCGGCATCAACGAA
	Gma 9647 1 S1_at	OMT-like	OMTF	TTTGTTGAAAGCCAGTGACAG
			OMTR	TTTTGCCTTTGTTGATGCTG

	Gma 17141 1 S1_at	Oxy	OXF	GCAAGTGGCTAACCGTCAAT
			OXR	AGATAGTTGGAGCTAGAGTTTGAAAAA
		PEPC	qPF	CATGCACCAAAGGGTGTTTT
			qPR	TTTTGCGGCAGCTATCTCTC
	Gma 1002 2 S1_at	UGT78K1	q3GTF	GGGTTTGTTGAGAGGACCAA
			q3GTR	CATCCCAATCTCCCAAACAT
	GmaAfx 71999 1 S1_at	UGT78K2	qUAF	AAGCTGCACAGGATTTTCGAC
			qUAR	ACACACACACACACACAAAAAC
	Gma 5621 1 S1_at	G4DT	P4F	GCAGACCGTTGTGTGAAGA
			P4R	CCAGGATACCAACTCCGAAA
	GmaAfx 12723 1 A1_at	PECTIN	PCTF	TGAAATAAAAGCCAATCCCATT
			PCTR	GATCAGTGTGCCCCAAAATC
	Gma 4530 1 A1_s_at	Ring Fing	RFF	CCATTACGGGATTGTTTGGA
			RFR	TCTCCATGCATACGGAACAA
	Gma 235 1 S1_at	SCOF TF	STFF	AGATCGAAATCCCCAATTT
			STFR	TGCAAATCTGCCACTGTTGT
	Gma 8472 1 S1_at	WD40	WDF	CAAGCTGGTTCAGTCGATCA
			WDR	GCCTTTCCTGTTCTGCAGTC
	Gma 17736 1 S1_at	C2H2 ZF	ZF	TGGACTTGGAGTTACACCTGTAGATA
			ZR	CAATGATCTTTATAAGGGAAAAGTCA
	Gma 4334 1 S1_at	Zinc Fing	ZF2F	ACTGTTGGTGGTCCACGATT
			ZF2R	AAGGCCAAATCCTCGTTCTT
qRT-PCR	Gma 1002 2 S1_at	UGT78K1	q3GTF	GGGTTTGTTGAGAGGACCAA
			q3GTR	CATCCCAATCTCCCAAACAT
		ANR1	qAF	CCCTCAATATAAAATTCCAACTGAA
			qAR	CAGAGCCCCTTTGCTCTTTA
		DFR1	qDF	GGGCCTGGACTTCATCACTA
			qDR	ACCTCCCTTCCACTTCTGGT
		GPAT	qGPF	ATAGGATCGTGCCTGTTGCT
			qGPR	CAGCCTTAACCGACATCTCC
		LAR1	qL1F	TAGGCTTTGGTGAGGAGGAA
			qL1R	TGACACCAAAACAGAGTGACAA
		PEPC	qPF	CATGCACCAAAGGGTGTTTT
			qPR	TTTTGCGGCAGCTATCTCTC
	GmaAfx 71999 1 S1_at	UGT78K2	qUAF	AAGCTGCACAGGATTTTCGAC
			qUAR	ACACACACACACACACAAAAAC
	GmaAfx 57777 1 S1_at	OMT5	OMT2F	CCTCACTGCATTACCATTCC
			OMT2R	TCTTTATCGGCATCAACGAA
cDNA cloning	GmaAfx 57777 1 S1_at	OMT5	HO5F	CACCcatatgGCCGAAGAGGAGAGGCAT
			HO5R	ACTCctcgagCCCCACTCCCCAAAGAGAG
	GmaAfx 71999 1 S1_at	UGT78K2	UHF	AAAAA CACCcatATGGATCATCAAAACAAACACG
			UAR	TAGCA ACTCggatccACACACACACACACACACA AAACATAAAAGT

[†]Restriction site in lowercase

^{*}Start codon underlined

APPENDIX B

Developmental properties of black (*iRT*) and brown (*irT*) soybean seed coats at the 300 – 400 mg fresh seed weight stage

Property	Black (<i>iRT</i>)	Brown (<i>irT</i>)	P-value ^a
Days post-anthesis	30	30	N/A
Pod length (cm) ^b	5.019 ± 0.20	5.206 ± 0.35	0.214
Pod color	yellowish-green	yellowish-green	N/A
Embryo morphology	transition/early maturation	transition/early maturation	N/A
<i>GPAT</i> relative expression	0.512 ± 0.23	0.510 ± 0.17	0.944
<i>DFR1</i> relative expression	0.601 ± 0.24	0.491 ± 0.06	0.220

^aStudent's t test.

^bPods containing three seeds.

^cqRT-PCR expression levels relative to *PEPC16*.

APPENDIX C

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 solvent 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 insoluble 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).

APPENDIX D

Gene probe sets up-regulated more than 2-fold in the seed coat of black (iRT) soybean compared to the brown (iRT) isolate^a

Probe Sets	Target Description	Black/brown fold change	SAM	
			P-value	d-score
GmaAffx 71212 1 A1_at	Glutathione S-transferase (GST26)	234 11	0 000003	21 22
GmaAffx 80720 1 S1_at	Dihydroflavonol-4-reductase (DFR2)	62 45	0 000002	24 70
GmaAffx 42116 1 S1_at	Chalcone synthase (CHS4/CHS5)	10 99	0 000020	8 45
GmaAffx 42116 1 S1_x_at	Chalcone synthase (CHS4/CHS5)	8 06	0 000026	7 12
GmaAffx 90450 1 S1_at	Similar to esterase/lipase domain (1 90e-44)	10 41	0 000011	13 04
GmaAffx 2469 1 S1_at	AP2/ERF transcription factor weakly similar to DDF1 (DWARF AND DELAYED FLOWERING 1)	7 45	0 001416	2 56
Gma 3745 1 S1_at	Moderately similar to A Thaliana polyamine oxidase (ATPAO1) NP_196874 1 (75 6%)	7 14	0 000028	7 11
Gma 1163 1 S1_at	Anthocyanidin synthase (ANS2/ANS3)	6 77	0 000018	8 77
Gma 17141 1 S1_at	Moderately similar to A Thaliana putative flavonone 3-β-hydroxylase clone U20744 (74%) (F3H-like)	6 43	0 004849	2 03
GmaAffx 67044 1 S1_at	Similar to cytochrome b5 superfamily domain (2 70e-14)	5 86	0 000008	14 10
GmaAffx 78720 2 S1_at	Similar to pectinacetyltransferase domain (1 28e-163)	5 78	0 000057	5 20
Gma 10642 1 S1_at	Similar to Mob1/phocein superfamily domain (3 34e-45)	5 69	0 000015	9 52
GmaAffx 73273 2 S1_s_at	Similar to esterase/lipase domain (1 90e-44)	5 29	0 000021	8 43
GmaAffx 12723 1 A1_at	Similar to leucine-rich repeat (LRR) domain (1 48e-03)	5 26	0 000010	13 11
GmaAffx 45454 1 S1_at	WD40 protein	4 34	0 000033	6 20
Gma 2605 1 S1_at	Clone JCVI-FLGm-9O12, similar to uncharacterized DUF1674 domain (3 12e-13)	4 02	0 000029	6 65
GmaAffx 14986 2 S1_at	Similar to phosphate-induced protein domain (1 01e-135)	3 98	0 003096	2 21
GmaAffx 14986 1 S1_at	Similar to phosphate-induced protein domain (1 93E-131)	3 76	0 000915	2 84
Gma 17736 1 S1_at	Moderately similar to A Thaliana ZAT11 C2H2 type zinc finger (48 4%) NP_181279 1	3 62	0 000379	3 57
Gma 2605 2 S1_at	Clone JCVI-FLGm-9O12, similar to uncharacterized DUF1674 domain (3 12e-13)	3 42	0 000064	5 03
Gma 17440 1 S1_at	Clone Gm-c1023, transcribed locus	3 32	0 000734	2 99
Gma 8411 1 S1_at	Weakly similar to XP_002323966 1 predicted protein [Populus trichocarpa]	3 20	0 001318	2 60
Gma 4530 1 A1_s_at	Weakly similar to A Thaliana PUB22 (PLANT U-BOX 22) (64 5%)	3 14	0 000038	5 81
Gma 4027 1 S1_at	Xyloglucan endotransglycosylase (TCH4)	3 13	0 000281	3 90
GmaAffx 71999 1 S1_at	Glycosyltransferase (UGT78K2)	3 09	0 000124	4 50
GmaAffx 24571 1 S1_s_at	Xyloglucan endotransglycosylase (TCH4)	3 05	0 000043	5 67
GmaAffx 2556 1 S1_at	Clone Gm-r1070	2 98	0 009617	1 79
GmaAffx 62542 1 S1_at	Clone Gm-r1070-2859	2 96	0 000759	2 96
Psojae_rRNA_785_at	Oomycota 18S rRNA	2 89	0 000925	2 83

Gma 2605 2 S1_a_at	Clone JCVI-FLGm-9O12, similar to uncharacterized DUF1674 domain (3 12e-13)	2 84	0 000262	3 96
Gma 1002 2 S1_at	UDP-glucose flavonoid 3-O-glucosyltransferase (UGT78K1)	2 81	0 000229	4 03
Gma 7423 2 S1_a_at	4-coumarate-CoA ligase-like protein (4CL-like)	2 80	0 005815	1 96
GmaAffx 30537 1 S1_at	Phosphate-induced protein (PHI-1)	2 80	0 000309	3 82
Gma 9672 1 S1_at	Transcribed locus clone Gm-c1023	2 80	0 008550	1 83
GmaAffx 32504 1 A1_at	Lipase (class 3) protein	2 76	0 000046	5 54
Gma 120 1 S1_at	Syringolide-induced protein B13-1-9	2 72	0 000935	2 83
GmaAffx 72190 1 S1_at	Weakly similar to Formin homology 1 (AFH1) (54%)	2 72	0 008756	1 82
Gma 4334 1 S1_at	Zinc finger protein, C2H2 type	2 71	0 002176	2 35
GmaAffx 51786 1 S1_s_at	Xyloglucan endotransglycosylase (TCH4)	2 67	0 000152	4 34
GmaAffx 17284 1 A1_s_at	WRKY56 transcription factor	2 64	0 000708	3 03
Gma 117 1 S1_at	Syringolide-induced protein 19-1-5	2 61	0 000154	4 34
GmaAffx 90491 1 A1_s_at	Weakly similar to A. Thaliana putative serine/threonine protein kinase NP_176864 1	2 58	0 000765	2 96
Gma 9647 1 S1_at	O-methyltransferase, putative (OMT-like)	2 58	0 000098	4 74
Gma 5935 2 S1_a_at	Clone Gm-c1009-2878, unknown protein	2 51	0 000376	3 58
Gma 3175 1 S1_at	Tyrosinase	2 49	0 000633	3 12
Gma 3526 1 S1_at	Xyloglucan endotransglycosylases (XET)	2 49	0 000713	3 02
Gma 13307 1 S1_at	Protein of unknown function (DUF1005)	2 48	0 000041	5 73
GmaAffx 57777 1 S1_at	Caffeoyl-CoA O-methyltransferase 5 (OMT5)	2 48	0 000157	4 33
GmaAffx 41334 1 A1_at	Clone Gm-c1079-1901	2 46	0 009429	1 80
Gma 7423 1 S1_a_at	Moderately similar to A. Thaliana AMP-dependent synthetase and ligase family protein NP_190468 1	2 44	0 003650	2 14
GmaAffx 18599 1 S1_at	Polyamine oxidase	2 38	0 000670	3 06
Gma 5621 1 S1_at	Pterocarpan 4-dimethylallyltransferase (G4DT)	2 36	0 009232	1 80
Gma 8236 1 S1_at	Xyloglucan endotransglycosylases (XET)	2 35	0 003203	2 19
GmaAffx 81605 1 S1_at	MYB transcription factor (MYB50)	2 34	0 002648	2 27
Gma 13607 1 S1_at	Mitochondrial oxoglutarate/malate carrier protein	2 29	0 000363	3 62
Gma 714 1 A1_at	Asparaginyl-tRNA synthetase	2 27	0 000680	3 05
Gma 8472 1 S1_at	4-Coumarate-CoA ligase 2 (4CL2)	2 27	0 000108	4 60
GmaAffx 61880 1 S1_at	Clone Gm-r1070-3972	2 26	0 000440	3 46
Gma 3586 1 S1_at	Unknown protein clone JCVI-FLGm-9L4	2 24	0 000177	4 25
GmaAffx 74814 1 S1_at	Inositol phosphate kinase (Itpk3)	2 23	0 000724	3 01
GmaAffx 90952 1 S1_at	Weakly similar to A. Thaliana ubiquitin-protein ligase PLANT U-BOX 22 (PUB22) (64 5%)	2 22	0 000582	3 20
GmaAffx 5393 1 S1_at	Clone JCVI-FLGm-20113 unknown mRNA	2 22	0 000623	3 13
Gma 15972 1 A1_at	EF-hand superfamily protein	2 19	0 003021	2 22
Gma 772 1 S1_at	Hypothetical protein clone Gm-r1070-486	2 18	0 002645	2 27
Gma 4531 2 S1_at	Moderately similar to A. Thaliana Exordium (EXO) (80 6%)	2 16	0 000163	4 30

GmaAffx 92641 1 S1_s_at	Clone sHB034L03	2 16	0 002336	2 32
Gma 53 1 S1_at	Maturation polypeptide (MP2)	2 13	0 005468	1 98
Gma 5935 1 S1_at	Unknown protein clone E01C10	2 12	0 000173	4 26
Gma 5475 1 S1_at	Weakly similar to A Thaliana Arabinogalactan protein 4 (AGP4) NP_196605 1 (51 1%)	2 11	0 001202	2 65
GmaAffx 79054 1 S1_at	Moderately similar to phosphate-induced 1 (PHI-1) (78 3%)	2 09	0 000240	4 00
GmaAffx 83213 2 S1_at	Ca ²⁺ -transporting ATPase-like protein	2 07	0 003554	2 15
Gma 5139 1 S1_at	Glutathione S-transferase (GST21)	2 05	0 000656	3 09
Gma 8309 1 A1_at	Transcribed locus	2 05	0 002549	2 29
Gma 9611 1 A1_at	Moderately similar to A Thaliana GH3 1 (NP_179101 1) (85 1%)	2 03	0 000544	3 25
GmaAffx 80258 1 S1_at	LEA_2 family protein	2 03	0 001766	2 45
GmaAffx 84528 1 S1_at	Protein of unknown function	2 03	0 000338	3 69
Gma 235 1 S1_at	Putative transcription factor SCOF-1	2 02	0 001831	2 43
GmaAffx 39483 1 A1_at	MYB transcription factor (MYB159)	2 02	0 000034	6 17
Gma 235 1 S1_x_at	Putative transcription factor SCOF-1	2 01	0 001525	2 52
Gma 9838 1 S1_at	Thioredoxin h1	2 00	0 007425	1 88

^aExpression values were obtained using RMA (Inzarry et al , 2003)

Appendix E

Distribution of gene function categories of probe sets that were up-regulated more than 2-fold in the seed coat of black (*IRT*) soybean relative to the seed coat of brown (*irt*) soybean.

Appendix F

Glycine max UGTs^{a,b}

Location	Gene
Chromosome 01	Glyma01g02700
	Glyma01g02740
	Glyma01g04250
	Glyma01g09160
	Glyma01g21580
	Glyma01g21590
	Glyma01g21620
	Glyma01g39570
Chromosome 02	Glyma02g03420
	Glyma02g11610
	Glyma02g11620
	Glyma02g11630
	Glyma02g11640
	Glyma02g11650
	Glyma02g11670
	Glyma02g11680
	Glyma02g11690
	Glyma02g11700
	Glyma02g11710
	Glyma02g25930
	Glyma02g32020
	Glyma02g32770
	Glyma02g39080
	Glyma02g39090
	Glyma02g39680
	Glyma02g39700
	Glyma02g44100
	Glyma02g47990
Chromosome 03	Glyma03g03830
	Glyma03g03840
	Glyma03g03850
	Glyma03g03870
	Glyma03g16160
	Glyma03g16250
	Glyma03g16290
	Glyma03g16310
	Glyma03g22640
	Glyma03g25000
	Glyma03g25020
	Glyma03g25030
	Glyma03g26890
	Glyma03g26940
	Glyma03g26980
	Glyma03g34410

	Glyma03g34420
	Glyma03g34440
	Glyma03g34460
	Glyma03g34470
	Glyma03g34480
	Glyma03g34490
	Glyma03g41730
Chromosome 04	Glyma04g10890
	Glyma04g36200
Chromosome 05	Glyma05g04200
	Glyma05g25160
	Glyma05g28330
	Glyma05g28340
	Glyma05g31500
Chromosome 06	Glyma06g33820
	Glyma06g35110
	Glyma06g36530
	Glyma06g39350
	Glyma06g40390
	Glyma06g43880
	Glyma06g47890
Chromosome 07	Glyma07g07320
	Glyma07g07330
	Glyma07g07340
	Glyma07g13130
	Glyma07g13560
	Glyma07g14510
	Glyma07g14530
	Glyma07g30180
	Glyma07g30190
	Glyma07g30200
	Glyma07g33880
	Glyma07g38460
	Glyma07g38470
Chromosome 08	Glyma08g07130
	Glyma08g11330
	Glyma08g11340
	Glyma08g13230
	Glyma08g19000
	Glyma08g19290
	Glyma08g26780
	Glyma08g26790
	Glyma08g26830
	Glyma08g26840
	Glyma08g38030
	Glyma08g38060
	Glyma08g38070
	Glyma08g44550

	Glyma08g44690
	Glyma08g44700
	Glyma08g44710
	Glyma08g44720
	Glyma08g44730
	Glyma08g44740
	Glyma08g44750
	Glyma08g44760
	Glyma08g46270
	Glyma08g46280
	Glyma08g48240
Chromosome 09	Glyma09g09910
	Glyma09g23310
	Glyma09g23330
	Glyma09g23600
	Glyma09g23720
	Glyma09g23750
	Glyma09g29160
	Glyma09g38130
	Glyma09g38140
	Glyma09g41690
	Glyma09g41700
Chromosome 10	Glyma10g07090
	Glyma10g07110
	Glyma10g07160
	Glyma10g15730
	Glyma10g15790
	Glyma10g16790
	Glyma10g33790
	Glyma10g33800
	Glyma10g40900
	Glyma10g42680
Chromosome 11	Glyma11g00230
	Glyma11g05680
	Glyma11g06880
	Glyma11g14260
	Glyma11g29480
	Glyma11g34720
	Glyma11g34730
Chromosome 12	Glyma12g06220
	Glyma12g14050
	Glyma12g15870
	Glyma12g28270
	Glyma12g34030
Chromosome 13	Glyma13g01220
	Glyma13g05580
	Glyma13g05590
	Glyma13g06170

	Glyma13g14190
	Glyma13g21040
	Glyma13g24230
	Glyma13g32770
	Glyma13g32910
	Glyma13g36490
	Glyma13g36500
Chromosome 14	Glyma14g00550
	Glyma14g04790
	Glyma14g04800
	Glyma14g35160
	Glyma14g35190
	Glyma14g35220
	Glyma14g35270
	Glyma14g37170
	Glyma14g37730
	Glyma14g37770
Chromosome 15	Glyma15g05600
	Glyma15g05700
	Glyma15g05710
	Glyma15g05980
	Glyma15g06370
	Glyma15g06390
	Glyma15g34720
	Glyma15g37520
Chromosome 16	Glyma16g03760
	Glyma16g27440
	Glyma16g29330
	Glyma16g29340
	Glyma16g29370
	Glyma16g29380
	Glyma16g29400
	Glyma16g29420
	Glyma16g29430
Chromosome 17	Glyma17g02270
	Glyma17g02280
	Glyma17g07340
	Glyma17g14640
	Glyma17g18220
Chromosome 18	Glyma18g00620
	Glyma18g01950
	Glyma18g03570
	Glyma18g29100
	Glyma18g29380
	Glyma18g43980
	Glyma18g44000
	Glyma18g44010
	Glyma18g48230

	Glyma18g48250
	Glyma18g50060
	Glyma18g50080
	Glyma18g50090
	Glyma18g50100
	Glyma18g50110
	Glyma18g50980
Chromosome 19	Glyma19g03580
	Glyma19g03610
	Glyma19g03620
	Glyma19g04570
	Glyma19g04610
	Glyma19g27600
	Glyma19g37120
	Glyma19g37130
	Glyma19g37140
	Glyma19g37150
	Glyma19g37170
	Glyma19g44350
Chromosome 20	Glyma20g05700
	Glyma20g24360
	Glyma20g26420
	Glyma20g33810
	Glyma20g33820
Scaffold 23	Glyma0023s00410
Scaffold 60	Glyma0060s00320

^aORFs were identified from the Glyma1 soybean genome sequence
(www.phytozome.net/soybean) by

TBLASTN of the UGT78K1 PSPG
(WAPQTHVLAHDSVGVFVTHCGANSVIESVSSGVPMICRPFFGDQ).

^bPSPGs without predicted ORFs or with PSPG sequence downstream of
stop codon were excluded.

Appendix G

Glycine max OMTs ^{a b}	
Location	Gene
Chromosome 01	Glyma01g00730
	Glyma01g39460
Chromosome 02	Glyma02g11530
	Glyma02g39930
Chromosome 04	Glyma04g36430
	Glyma04g40580
	Glyma04g40590
Chromosome 05	Glyma05g02810
	Glyma05g27960
	Glyma05g36210
	Glyma05g36220
Chromosome 06	Glyma06g14200
	Glyma06g14210
	Glyma06g14220
	Glyma06g18460
	Glyma06g43940
	Glyma06g43950
	Glyma06g43970
	Glyma06g44010
	Glyma06g45050
	Glyma07g05460
Chromosome 07	Glyma07g05470
	Glyma07g05480
	Glyma07g05730
	Glyma07g06140
Chromosome 08	Glyma07g33780
	Glyma08g03430
	Glyma08g03440
	Glyma08g10930
	Glyma08g10940
	Glyma08g18740
	Glyma08g27050
	Glyma08g27090
	Glyma08g27110
	Glyma08g27260
Chromosome 09	Glyma09g00590
	Glyma09g12440
	Glyma09g12480
	Glyma09g41840
Chromosome 10	Glyma09g41850
	Glyma10g31990
	Glyma10g32010
	Glyma10g32020
	Glyma10g32030

	Glyma10g35980
Chromosome 11	Glyma11g05800
	Glyma11g21080
	Glyma11g36410
Chromosome 12	Glyma12g12230
	Glyma12g13980
	Glyma13g24210
Chromosome 14	Glyma13g33830
	Glyma14g00800
	Glyma14g38080
	Glyma14g38090
	Glyma14g38100
Chromosome 15	Glyma14g38110
	Glyma15g38540
Chromosome 16	Glyma16g02000
	Glyma16g02750
Chromosome 17	Glyma17g13480
	Glyma17g18750
	Glyma17g18800
Chromosome 18	Glyma18g00340
	Glyma18g49870
	Glyma18g50260
	Glyma18g50280
	Glyma18g50290
Chromosome 19	Glyma18g50470
	Glyma19g17430
	Glyma19g39890
	Glyma19g44490
	Glyma19g45000
Chromosome 20	Glyma20g00590
	Glyma20g00600
	Glyma20g04780
	Glyma20g31600
	Glyma20g31610
	Glyma20g31700
	Glyma20g35610
	Glyma20g35620
	Glyma20g35630
	Glyma20g35640
Scaffold 335	Glyma0335s00200

^aORFs were identified from the Glyma1 soybean genome sequence (www.phytozome.net/soybean) by TBLASTN of the conserved OMT domain (PLN02589) sequence from OMT5 (KHRMSSNPVILQSVNLTKYV LETSVYPREEETLKELRKATAGHPWGFMGAAAPDAGQLMTLLLKLLNAKKTIEVGVFTGYSLLLTALTIPDDGK IIALDPDREAYEIGLPFIKKAGVEHKIDFIESPALPVL KLEDPNKNESFDFAFVDADKDNWNYHERLLKLV).

^bPSPGs without predicted ORFs or with the conserved OMT domain interrupted by a stop codon were

Appendix H

Gene probe sets downregulated more than 2-fold in the seed coats of black (iRT) soybean compared to the seed coats of brown (iRT) soybean at the 300 - 400 mg fresh seed weight stage of development^a

Probe Sets	Target Description	Black/brown fold change	SAM	
			P-value	d-score
Gma 17605 1 S1_at	Chalcone synthase (CHS1)	0.028	0.000007	-16.45
GmaAffx 92491 1 S1_s_at	Chalcone synthase (CHS9)	0.047	0.000005	-19.36
Gma 16971 2 A1_s_at	Ribulose-1,5-bisphosphate carboxylase small subunit rbcS2 (LOC547605)	0.101	0.000031	-6.35
Gma 571 1 S1_at	Weakly similar to A. thaliana Thioredoxin 2 (AtTRX2) NP_198811.1 (61.5%)	0.141	0.000013	-9.87
GmaAffx 14886 1 S1_at	Moderately similar to A. thaliana branched-chain amino acid transaminase 2 (AtBCAT-2) (72.5%)	0.172	0.001301	-2.61
GmaAffx 49936 1 S1_at	Transcribed locus	0.249	0.000103	-4.71
GmaAffx 19055 1 S1_at	5'-clone Gm-c1036-8049	0.270	0.000226	-4.03
GmaAffx 89828 1 S1_s_at	Highly similar to isoflavone reductase homolog 1 (IFR1) (93%)	0.272	0.000132	-4.46
Gma 3405 1 S1_s_at	Moderately similar to A. thaliana branched-chain amino acid transaminase 2 (AtBCAT-2) (72.5%)	0.294	0.003461	-2.16
GmaAffx 77655 1 S1_at	Transcribed locus	0.306	0.000180	-4.23
GmaAffx 85444 1 S1_at	Moderately similar to Phosphate 2 ubiquitin-protein ligase (PHO2) NP_850218.1 (70.1%)	0.326	0.000454	-3.43
Gma 9776 1 S1_at	Kinesin heavy chain	0.332	0.000023	-7.83
Gma 1299 1 S1_at	Weakly similar to Phosphate 2 ubiquitin-protein ligase (PHO2) NP_850218.1 (60.1%)	0.339	0.001125	-2.69
Gma 7958 1 S1_at	Transcribed locus	0.341	0.000147	-4.36
GmaAffx 14886 1 S1_at	Kinesin heavy chain	0.342	0.001491	-2.53
Gma 3801 1 S1_at	Moderately similar to Phosphate 2 ubiquitin-protein ligase (PHO2) NP_850218.1 (70.1%)	0.351	0.000036	-5.95
GmaAffx 40574 1 S1_at	Weakly similar to Populus trichocarpa predicted protein XP_002311637.1 (65.1%)	0.376	0.000832	-2.90
Soybean_rRNA_918_at	Plant 18S ribosomal RNA	0.382	0.000536	-3.28
Gma 14852 1 S1_at	Clone JCVI-FLGm-707 unknown mRNA	0.388	0.000093	-4.77
Gma 5402 1 S1_at	Moderately similar to A. thaliana serine carboxypeptidase-like 40 (scpl40) (71.4%)	0.394	0.000736	-2.99
Gma 8455 1 S1_at	Flavonoid 3'-hydroxylase (F3'H)	0.400	0.000646	-3.10
GmaAffx 93221 1 S1_s_at	Moderately similar to Rosa hybrid cultivar orcinol O-methyltransferase (72%)	0.410	0.003428	-2.17
Gma 10502 1 S1_at	3'-mRNA sequence clone Gm-r1088-1474	0.410	0.000368	-3.59
GmaAffx 39265 1 S1_at	Weakly similar to A. thaliana antiporter/drug transporter MATE family protein (69.8%)	0.414	0.000265	-3.95
Gma 9164 1 S1_at	Histone H4	0.419	0.000602	-3.16
GmaAffx 42587 1 A1_at	Moderate similarity to A. thaliana MYB30 (71.5%)	0.427	0.000472	-3.40
Gma 9786 1 S1_at	Transcribed locus	0.433	0.000414	-3.50
GmaAffx 74238 1 S1_at	Putative RAS protein	0.439	0.006397	-1.93

GmaAffx 34868 1 A1_at	Highly similar to Phaseolus coccineus leucoanthocyanidin reductase (LAR) (88%)	0 443	0 000016	-9 02
Gma 13465 1 A1_at	Hypothetical protein	0 444	0 000266	-3 95
Gma 9511 1 S1_at	Clone JCVI-FLGm-4E15 unknown mRNA	0 451	0 000719	-3 01
Gma 17323 1 S1_at	5'-mRNA sequence clone Gm-r1030-2753	0 456	0 001666	-2 48
GmaAffx 34657 1 A1_s_at	Hydroxyindole-O-methyltransferase-related	0 457	0 001815	-2 43
GmaAffx 66066 1 A1_at	Weakly similar to Populus trichocarpa AP2/ERF domain-containing transcription factor XP_002336822 1 (67 7%)	0 459	0 007008	-1 90
GmaAffx 42543 1 A1_at	Transcribed locus	0 462	0 001638	-2 49
Gma 4340 2 S1_a_at	Moderately similar to P. trichocarpa flavonoid o-methyltransferase-related (73 9%)	0 462	0 004180	-2 08
GmaAffx 40267 1 A1_at	cDNA clone Gm-r1070-7291 3-, mRNA sequence	0 466	0 001952	-2 40
GmaAffx 77278 1 A1_at	Transcribed locus clone Gm-r1088-3517	0 467	0 001002	-2 77
Gma 17559 1 S1_at	Weakly similar to A. thaliana heavy-metal-associated domain-containing protein (51 7%)	0 469	0 004262	-2 08
GmaAffx 9618 1 S1_at	Calcium/calmodulin-dependent protein kinase	0 472	0 000025	-7 57
GmaAffx 85409 1 S1_at	Moderately similar to Ricinus communis putative metal ion binding protein (75%)	0 474	0 000647	-3 09
GmaAffx 51888 1 S1_at	Putative RAD50 DNA repair protein	0 479	0 000889	-2 86
GmaAffx 79131 1 S1_at	Splicing factor U2AF 50 KD subunit	0 481	0 002673	-2 26
Gma 5949 1 A1_at	Histone H3	0 483	0 001154	-2 67
Gma 10876 1 S1_at	Strongly similar to A. thaliana mannan synthase/transferase (AtCSLA02) NP_197666 1 (91 5%)	0 485	0 000667	-3 07
GmaAffx 19996 1 S1_at	IFA-binding protein	0 486	0 000190	-4 20
Gma 4884 2 S1_a_at	Moderately similar to Citrus sinensis ethylene-responsive element-binding protein (EREBP) mRNA (74 0%)	0 487	0 002694	-2 26
GmaAffx 78543 2 S1_at	Transcribed locus	0 487	0 007151	-1 89
GmaAffx 57899 1 S1_at	Zinc finger, C3HC4 type (RING finger) protein	0 494	0 002101	-2 37
Gma 10949 1 S1_s_at	Moderately similar to A. thaliana 3-chloroallyl aldehyde dehydrogenase/aldehyde dehydrogenase (NAD)/coniferyl-aldehyde dehydrogenase (ALDH2C4) (75 5%)	0 495	0 000273	-3 92
GmaAffx 7217 1 A1_at	Transcribed locus	0 496	0 001277	-2 62
GmaAffx 27586 2 S1_at	26S proteasome regulatory subunit	0 496	0 000562	-3 23

^aExpression values were obtained using RMA (Irizarry et al , 2003)

Appendix I

Distribution of gene function categories of probe sets that were down-regulated more than 2-fold in the seed coat of black (*irt*) soybean relative to the seed coat of brown (*irt*) soybean.

Appendix J

Genes located on chromosome 9 of the soybean genome sequence Glyma1 that are associated with probe sets differentially-regulated more than 2-fold in the seed coat of black (IRT) soybean variety Clark

Probes Sets	Gene Identifier	Genomic location	Target Description
Up-regulated	GmaAffx 90491 1 A1_s_at	None	Gm09 38,123,568 38,127,784 Weakly similar to A thaliana putative serine/threonine protein kinase NP_176864 1
	Gma 2605 1 S1_at	Glyma09g32240	Gm09 38,788,863 38,791,460 Similar to uncharacterized DUF1674 domain (3 12e-13)
	Gma 2605 2 S1_at		Similar to uncharacterized DUF1674 domain (3 12e-13)
	Gma 2605 2 S1_a_at		Similar to uncharacterized DUF1674 domain (3 12e-13)
	GmaAffx 45454 1 S1_at	Glyma09g33060	Gm09 39,592,425 39,601,531 WD40 protein
	GmaAffx 12723 1 A1_at	Glyma09g34200	Gm09 40,647,736 40,650,017 Similar to leucine-rich repeat (LRR) domain (1 48e-03)
	GmaAffx 78720 2 S1_at	Glyma09g35050 1	Gm09 41,280,276 41,287,219 Similar to pectinacetyltransferase domain (1 28e-163)
	Gma 10642 1 S1_at	Glyma09g35050 5	Gm09 41,280,276 41,287,596 Similar to Mob1/phocein superfamily domain (3 34e-45)
Down-regulated	Gma 3745 1 S1_at	Glyma09g36150	Gm09 41,989,507 41,996,277 Moderately similar to A thaliana polyamine oxidase (ATPAO1) NP_196874 1 (75 6%) Polyamine oxidase
	GmaAffx 18599 1 S1_at		
	Gma 9786 1 S1_at	None	Gm09 38,200,643 38,200,971 Transcribed locus
	Gma 10949 1 S1_s_at	Glyma09g32170	Gm09 38,729,659 38,730,312 Moderately similar to A thaliana 3-chloroallyl aldehyde dehydrogenase/aldehyde dehydrogenase (NAD)/coniferyl-aldehyde dehydrogenase (ALDH2C4) (75 5%)
	Gma 9776 1 S1_at	Glyma09g32280	Gm09 38,820,324 38,820,731 Kinesin heavy chain
	GmaAffx 14886 1 S1_at		Gm09 38,819,390 38,819,711 Kinesin heavy chain
	Gma 14852 1 S1_at	Glyma09g34330	Gm09 40,741,617 40,742,478 Clone JCVI-FLGm-707 unknown mRNA
	Gma 5402 1 S1_at	Glyma09g36080	Gm09 41,955,543 41,955,967 Moderately similar to A thaliana serine carboxypeptidase-like 40 (scpl40) (71 4%)
	GmaAffx 66066 1 A1_at	Glyma09g36840	Gm09 42,442,582 42,443,019 Weakly similar to Populus trichocarpa AP2/ERF domain-containing transcription factor XP_002336822 1 (67 7%)
	GmaAffx 42587 1 A1_at	Glyma09g37340	Gm09 42,909,762 42,910,176 Moderate similarity to A thaliana MYB30 (71 5%)
	Gma 571 1 S1_at	Glyma09g37600	Gm09 43,114,292 43,122,807 Weakly similar to A thaliana Thioredoxin 2 (AtTRX2) NP_198811 1 (61 5%)

Appendix K

Semi-qRT-PCR validation of the expressions of select genes found to be differentially expressed in black (*iRT*) and brown (*irT*) seed coats by microarray analysis. An asterisk (*)

represents genes located on Chromosome Gm09 of the soybean Glyma1 genome sequence. Genes and corresponding differentially regulated probe sets: 4CL-L, Gma.7423.2.S1_a_at; 4CL-2, Gma.8472.1.S1_at; CHS4 and CHS5, GmaAffx.42116.1.S1_at; LAR1, GmaAffx.34868.1.A1_at; DFR2, GmaAffx.80720.1.S1_at; ANS2/ANS3, Gma.1163.1.S1_at; UGT78K1, Gma.1002.2.S1_at; UGT78K2, GmaAffx.71999.1.S1_at; OMT-like, Gma.9647.1.S1_at; OMT5, GmaAffx.57777.1.S1_at; GST26, GmaAffx.71212.1.A1_at; GST21, Gma.5139.1.S1_at; MYB50, GmaAffx.81605.1.S1_at; MYB159, GmaAffx.39483.1.A1_at; C2H2 ZF, Gma.17736.1.S1_at; WD40, GmaAffx.45454.1.S1_at; SCOF-1, Gma.235.1.S1_at; AP2, GmaAffx.2469.1.S1_at; EF-hand, Gma.15972.1.A1_at; ProtK, GmaAffx.90491.1.A1_s_at; LRR, GmaAffx.12723.1.A1_at; G4DT, Gma.5621.1.S1_at; PAO1-L, Gma.3745.1.S1_at; Am Oxy, Gma.3745.1.S1_at; PCT, GmaAffx.78720.2.S1_at; PUB22, Gma.4530.1.A1_s_at; 9O12a, Gma.2605.1.S1_at; 9O12b, Gma.2605.2.S1_at; Lipase, GmaAffx.90450.1.S1_at.

Appendix L

UGT78K2	MDHQ ^Q NKHVAVFAFPFGSHLMPLLNVLKLAHSLPNC ^S FSFIGTDKSNAILFPKPHIPNNI 60
UGT78K1	MDHQ ^Q NKHVAVFAFPFGSHLMPLLNVLKLAHSLPNC ^S FSFIGTHKSNAILFPKPHIPNNI 60
UGT78K2	KAYSISDGIPEGHVLGKNPTEKLNLFLOTGPENLHKGIELAEAETKKRVTCI ^V ADAFVTS 120
UGT78K1	KAYSISDGIPEGHVLGKNPTEKLNLFLOTGPENLHKGIELAEAETKKRVTCI ^L ADALVTS 120
UGT78K2	SL ^F VAQTLNVPWIALWLPNSC ^S LSLYFYTELIRQHCAN ^H HAGNTTLD ^F LPGLSKLRVEDMP 180
UGT78K1	SL ^L VAQTLNVPWIALWLPNSC ^S LSLYFYTD ^L IRQH ^C CASRAGNK ^T LD ^F IPGLSKLRVEDMP 180
UGT78K2	QDLLDVGEKETVFA ^R RELNSLGKVL ^P QAKVVVMNFFEELEPPLFVQDMRSKLQSLLYVVPL 240
UGT78K1	QDLLDVGEKETVFS ^R RELNSLGKVL ^P QAKVVVMNFFEELEPPLFVQDMR ^N NKLQSLLYVVPL 240
UGT78K2	PSTLLPPSDTDSSGCLSWL ^D TKNSKSVAYVCFGTVVAPPPHELVAVAEAEESGFPFLWS 300
UGT78K1	PSTLLPPSDTDSSGCLSWL ^G MKNKSVAYVCFGTVVAPPPHELVAVAEAEESGFPFLWS 300
UGT78K2	LKEGLIGLLPNGFVERTKK ^H GKIVSWAPQT ^V VLAHDSVGVFVTHCGANSVIESVSSGVPM 360
UGT78K1	LKEGLMSLLPNGFVERTKK ^R GKIVSWAPQT ^H VLAHDSVGVFVTHCGANSVIESVSSGVPM 360
UGT78K2	ICK ^P PFFGDQVVA ^A ARVIEDVWEIGV ^I MEGKVF ^T KNGLVKS ^L DLILVH ^Q EGKKIRDNAL ^K VK 420
UGT78K1	ICK ^P PFFGDQGV ^V ARVIEDVWEIGM ^I EGKM ^F TKNGLVKS ^L NLILVH ^Q EGKKIRDNAL ^R VK 420
UGT78K2	KTVEDAGRPEGQAA ^Q DFDTLVEVISRS 447
UGT78K1	KTVEDAGRPEGQAT ^Q DFNTLVEVISRS 447

Alignment of *G. max* UG3GT proteins UGT78K2 and UGT78K1 from variety Clark using the ClustalW program with default parameters. Amino acid differences are shown with grey background.

Appendix M

Primers				
Experiment	Corresponding probe set	Target	Primer	Sequence (5' to 3') ^{†‡}
Semi-qRT-PCR	GmaAffx 39483 1 A1_at	MYB159	159F	TGACTCAGCCTCACCCTTG
			159R	CAAAGGCATTGACCCAAATAA
	Gma 15972 1 A1_at	4CL2	4C2F	ACAAACATGGTGGTGGACAA
			4C2R	GGTAGAAACCGCTGCCACT
	Gma 7423 2 S1_a_at	4CL	4CF	GCAAGACCAATCTTGCATCC
			4CR	TTTTGCCCAGGCATATATGTAA
	Gma 2605 1 S1_at	9012b	901F	CACTTTGACGTCGATCCAAAC
			901R	GAATCAGATGTCAAACCGTTCA
	Gma 2605 2 S1_at	9012a	902F	GCACGAGGGAAAATGTGTTT
			902R	GTTGGAAACGGATTTGGTGT
		ANR1	qAF	CCCTCAATATAAAAATTCCAAGTAA
			qAR	CAGAGCCCCCTTGCTCTTTA
	GmaAffx 42116 1 S1_at	CHS4	CHS4F	CCTTCCAAGCCACTTTGCA
			CHS4R	CTGGAGCAAAGGATGAAAGTGA
	GmaAffx 42116 1 S1_at	CHS5	CHS5F	CACTTTGCCACATTCAATCC
			CHS5R	TGTGAATGAACTAATGAAGCTATAGC
		CHS7/ CHS8	CHS78F	TAGGCAAGACATGGTGGTGG
			CHS78R	CTTTGACTTTGGCTGGCCC
	GmaAffx 80720 1 S1_at	DFR2	DFR2F	CCCCTTTCTTATGCCAACAA
			DFR2R	ATGTACCTCCCTTCGGCTTT
	Gma 15972 1 A1_at	EF-Hand	EHF	ACGCCATCTCCATTCAAATC
			EHR	CAGCAGGGGAAGATGAGAAG
	Gma 5139 1 S1_at	GST 21	G21F	ACTTGATGCAGACCCCTTGT
			G21R	TTCAAGAACACCCAACACCA
	GmaAffx 71212 1 A1_at	GST1	GST1F	CCAGGGCTTGAACATCTCAT
			GST1R	TCCACGCCATGTATTTGTTG
	GmaAffx 90491 1 A1_s_at	Protein kinase	KIF	GAAAGGTTTGAACGCACCAT
			KIR	ATTTTGGCGGAGTTTCACAC
	GmaAffx 73273 2 S1_s_at	Lipase	LIPF	CCGCATGGTTCTATCTTGGT
			LIPR	TGCATTTGACAAACCTTCA
	GmaAffx 45454 1 S1_at	LRR	LRF	TTACATCAATGGCGGGATCT
			LRR	TTGGTGGGAACCTCTTTTGG
	GmaAffx 81605 1 S1_at	MYB50	M50F	TTTCAATCTGGAATTGGTTCG
			M50R	TTTACTCATTCAACCAATAACAACAC
	GmaAffx 57777 1 S1_at	OMT5	OMT2F	CCTCACTGCACCTTACCATTCC
			OMT2R	TCTTTATCGGCATCAACGAA
	Gma 9647 1 S1_at	OMT-like	OMTF	TTTGTTGAAAGCCAGTGCA
			OMTR	TTTTGCCCTTTGTTGATGCTG
	Gma 17141 1 S1_at	Oxy	OXF	GCAAGTGGCTAACCGTCAAT
			OXR	AGATAGTTGGAGCTAGAGTTTGAAAAA
		PEPC	qPF	CATGCACCAAAGGGTGT
			qPR	TTTTGCGGCAGCTATCTCTC
	Gma 1002 2 S1_at	UGT78K1	q3GTF	GGGTTTGTTGAGAGGACCAA
			q3GTR	CATCCCAATCTCCCAACAT

	GmaAffx 71999 1 S1_at	UGT78K2	qUAF	AAGCTGCACAGGATTTTCGAC
			qUAR	ACACACACACACACACACAAAAC
	Gma 5621 1 S1_at	G4DT	P4F	GCAGACCGTTGTGTTGAAGA
			P4R	CCAGGATACCAACTCCGAAA
	GmaAffx 12723 1 A1_at	PECTIN	PCTF	TGAAATAAAAGCCAATCCCATT
			PCTR	GATCAGTGTGCCCCAAAATC
	Gma 4530 1 A1_s_at	Ring Fing	RFF	CCATTACGGGATTGTTTGGA
			RFR	TCTCCATGCATACGGAACAA
	Gma 235 1 S1_at	SCOF TF	STFF	AGATCGAAATCCCCCAATTT
			STFR	TGCAAATCTGCCACTGTTGT
	Gma 8472 1 S1_at	WD40	WDF	CAAGCTGGTTCAGTCGATCA
			WDR	GCCTTTCCTGTTCTGCAGTC
	Gma 17736 1 S1_at	C2H2 ZF	ZF	TGGACTTGGAGTTACACCTGTAGATA
			ZR	CAATGATCTTTATAAGGGAAAAGTCA
	Gma 4334 1 S1_at	Zinc Fing	ZF2F	ACTGTTGGTGGTCCACGATT
			ZF2R	AAGGCCAAATCCTCGTTCTT
qRT-PCR	Gma 1002 2 S1_at	UGT78K1	q3GTF	GGGTTTGTTGAGAGGACCAA
			q3GTR	CATCCCAATCTCCCAAACAT
		ANR1	qAF	CCCTCAATATAAAATTCCAAGTAA
			qAR	CAGAGCCCCTTTGCTCTTTA
		DFR1	qDF	GGGCCTGGACTTCATCACTA
			qDR	ACCTCCCTTCCACTTCTGGT
		GPAT	qGPF	ATAGGATCGTGCCTGTTGCT
			qGPR	CAGCCTTAACCGACATCTCC
	GmaAffx 34868 1 A1_at	LAR1	qL1F	TAGGCTTTGGTGAGGAGGAA
			qL1R	TGACACCAAAACAGAGTGACAA
		PEPC	qPF	CATGCACCAAAGGGTGT TTT
			qPR	TTTTGCGGCAGCTATCTCTC
cDNA cloning	GmaAffx 71999 1 S1_at	UGT78K2	qUAF	AAGCTGCACAGGATTTTCGAC
			qUAR	ACACACACACACACACACAAAAC
	GmaAffx 57777 1 S1_at	OMT5	OMT2F	CCTCACTGCACTTACCATTCC
			OMT2R	TCTTTATCGGCATCAACGAA
	GmaAffx 57777 1 S1_at	OMT5	HO5F	CACCcatatgGCCGAAGAGGAGAGGCAT
			HO5R	ACTCctcgagCCCCACTCCCCAAAAGAGA
				GAAAAA
				CACCcatATGGATCATCAAAACAAACAC
	GmaAffx 71999 1 S1_at	UGT78K2	UHF	GTAGCA
			UAR	ACTCggaatccACACACACACACACACAC
				AAAACATAAAAGT

[†]Restriction site in lowercase

^{*}Start codon underlined

Appendix N

Primers.

Experiment	Target	Primer	Sequence (5' to 3') ^{†,‡}
5'-RACE of <i>ANR</i>	EV264997	AEV1R	GCAAAGTTCACAGGTGTAGCAAGCTGGA
	BM092813	AEB4R	GAGCACAGCAAATGTATCGACCAGAAGC
3'-RACE of <i>ANR</i>	EV264997	AEV1F	GATTGGAGGGAGTGGATTTATGGCCTCT
	BM092813	AB4F	GCTTCTGGTCGATACATTTGCTGTGCTC
qRT-PCR	<i>ANR1</i>	qAF	CCCTCAATATAAAATTCCAAGTAA
		qAR	CAGAGCCCCTTTGCTCTTTA
	<i>ANR2</i>	qA2F	AAGCAAATCGGAAAGAAGCA
		qA2R	TCAATTCGCCCAGATTTTTC
	<i>UGT78K2</i>	qUAF	AAGCTGCACAGGATTTGAC
		qUAR	ACACACACACACACACAAAAAC
	<i>ANS2/ANS3</i>	A23F	CTGAGCAACGGCAAGTACAA
		A23R	CAATTTGGGAGACCTTCCTG
	<i>LAR1</i>	qL1F	TAGGCTTTGGTGAGGAGGAA
		qL1R	TGACACCAAACAGAGTGACAA
	PEPC16	qPF	CATGCACCAAAGGGTGTTTT
		qPR	TTTTGCGGCAGCTATCTCTC
cDNA cloning	<i>ANR1</i>	AHF	CACCcat ATG GCCGCTATCAAGCCAACTG
		AHR	ACTCggatccACAGAGATGGTTGCTATTCCCCTTGATT
	<i>ANR2</i>	AH2F	CACCcat ATG GCCAATATCAAGCAAATCG
		AH2R	ACTCggatccTTAGTTGAGCAGGGCCCCC
Genomic cloning	<i>ANR1</i>	OGF	CACCGAATTCCTTCTCTCCTGAGGGAGGATT
		OGR	ACTCGGATCCTCCAACTTAATAAAGCGACTGA

[†]Restriction site underlined.

[‡]Start codon bold.

Appendix O

Primers.

Experiment	Target	Primer	Sequence (5' to 3') [†]
PCR genotyping	GUS linker	GUSF	CATGAAGATGCGGACTTACG
		GUSR	ATCCACGCCGTATTCCG
ANR-RNAi cloning	ANR1 3'-CDS	ASF	CACCGGTACCCTCTCACAACCTGGTCCTTCTG TCACTAC
		ASR	AGTTGGATCCGCTGAGAACTTTGCAAGCTC AGG
qRT-PCR	ANR1	qAF	CCCTCAATATAAAAATTCCAAGTAA
		qAR	CAGAGCCCCTTTGCTCTTTA
	ANR2	qA2F	AAGCAAATCGGAAAGAAGCA
		qA2R	TCAATTCGCCCAGATTTTTC
	ANS2/ANS3	A23F	CTGAGCAACGGCAAGTACAA
		A23R	CAATTTGGGAGACCTTCCTG
	DFR1	qDF	GGGCCTGGACTTCATCACTA
		qDR	ACCTCCCTTCCACTTCTGGT
	GST26	GST1F	CCAGGGCTTGAACATCTCAT
		GST1R	TCCACGCCATGTATTTGTTG
	GUS linker	qGF	CCCTTACGCTGAAGAGATGC
		qGR	GAGGTTAAAGCCGACAGCAG
	LAR1	qL1F	TAGGCTTTGGTGAGGAGGAA
		qL1R	TGACACCAAAACAGAGTGACAA
	PEPC16	qPF	CATGCACCAAAGGGTGTTTT
		qPR	TTTTGCGGCAGCTATCTCTC
	UGT78K1	q3GTF	GGGTTTGTTGAGAGGACCAA
		q3GTR	CATCCCAATCTCCCAAACAT
	UGT78K2	qUAF	AAGCTGCACAGGATTTTCGAC
		qUAR	ACACACACACACACACAAAAC

[†]Restriction site underlined.

Appendix P

Multiple reaction monitoring specifications for quantification of differentially accumulated flavonoid compounds from the seed coats of transgenic soybeans expressing the ANR-RNAi construct using a 3200 QTRAP[†].

Compound	Q1	Q3	Time (msec)	DP (Volts)	EP (Volts)	CEP (Volts)	CE (Volts)	CXP (Volts)
(-)- Epicatechin	291.0	139.1	20	26	6	26	21	4
Isorhamnetin- 3-O- glucoside	479.0	317.1	20	16	6	24	25	6
Procyanidin B2	579.8	126.9	20	36	7	30	35	4
Quercetin-3- O-glucoside	579.8	123.0	20	36	7	30	75	4
Cyanidin-3- O-galactoside	465.0	303.2	20	21	4	24	21	4
Cyanidin-3- O-glucoside	465.0	85.1	20	21	4	24	45	4
Cyanidin-3- O-galactoside	449.0	288.1	20	70	10	23	50	3
Cyanidin-3- O-glucoside	449.0	287.2	20	70	10	23	50	3
Cyanidin-3- O-glucoside	449.0	288.2	20	70	10	23	50	3
Cyanidin-3- O-glucoside	449.0	287.1	20	70	10	23	50	3

[†]See Materials and Methods section for detailed description of the LC-MS/MS system.

Appendix Q

Developmental properties of T2 lines compared to the untransformed control (ir) at two stages of development.

Property	ir	A1A1-1-2	A1A1-2-2	A1A1-3-4	A1A1-4-4
25 - 50 mg FSW					
Days post-anthesis	30	30	30	30	30
Embryo morphology	cotyledon	cotyledon	cotyledon	cotyledon	cotyledon
<i>GPAT</i> relative	4.371 ±	5.357 ±	3.950 ±	4.430 ±	4.636 ±
expression ^a	0.19	0.64	0.97	0.29	0.58
T-Test ^b	N/A	0.104	0.41	0.802	0.524
400 - 500 mg FSW					
Days post-anthesis	40	40	40	40	40
	early	early	early	early	early
Embryo morphology	maturation	maturation	maturation	maturation	maturation
<i>GPAT</i> relative	0.639 ±	0.672 ±	0.631 ±	0.635 ±	0.650 ±
expression ^a	0.04	0.02	0.04	0.04	0.05
T-Test ^b	N/A	0.271	0.838	0.917	0.759

^aqRT-PCR relative to *PEPC16* using seed coat cDNA.

^bTwo-tailed student's *t* test compared to the untransformed control (ir).

Appendix R

Percent sequence similarity of *ANR1*-
RNAi to RED superfamily genes.

Gene	Genbank Accession	Percent Identity
<i>ANR1</i>	JF433915	100
<i>ANR2</i>	JN098512	86
<i>LAR1</i>	JF433916	55
<i>DFR1</i>	AF167556	53
<i>DFR2</i>	DQ026299	51
<i>IFR1L</i>	JF433916	47